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LIFELONG EXPOSURE TO DIETARY SOY ISOFLAVONES – IMPACT ON MAMMARY GLAND AND SMALL INTESTINE OF FEMALE RATS

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Abstract

Introduction: The incidence of breast cancer in Eastern Asia is approximately 3-times lower than in western countries, and nutrition seems to play an important role. The traditional East Asian diet is rich in soy, which is the main source of the isoflavones (ISO) genistein (GEN), daidzein (DAI), and glycitein (GLY). Some evidence suggests that ISO intake must be high during certain windows of development to exert anti-cancerogenic action. The aim of the present thesis was to investigate the effects of soy ISO exposure in different periods of life on the estrogen sensitivity of the mammary gland and homeostasis of the small intestine in female rats.

Methods: In a first experiment, animals were exposed to an ISO-free (IDD), an ISO-rich (IRD; 248 µg GEN, 213 µg DAI, 59 µg GLY per g diet) or an IDD diet supplemented with GEN (GRD; 700 µg GEN per g diet), throughout their whole lifetime. At the age of 50 (PND 50) and 80 days (PND 80) proliferative activity was analysed in the mammary gland and compared to ISO serum concentrations. Additionally, the tissue homeostasis of the small intestine was determined. In order to analyze estrogen sensitivity of the mammary gland IDD, IRD and GRD rats (PND 80) were ovariectomized and treated either with vehicle (OVX), estradiol (E_2; 7.8 µg/g BW/day), or GEN (19.6 mg/kg BW/day). In a second experiment, two group of rats were lifelong exposed to either an IDD or to an ISO-containing diet (ISD; 147 µg GEN, 114 µg DAI, 36 µg GLY per g diet). A third group received the ISD only from shortly before the onset of puberty (PND 30) up to the end of puberty (PND 60; pISD). Animals were treated with vehicle (OVX), E_2 (4 µg/g BW/day) or GEN (10 mg/kg BW/day). As in the first experiment, the responsiveness of the mammary gland was analysed.

Results: At PND 50, PCNA expression showed an increase in the mammary gland of both IRD and GRD animals compared to the IDD group. In the small intestine an increased PCNA and PS2-expression could be measured. Differences in the expression of PCNA in the mammary gland could neither be detected in intact rats at PND 80, nor at PND 97 for OVX and GEN treated rats, whereas treatment with E_2 resulted in a significant lower proliferative (PCNA expression) and estrogenic (PR and ERα expression) response of the mammary gland in the IRD and GRD group compared to the IDD animals. In contrast, the expression of estrogen receptor beta (ERβ) and PS2 was significantly higher in IRD and GRD fed animals compared to the IDD group. In the second experiment, the proliferative activity in the mammary gland was not affected by IDD and ISD, while a significant increase could be detected for pISD animals at day 50. The analysis of Ki-67 and PCNA mRNA expression showed that the proliferative response to E_2 was significantly reduced in the pISD and ISD group compared to IDD. The induction of PR mRNA expression was significantly increased in both IDD and pISD animals compared to ISD.

Conclusions: The data of the present studies provide evidence that lifelong exposure to soy ISO reduces the sensitivity of the mammary gland towards E_2 and seems to improve protective mechanism of the small intestine. Additionally, it could be shown that ISO exposure starting first shortly pre-pubertal appears sufficient to reduce the proliferative response of the mammary gland towards estrogens.

Kurzfassung

Einleitung: Die Inzidenz von Brustkrebs ist in Ostasien um ein 3-faches niedriger als in westlichen Ländern und die Ernährung scheint hierbei eine bedeutende Rolle zu spielen. Die traditionelle ostasiatische Ernährung ist reich an Soja, welches die Hauptquelle für die Isoflavone (ISO) Genistein (GEN), Daidzein (DAI) und Glycitein (GLY) ist. Einige Studien deuten an, dass die ISO-Aufnahme zu bestimmten Entwicklungsstadien erfolgen muss, damit antikanzerogene Effekte erzielt werden können. Ziel der vorliegenden Dissertation war es zu untersuchen, wie sich eine ISO-reiche Ernährung zu verschiedenen Zeitpunkten des Lebens auf die Estrogensensitivität der Brustdrüse und die Gewebehomöostase des Dünndarms weiblicher Ratten ausübt.

Methoden: In einem ersten Experiment erhielten die Tiere lebenslang entweder eine ISO-freie Diät (IDD), eine ISO-reiche Diät (IRD; 248 µg GEN, 213 µg DAI, 59 µg GLY pro g Futter) oder eine IDD supplementiert mit Genistein (GRD; 700 µg GEN pro g Futter). Im Alter von 50 (PND 50) und 80 Tagen (PND 80) wurde die proliferative Aktivität in der Brustdrüse mit den ISO-Konzentrationen im Serum verglichen. Zusätzlich wurde die Gewebehomöostase im Dünndarm bestimmt. Um die estrogene Sensitivität der Brustdrüse zu analysieren wurden IDD, IRD und GRD Ratten ovariektomiert und entweder mit dem Lösungsmittel (OVX), Estradiol (E_2; 7.8 µg/g KG/Tag) oder GEN (19.6 mg/kg KG/Tag) behandelt. In einem zweiten Experiment erhielten zwei Tiergruppen entweder eine IDD oder eine ISO-haltige Diät (ISD; 147 µg GEN, 114 µg DAI, 36 µg GLY pro g Futter). Eine dritte Ernährungsgruppe wurde erst ab kurz vor Beginn der Pubertät (PND 30) bis zum Ende der Pubertät (PND 60) mit einer ISD gefüttert (pISD). Die Tiere wurden entweder mit dem Lösungsmittel (OVX), E_2 (4 µg/g KG/Tag) oder GEN (10 mg/kg KG/Tag) behandelt. Auch in diesem Experiment wurde die Responsivität der Brustdrüse untersucht.

Ergebnisse: An PND 50 war die PCNA Expression in der Brustdrüse von IRD und GRD Tieren, verglichen mit der IDD-Gruppe, erhöht. Im Dünndarm resultierten IRD und GRD in erhöhter Proliferation und PS2-Expression. An PND 80 waren keine Unterschiede in der PCNA Expression in der Brustdrüse von intakten, an PND 97 in OVX und GEN Tieren zu beobachten, wohingegen eine Behandlung mit E_2 in einer signifikant niedrigeren proliferativen (PCNA Expression) und estrogenen (PR und ERα Expression) Responsivität der Brustdrüse in der IRD und GRD Gruppe verglichen mit der IDD resultierte. Im Gegensatz dazu war die Expression des Estrogenrezeptor Beta (ERβ) und von PS2 in Tieren, die eine IRD oder GRD erhielten signifikant höher als in der IDD-Gruppe. Im zweiten Experiment war die proliferative Aktivität an PND 50 durch IDD und ISD nicht beeinflusst, wobei ein signifikanter Anstieg bei pISD-Tieren beobachtet wurde. Die Analyse von Ki-67 und PCNA mRNA zeigte, dass die proliferative Antwort auf E_2 in der pISD und ISD-Gruppe, verglichen mit der IDD, signifikant reduziert war. Die Induktion der PR mRNA Expression war sowohl in IDD als auch in pISD-Tieren signifikant erhöht.

Schlussfolgerungen: Die Ergebnisse der vorliegenden Studien belegen, dass eine lebenslange Exposition gegenüber ISO die Sensitivität der Brustdrüse gegenüber E_2 reduziert und wahrscheinlich den Schutzmechanismus des Dünndarms verbessert. Zusätzlich wurde gezeigt, dass eine ISO-reiche Ernährung, die erst kurz vor Einsetzen der Pubertät beginnt, ausreichend scheint, um in der Brustdrüse die proliferative Reaktion auf Estrogene zu reduzieren.

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ABBREVIATIONS

µg/ kg BW	microgram per kilogram body weight
DAI	daidzein
ER	estrogen receptor
E_2	estradiol
GD	gestational day
GRD	genistein-rich diet
GEN	genistein
GLY	glycitein
HRT	hormone replacement therapy
IDD	isoflavone-depleted diet
IRD	isoflavone-rich diet
ISD	isoflavone-containing diet
ISO	soy isoflavone
OVX	ovariectomized control group
PCNA	proliferating cell nuclear antigen
pISD	pre- to post-pubertal ISD
PND	postnatal day
PR	progesterone receptor
s. c.	subcutaneously
TEB	terminal end bud

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CHAPTER ONE

General Introduction

The most common hormone dependent cancer occurring to women is breast cancer, with increasing incidence in postmenopausal women. The second most common is bowel cancer. Although bowel cancer has not been regarded as being hormone-dependent for a long time, the observation that 2-times more men than premenopausal women are suffering from this disease indicates a protective role of female sex hormones. Underlined is this assumption by the observation that bowel cancer risk for women increases after menopause (Calle *et al.*, 1995; Messina & Bennink, 1998; Di Leo *et al.*, 2001). Furthermore, treatment with estrogens in the context of hormone replacement therapy decreases the risk of bowel cancer in postmenopausal women, whereas breast cancer risk increases at the same time (Chen *et al.*, 1998; Nelson *et al.*, 2002; Beral, 2003; Chlebowski *et al.*, 2004; Wada-Hiraike *et al.*, 2006; Chlebowski *et al.*, 2009).

That the incidence of these hormone-dependent diseases is much lower in Eastern Asia compared to Europe or the United States has been characterized many years ago (Adlercreutz *et al.*, 1992; Knight & Eden, 1996; Setchell & Cassidy, 1999; Messina & Wood, 2008). Questions arose about the factors reducing the cancer risk in the Asian population and migration studies were performed to analyse whether genetic differences were responsible for these effects. In respect to breast cancer it has been shown that Asian women which migrated to the United States lost the cancer protection if adapting the American diet (Ziegler *et al.*, 1993; Stanford *et al.*, 1995). Further studies showed that the cancer protection persists if the migration occurred after puberty (Shu *et al.*, 2001; Wu *et al.*, 2002). These observations led to the assumption that nutrition in early life plays a key role in the prevention of hormone dependent cancers.

The traditional East Asian diet is rich in soy-based food products. Soy is the main source of isoflavones (ISO; 10–30 mg ISO/g protein) and contains, in descending concentrations (10:8:1), the ISO genistein (GEN), daidzein (DAI), and glycitein (GLY) (Walz, 1931; Horn-Ross *et al.*, 2000). ISO belong to the class of phytoestrogens, and because of their structural similarity to endogenous estrogens, ISO are able to bind to both estrogen receptors and subsequent initiate estrogen action. Although the estrogenic activity of ISO is 100 to 10,000-times lower compared to the endogenous estrogen 17β-estradiol (E_2), the plasma concentrations of biological active ISO can reach 100 to 1,000-times of the level of free estrogens. This is due to the lower binding affinity of ISO to sexual-hormone-binding globulines (Limer & Speirs, 2004).

Soy intake in the Asian population consuming a traditional diet range between 6 and 11 g soy protein per day, resulting in 25–50 mg ISO (calculated as aglykone). About 10 % of the Asians reach a high intake of 25 g soy protein per day (100 mg ISO as aglykone equivalent). In contrast, consumption of less than 1 mg soy protein per day is common in Europe (Adlercreutz, 1998; Setchell & Cassidy, 1999; Munro *et al.*, 2003; Sirtori *et al.*, 2005). Beside differences in the daily ISO uptake, the exposure pattern differs between both populations. In Asia, relative large amounts of soy products are consumed throughout life, which result in an ISO exposure already in utero, because the fetus is exposed to the same ISO levels as found in the maternal circulation during pregnancy (Doerge *et al.*, 2006). On the contrary, most European people do not consume large amounts of soy products in their life. In the last years, soy-based supplements are advertised as an effective and side-effect-free alternative to hormone replacement therapy in postmenopausal women. The users of these supplements are exposed to high doses of ISO in a purified form for the first time in their life in a late adult stage. The dosage of the soy-based dietary supplements varies between 20 and 80 mg ISO/day, but sometimes doses up to 150 mg ISO/day are recommended (Wei *et al.*, 2012; Ferrari, 2009).

Although no harmful effects of soy ISO has been described so far if the consumption occurred as a regular part of the diet, the effects of isolated and/or high-dosed ISO consumption are discussed controversial. Health beneficial declarations of soy-based dietary supplements usually refer to Asian populations and their consumption of soy, whereas epidemiologic studies in European populations have shown that the efficiency of ISO to treat hormone dependent diseases is questionable (Tomar & Shiao, 2008; Wu *et al.*, 2008).

Cell culture and animal studies even indicate that ISO increase the risk of breast cancer, especially estrogen-dependent carcinomas, because of their estrogenic activity. Furthermore, it has been shown that the stimulating effects of GEN on tumor growth depend on ISO concentration and estrogen status (Wang *et al.*, 1996; Ju *et al.*, 2006). Also, results of animal studies have indicated that ISO may either prevent or promote carcinogenesis, depending partly on the developmental stage of the animal and mostly on factors that have not yet been understood. Additionally, animal studies indicate that ISO exposure during pre-puberty provide protection against breast cancer later in life. In rats, pre-pubertal exposure to GEN until postnatal day 20 resulted in altered morphology of mammary glands, reduced numbers of terminal end buds, increased lobular differentiation, and reduced breast

cancer incidence when tumor growth was subsequently induced by carcinogen treatment (Warri *et al.*, 2008).

Regarding the intestine, several in vitro and in vivo studies showed that GEN and E_2 are able to influence intestinal homeostasis, whereas the results of these studies remain conflicting (Booth *et al.*, 1999a; Booth *et al.*, 1999b; Chen *et al.*, 2005; Javid *et al.*, 2005; Weige *et al.*, 2009). It is assumed that GEN and E_2 exert their effects in the intestine via activating the ERβ. It has been shown that over-expression of ERβ resulted in a reduction of proliferation in human cancer cells, suggesting that some key regulators of the cell cycle are modulated by the ERβ (Martineti *et al.*, 2005). Altogether, GEN as a ligand of the ERβ may serve as potential regulators of intestinal tissue, whereas the underlying mechanisms are still unclear.

Given the challenge to transfer data from animal models to the human situation, a number of clinical studies have investigated the effects of ISO exposure on human breast and intestine so far, but the results of these studies are conflicting and did not provide clear evidence with respect to health risks or benefits of ISO uptake (Nagata, 2010; Adlercreutz & Mazur, 1997; Setchell & Cassidy, 1999; Messina & Loprinzi, 2001; Adams *et al.*, 2005; Duffy *et al.*, 2007; Mense *et al.*, 2008; Yang *et al.*, 2009). This may be due to limitations of the study designs, such as the lack of suitable control groups, variations in or even unknown composition of the consumed products or limited number of readouts. It is questionable whether further clinical and epidemiologic studies alone will provide sufficient data to answer all the questions regarding the effect of ISO exposure on risk of breast and bowel cancer. To improve the knowledge about the apparently contradictory effects of ISO the underlying molecular mechanisms have to be investigated.

To our knowledge, no data exists regarding the impact of lifelong exposure to ISO on estrogen sensitivity of the mammary gland and on tissue homeostasis of the small intestine of females. The present thesis should make an important contribution to understand molecular mechanisms that are affected by ISO and whether intake of ISO may be protective or adverse with respect to the risk of developing breast and bowel cancer.

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OBJECTIVES OF THE THESIS

The main research objective of the present thesis is to improve knowledge about the beneficial or adverse effects of ISO on the mammary gland and the small intestine. In order to make a contribution to the elucidation of involved molecular mechanisms, the major aim of this thesis was to investigate effects of lifelong ISO exposure on the estrogen sensitivity of the mammary gland and the small intestine of female rats. For this purpose, the thesis addresses the following three key research questions:

- CHAPTER TWO:

  Is the estrogen sensitivity of the mammary gland influenced by in utero and postnatal exposure to soy ISO or to solely GEN via diet?

- CHAPTER THREE:

  In which way is the tissue homeostasis of the small intestine of female rats modulated by lifelong exposure to ISO or to GEN?

- CHAPTER FOUR:

  How important is the time of ISO exposure for their protective effects on the mammary gland?

CHAPTER TWO

In utero and postnatal exposure to isoflavones results in a reduced responsivity of the mammary gland towards estradiol*

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Abstract:

Scope: Exposure scenarios during different stages of development of an organism are discussed to trigger adverse and beneficial effects of isoflavones (ISO). The aim of this study was to investigate how in utero and postnatal ISO exposure modulates the estrogen sensitivity of the mammary gland and to identify underlying molecular mechanisms.

Methods and results: Therefore rats were exposed to either ISO-free (IDD), ISO-rich (IRD) or genistein rich diet (GRD), up to young adulthood. Proliferative activity (PCNA expression) in the mammary gland at different ages and the estrogen sensitivity of the mammary gland to estradiol (E_2) or genistein (GEN) in adult ovariectomized animals was determined and compared to the different treatments. Treatment with E_2 resulted in a significant lower proliferative and estrogenic response of the mammary gland in IRD and GRD compared to IDD. This correlates to a change in the gene expression pattern and a decrease in the ratio of estrogen receptor alpha (ERα) beta (ERβ).

Conclusion: Our results provide evidence that in utero and postnatal exposure to a diet rich in ISO but also to GEN reduces the sensitivity of the mammary gland towards estrogens and support the hypothesis that in utero and postnatal ISO exposure reduces the risk to develop breast cancer.

Introduction:

The incidence of breast cancer in Eastern Asia is approximately 3-time lower than in western countries (Mense *et al.*, 2008). It has been hypothesized that nutrition plays an important role in the prevention of hormone dependent breast cancer (Adlercreutz & Mazur, 1997). The traditional East Asian diet includes soy, which is the main source of isoflavones (ISO), belonging to the class of phytoestrogens. Soy ISO show a polyphenolic non-steroidal structure and their hydroxyl groups at C-7 of the A-ring and C-4´of the B-Ring of the ISO skeleton exhibit a distance similar to those of the endogenous estrogens. Because of this similarity, ISO exert the ability to bind to both estrogen receptor subtypes (ER), with a higher affinity to the ERβ, and subsequently initiate estrogen-dependent transcription (Newbold *et al.*, 2001).

The estrogenic potency of ISO is much lower compared to estrogens, but the circulating concentration of ISO can reach much higher levels after ingestion of soy food or soy-derived dietary supplements. The daily uptake of soy ISO in the various Asian countries range around 20–50 mg, resulting in ISO plasma concentrations of 870 nM, while the Western population ingests 1 mg ISO per day (Setchell & Lydeking-Olsen, 2003), which lead to plasma concentrations of ISO as low as 10 nM (Adlercreutz *et al.*, 1993). Intake of ISO via supplements normally range between 20 and 80 mg/day, resulting in ISO plasma concentrations in the range of 1–3 µM.

Whether effects of ISO are beneficial or not is controversially discussed. A protective effect of ISO towards bone health is described in animal studies (Branca, 2003; Hertrampf *et al.*, 2009a; Hertrampf *et al.*, 2009b), although questionable for the human situation as neither a randomized double blind placebo controlled study nor a recent meta-analysis were able to provide evidence (Brink *et al.*, 2008; Liu *et al.*, 2009). A benefit of ISO on hot flushes is also controversially discussed (Ferrari, 2009; Jacobs *et al.*, 2009).

The same controversy exists regarding the outcome of ISO intake in respect to breast cancer. For example in an animal model which mimics key aspects of postmenopausal conditions by inoculating MCF-7 breast cancer cells into immunodeficient nude mice an increase in breast tumor growth was detected after treatment with GEN (Ju *et al.*, 2006). In vitro for MCF-7 breast cancer cells a biphasic effect on cell growth dependent on concentration could be observed in response to GEN (Wang *et al.*, 1996). The National Toxicology Program (NTP) conducted a two years multigenerational GEN study in Sprague Dawley rats, which was recently reviewed by Doerge (Doerge, 2011). The key results relevant to this

study were that depending on the study arm a trend for increased incidences of mammary adenoma and adenocarcinoma could be observed in the 5 ppm and 100 ppm GEN feeding group, which although the absolute numbers were relatively low, reached statistical significance in the 500 ppm feeding group (Doerge, 2011; 2008a; 2008b). However, there is also evidence that in utero and postnatal or neonatal ISO or GEN exposure may protect against breast cancer (Whitsett & Lamartiniere, 2006; Lee *et al.*, 2009). In addition, soy ISO intake was found to be inversely correlated to mortality and recurrence from breast cancer.

To understand these controversial results it is important to have a closer look towards the risk factors involved in the development of breast cancer. Risk factors for breast cancer are early onset of menarche, late onset of menopause and high amounts of free circulating E_2 in the serum of post-menopausal women (Toniolo *et al.*, 1995; Lu *et al.*, 1996; Hulka & Moorman, 2001). ISO has the ability to increase the amount of sex hormone binding globuline, which results in a reduction of free, bioavailable circulating estrogens (Adlercreutz *et al.*, 1987). This conforms to the fact that compared to Western women the free serum-estradiol levels of Asian women are 40 % lower (Peeters *et al.*, 2003). Beside the concentration of circulating estrogens also the estrogen sensitivity of the non-malignant breast tissue may be altered by ISO. There is evidence that ISO may influence the development of the mammary gland, starting already in utero. A rudimentary gland is present at birth, and during puberty, hormone-dependent development of the mammary gland occurs (Lanigan *et al.*, 2007). Various studies suggest a protective effect of ISO if the ingestion starts before onset of puberty (Limer & Speirs, 2004; Browning *et al.*, 2005). Furthermore, it has been shown that pre-pubertal exposure to GEN alters the development of the mammary gland (Warri *et al.*, 2008). Hence, it seems reasonable that the exposure to ISO has to begin before pubertal breast development starts in order to exert protective effects as recently proposed (Messina & Hilakivi-Clarke, 2009).

Information whether the ISO exposure during puberty and in further developmental stages alters the sensitivity of breast tissue towards estrogens or ISO in postmenopausal woman is still limited. However, as recently reviewed the degree of maturation of terminal end buds appears to play a key role (Jenkins *et al.*, 2012). While studies investigating environmental endocrine disrupters, e.g. bisphenol A, suggest that early embryonic exposure significantly impacts on tissue (patho)physiology and hormone responsiveness later in life by delaying maturation (Betancourt *et al.*, 2010), the tumor preventive effect of neonatally administered

GEN appears to occur through advanced differentiation of these glandular structures which is paralleled by alterations in cell proliferation and apoptosis as well as up-regulation of tumor-suppressor genes (reviewed in (Jenkins *et al.*, 2012; Whitsett & Lamartiniere, 2006)). Taking into consideration that manufacturers advertise dietary soy supplements as an effective alternative to conventional HRT without adverse side effects, the understanding of the consequences of an early ISO exposure seems of fundamental importance.

The aim of this study was to investigate the effect of in utero and postnatal ISO exposure on estrogen sensitivity of the mammary gland in adult intact and ovariectomized female rats. The animals were fed diets containing different amounts of ISO. The ISO exposure was initiated in utero, maintained during neonatal, and pre-pubertal stages of development and adhered until adulthood. It is well known that an increased cell proliferation rate is associated with a high risk of developing breast cancer. For that reason the proliferation in the mammary gland was determined as a biological endpoint for estrogen sensitivity. In addition, the expression of the progesterone receptor (PR), the estrogen receptor α (ERα) and estrogen receptor β (ERβ) and PS2 was investigated.

Materials and Methods

Experimental animals and study design

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 50.203.2-K15).

Wistar rats were obtained from Janvier (Le Genest St Isle, France) and kept under controlled conditions of temperature (20℃ ± 1), relative humidity (50–80 %) and illumination (12 h dark, 12 h light). Female rats were mated and the dams (7 in each dietary group) were fed one of the three diets during pregnancy and nursing. After weaning the female offspring of each nutrition group (30 each group) was randomly assigned to five treatment groups (6 each group), to make sure that pups from different mothers generate a group. These rats had ad libitum access to the appropriate diet and water.

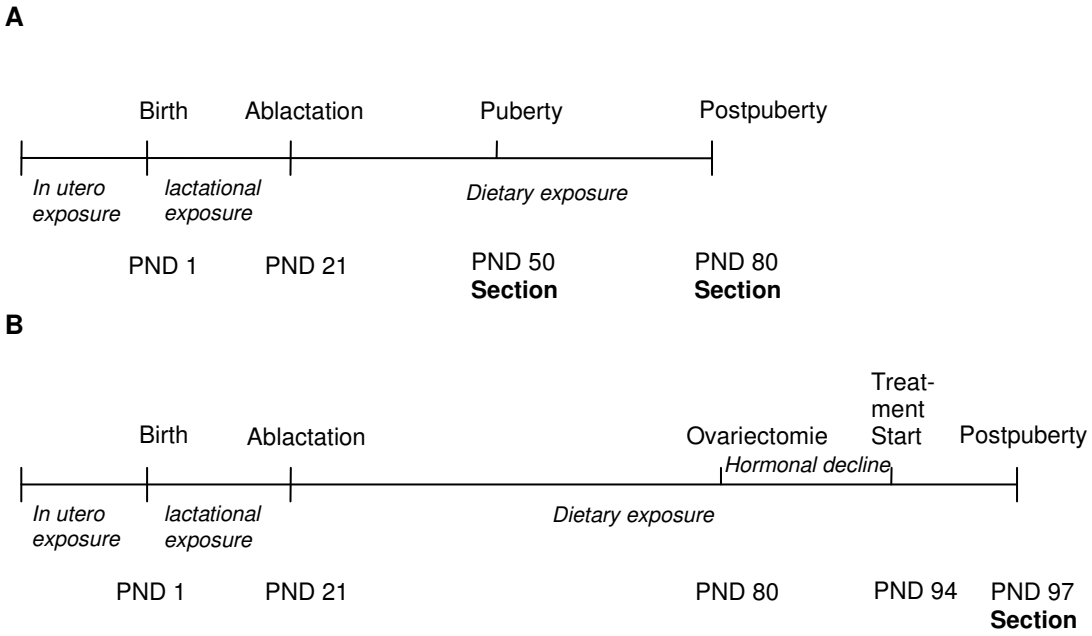

Fig. 1: Timeline and experimental setting of the study depicting modes of isoflavone exposure (in utero, lactational, and dietary) and points of time for analysis. **A** In intact PND 50 and PND 80 the ISO content were measured in the serum and the proliferation were determined in the mammary gland. **B** After ovariectomie on PND 80, animals were treated with E_2, GEN, or the vehicle from PND 94 up to PND 97 and were sacrified afterwards. In these animals the proliferation and the estrogenic response of the mammary gland were determined.

The first subgroup was sacrificed on postnatal day (PND) 50 (puberty), the second on PND 80 (adult). The other three subgroups of each nutrition group were ovariectomized at day 80. After 14 days of hormonal decline an uterotrophic assay was performed at day 94. These rats then received 17β-estradiol (E_2; 7.8 µg/kg BW/day), genistein (GEN; 19.6 mg/kg BW/day), or the vehicle solely (OVX) subcutaneously for three days before they were sacrificed (PND 97). The estrogenic compounds were solved in 20 % DMSO/peanutoil.

Diets

The animals had free access to one of three diets containing different amount of ISO: an ISO-depleted diet (IDD; Ssniff R/M-H Ssniff GmbH, Soest, Germany), an ISO-rich diet (IRD; Harlan Teklad 8604 rodent diet, Harlan Winkelmann, Borchen, Germany), or an IDD supplemented with 700 µg GEN (GRD; 4´,5,7-trihydroxyisoflavone, LC Laboratories, Woburn, USA). The compositions of the diets are depicted in Table 1.

	IDD	**IRD**
Brutto energy (kcal/g)	4	3.93
Metabolizable Energy (kcal/g)	3	3.3
Crude protein (%)	19.3	24.0
Crude fat (%)	3.3	4.0
Crude fiber (%)	4.4	4.5
N-free extractive (%)	55.1	46.64
C18:2 (Linols) (%)	1.49	1,87

Tab. 1: Composition of the different diets. IDD = Isoflavone depleted diet (Ssniff). IRD = Isoflavone rich diet (Harlan Teklad)

Indicated by the manufactures the protein source in the Ssniff R/M-H diet is cereals and potatoes, in the Harlan Teklad 8604 rodent diet soy, fish meal and yeast. The choice of these diets and the respective ISO content was based on results of former studies. In previous experiments of our laboratory, an oral dose up to 50 mg GEN per kg did not affect the uterine wet weights (Diel *et al.*, 2001), while an oral GEN

dose of 100 mg/kg results in effects on several tissues in the animals (Seibel *et al.*, 2009). This very high dose is representative for an exposure scenario depicting supplementation with soy extracts or pure GEN. In contrast, in the used IRD the source of the high content of ISO is dehulled soybean meal. Based on the data from previous studies, where ISO-content of rodent diets from different vendors was analysed and compared (Degen *et al.*, 2002), the ISO-rich diet (IRD) from Harlan-Winkelmann was chosen because of its high ISO content. This diet depicts more an exposure scenario of an ISO-rich nutrition like consumed in Eastern Asia.

Given the ISO content and daily food consumption (18–20 g/d/animal), the average oral intake resulted in 13.5 mg/kg BW for DAI and 15.7 mg/kg BW for GEN per adult animal in the IRD group, 42 mg/kg BW per adult animal and day for GEN in the GRD group, and less than 0.1 mg DAI or GEN/kg BW per day in the IDD group.

ISO Standards

The ISO aglycones daidzein, genistein, glycitein, their corresponding 7-*O*-ß-glucosides daidzin, genistin, glycitin, as well as 6”-*O*-acetyl-daidzin, -genistin and -glycitin and 6”-*O*-malonyl-daidzin, -genistin and -glycitin were obtained from Wako Chemicals GmbH (Neuss, Germany). Purity of the standard compounds was not less than 97 % (determined by HPLC/DAD analysis at 250 nm).

Tissue preparation

After weighing, animals were decapitated and blood was collected. The mammary gland and the uterus were removed and the uterus wet weights were determined. Specimen of each tissue were either snap frozen in liquid nitrogen for mRNA and protein preparation or fixed and embedded in paraffin for histological analysis.

Quantification of ISO derivatives in the diet by HPLC/DAD analysis

The pelleted diets IRD and IDD were crushed using mortar and pestle. Approx. 250 mg of an accurately weighted sample of each homogenized diet powder were vortexed for 30 s in exactly 40 mL 65 % (v/v) methanol and extracted gently for 60 min at room temperature using an overhead rotation shaker. The suspensions were centrifuged at 8600 g for 5 min and filtered using 15 mm 0.45 um PTFE syringe

filters. The filtrate was diluted by pipetting 1 ml of the solution into a 50 ml graduated flask and make up to the mark with 65 % (v/v) methanol.

The LC-DAD analyses were performed on a Shimadzu LC system equipped with a controller (CBM-20A), a degasser (DGU-20A3), two pumps (LC-20AD), an autosampler (SIL-20AC HT), a column oven (CTO-20AC) and a diode array detector (SPD-M20A). The LC system was controlled by the software LC solution 1.24. Separation of the ISO derivatives was performed on a Phenomenex Kinetex PFP column (3.0 mm internal diameter, 100 mm length, 2.6 µm) with an oven temperature of 35°C. Solvent A was 0.1 % (v/v) formic acid in bidest. water and solvent B was acetonitrile (VWR, LC grade). Flow rate was 0.7 ml/min, the injection volume 10 µl. The LC gradient started with an initial period of 3 min at 10 % B, increasing linearly to 45 % B at 12 min, and finally to 100 % at 12.5–15.5 min, re-equilibrating the system in a 7.5 min postrun (10 % B). Eluent was monitored between 200 and 500 nm using diode array detection. Peaks were integrated at 250 nm. The identity of each compound was confirmed by the retention time and the UV-Vis spectra. The limit of quantification (LOQ) and lowest calibration point was 0.8 nmol for all target analytes.

Measurement of the GEN and DAI serum concentrations

GEN and DAI were quantified in the serum samples (100 µl) by capillary gas chromatography-mass spectrometry (1200 Varian Triple Quadruole system) as previously described in detail (Rufer *et al.*, 2008) with the exception that the derivatization of the ISO was performed using *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide with 1 % *tert*-butyldimethylchlorosilane (Sigma-Aldrich, Taufkirchen, Germany). The two stable isotopically labeled ISO, [3,4,8-$^{13}C_3$]daidzein, and [3,4,1′-$^{13}C_3$]genistein (Nigel Botting, University of St Andrews, UK) were used as internal standard compounds.

Western Blot analysis

Frozen mammary gland tissue specimen was powdered and homogenized in buffer (623.5 nM Tris pH 8 EDTA) containing enzyme inhibitors (5 mg/ml aprotonin, 5 mg/ml leupeptin, 1 mg/ml pepstatin-A, 5 mg/ml antipain, 100 mM pefac in 0.5 M EDTA pH 8). Protein concentrations were measured with the method of Lowry

(Lowry *et al.*, 1951) (D_c Protein Assay, Bio-Rad). Equal amounts of samples (40 µg protein) were loaded on 4–12 % Bis-Tris NUPAGE® Novex Gels (Invitrogen Life Technologies, Karlsruhe, Germany). For the electrophoresis the MES Buffer was used (Invitrogen Life Technologies, Karlsruhe, Germany). After electrophoresis the proteins were transferred onto nitrocellulose membranes and blocked with 5 % BSA in phosphate buffered saline solution at room temperature for 1 hour. The protein expressions of actin and PCNA were quantitative detected using specific antibodies (Anti Actin A 5060, Sigma-Aldrich, Taufkirchen, Germany; Anti PCNA M0879, Dako, Glostrup, Denmark). As species specific antibodies the Horseradish Peroxidase conjugated Polyclonal Rabbit Anti Mouse (Dako, Glostrup, Denmark) and Polyclonal Swine Anti Rabbit (Dako, Glostrup, Denmark) were used. The visualization of the blot signals was performed with the chemiluminescent POD-substrate and a Fluorchem Luminescent Imager. The densitometrical analysis was performed with the Image J program (ImageJ 1.33u, National Institute of Health, USA, http://rsb.info.nih.gov/ij/). Actin was used as reference protein and served as loading control.

Immunhistochemical analysis

The paraffin-embedded mammary glands were cut in 7 µm sections and were mounted on slides coated with polylysine (Menzel Gläser, Hilden, Germany). The mammary gland tissue was cleared, hydrated and antigen retrieval was performed using TrisEDTA. After overnight incubation in TrisEDTA at 60°C, the mammary gland tissues were washed four times with phosphate buffered saline (PBS). Then the tissue was incubated with a solution consisting of 0.5 M ammoniumchloride in 0.25 % Triton-X/PBS for 10 min. After four wash steps the unspecific binding sites were blocked with 5 % BSA for one hour. Then the mammary gland tissue sections were incubated with the first antibody (Anti PCNA M0879, Dako, Glostrup, Denmark; Anti PR 1408, Beckman Coulter, Marseille, France; Anti ERβ (H-150) sc-8974, Santa Cruz Biotechnology, Santa Cruz, USA) at 4°C overnight. After 4 washsteps the second antibody (Polyclonal Rabbit Anti Mouse biotinylated, Dako, Glostrup, Denmark; Polyclonal Goat Anti Rabbit biotinylated, Dako, Glostrup, Denmark) was incubated for one hour at room temperature. To visualize the binding of the PCNA antibody, the tissue sections were incubated with FluoroLink™ Cy™3 labelled streptavidin (PA 43001, Amersham Biosciences). To depict the percentage of proliferating nuclei, all nuclei were stained with DAPI (4´,6-Diamidino-2-

phenylindole, D 9542, Sigma-Aldrich, Taufkirchen, Germany). Per slide 300 nuclei were analysed for PCNA expression and the percentage of PCNA-positive nuclei was calculated. In order to visualize the binding of the PR and the ERβ antibody, the tissue sections were incubated with Streptavidin-Biotinylated Horseradish Peroxidase Complex (RPN1051, GE Healthcare, Buckinghamshire, UK) and stained with Diaminobenzidin (DAB). 300 nuclei were counted per slide, and the percentage of PR positive nuclei was calculated. Staining of ERβ was utilized by densitometrical analysis using the Image J program (ImageJ 1.33u, National Institute of Health, USA, http://rsb.info.nih.gov/ij/).

RNA preparation

Frozen mammary gland tissue specimen was powdered and homogenized in TRIzol®. Total RNA was isolated from cells using the TRIzol® (Invitrogen Life Technologies, Karlsruhe, Germany) standard protocol (Chomczynski & Sacchi, 1987) followed by cDNA synthesis with the Quantitect® Reverse Transcription Kit (Quiagen, Hilden, Germany).

Real-time RT-PCR

Quantitative Real-time RT-PCR was performed in the MxPRO (Stratagene) with Platinum® Taq DNA Polymerase (Invitrogen, Karlsruhe, Germany). SybrGreen I® was used as detection dye. The Cytochrome-C-oxidase subunit 1A (1A) was used as housekeeping gene, and the expression of all genes was normalized to 1A. Specific primers were designed with the primer3 software (Whitehead Institute for Biomedical Research, Cambridge, USA) based on the cDNA sequences available at the EMBL database: 1A: up: 5'-CGTCACAGCCCATGCATTCG-3 ', dw: 5'-CTGTTCATCCTGTTCCAGCTC-3 '; PR: up: 5'-CATGTCAGTGGACAGATGCT-3' ', dw. 5'-ACTTCAGACATCATTTCCGG-3 '; PS2: up: 5´-GGAAAGGGTTGCTGTTTTG-3´, dw: 5´-ACAGGTGTGTATGAAGCAGGTG-3´; ERα: up: 5´-GGAAGCACAAGCGTCAGAGAGAT-3´, dw: 5´-AGACCAGACCAATCATCAGGAT-3´. The PCR program consisted of a first denaturation step at 95°C for 4 min, followed by 45 cycles of 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. The fluorescence was quantified during the 72°C elongation step and the product formation was confirmed by melting curve analysis

(55–95°C). For calculation of relative rates of gene expression the $\Delta\Delta C_T$ method was used (Pfaffl, 2001). Gene expressions were compared to those of control animals fed with the ISO-free diet.

Statistical analysis

Statistical analysis was performed using the SPSS Statistical Analysis System, SAS, Version 12.0. All data are expressed as arithmetic means with their standard deviations. First a global Kruskal Wallis-H-Test was performed to analyse if there are significant differences between the groups. In case of differences, a Mann-Whitney U-Test was additionally performed to identify the groups with statistical significant variance. Statistical significance was established at $p < 0.05$.

Results

The experimental design of this study is depicted in Figure 1. The animals of this experiment received one of three different diets. The IDD contains no detectable amounts of ISO (< 10 µg/g). The IRD contains 248 µg GEN/g, 213 µg DAI/g and 59 µg GLY/g (each calculated as aglykone). ISO in the diet were mainly (> 90 %) present as the respective glucoside and malonyl-glucoside derivatives. A detailed analysis of the ISO derivatives in the diet is given in Table 2. The GRD based on an IDD, enriched with 700 µg GEN/g (Hertrampf *et al.*, 2009a).

ISO derivative	**MW** [g/mol]	**mean value** µg/g IRD	**mean value** µM/g IRD	**calculated as aglycone** µg/g IRD
Daidzein	254.24	8.83	0.035	8.83
Genistein	270.24	8.59	0.032	8.59
Glycitein	284.27	n.d.	-	-
Daidzin	416.38	191.96	0.461	117.21
Genistin	432.38	210.63	0.487	131.64
Glycitin	446.40	58.82	0.132	37.46
6''-*O*-Acetyl-Daidzin	458.41	48.21	0.105	26.73
6''-*O*-Acetyl-Genistin	474.41	51.18	0.108	29.17
6''-*O*-Acetyl-Glycitin	488.44	12.68	0.026	7.39
6''-*O*-Malonyl-Daidzin	502.42	119.25	0.237	60.32
6''-*O*-Malonyl-Genistin	518.42	150.04	0.289	78.19
6''-*O*-Malonyl-Glycitin	532.45	26.28	0.049	14.04

Sum of daidzein derivatives calculated as Daidzein aglycone in µg/g IRD 213.09

Sum of genistein derivatives calculated as genistein aglycone in µg/g IRD 247.59

Sum of glycitein derivatives calculated as glyciteinaglycone in µg/g IRD 58.89

Tab. 2: Content of ISO derivatives in the ISO-rich diet (IRD). (nd = not detected)

Serum concentrations of GEN and DAI were measured at PND 50 and PND 80 (Tab. 3). As expected, the serum concentrations of GEN and DAI significantly increased with the increasing ISO content in the diet. No differences were detected between PND 50 and PND 80.

		DAI (µM)	**GEN (µM)**
PND 50	IDD	n.d.	0.24 ± 0.01
	IRD	1.70 ± 0.67	1.09 ± 0.13*
	GRD	n.d.	2.55 ± 0.23**$^{++}$
PND 80	IDD	0.11 ± 0.24	0.26 ± 0.02
	IRD	2.15 ± 1,75	1.17 ± 0.31*
	GRD	0.21± 0.29	2.49 ± 0.44**$^{+}$

Tab. 3: Genistein and Daidzein concentration (in µM) in the blood serum of 50 (PND 50) and 80 (PND 80) days old rats. * = sign. vs. IDD ($p < 0.05$),** = sign. vs. IDD ($p < 0.01$), $^{+}$ = sign. vs IRD ($p < 0.05$), $^{++}$ = sign. vs. IRD ($p < 0.01$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

As depicted in Table 4, neither treatment nor nutrition had influenced the body weights or the heart weights. The wet weights of the uteri significantly increased after treatment with E_2 in all groups compared to their OVX groups. Additionally, IRD and GRD animals treated with E_2 showed a significant increase in the wet weights of the uteri compared to the IDD group. Treatment with GEN had no significant effect on uterine wet weights in any group.

		Body Weight (g)	**Uterus (mg/kg BW)**	**Heart (mg/kg BW)**
PND 50	IDD	156 ± 7	1058 ± 73	3971 ± 178
	IRD	159 ± 6	1687 ± 171	3943 ± 283
	GRD	170 ± 7	1395 ± 223	3912 ± 265
PND 80	IDD	251 ± 14	1110 ± 179	3347 ± 272
	IRD	227 ± 3	1254 ± 222	3199 ± 215
	GRD	249 ± 10	1237 ± 206	2970 ± 272
OVX	IDD	296 ± 7	469 ± 23	3370 ± 104
	IRD	299 ± 10	636 ± 39	3365 ± 122
	GRD	294 ± 11	553 ± 43	3273 ± 34
E_2	IDD	269 ± 13	1804 ± 124 **	3443 ± 112
	IRD	286 ± 10	5620 ± 750 **$^{++}$	3533 ± 57
	GRD	295 ± 6	5104 ± 372 **$^{++}$	3470 ± 68
GEN	IDD	264 ± 8	527 ± 44	3615 ± 124
	IRD	280 ± 10	712 ± 68	3475 ± 80
	GRD	320 ± 10	691 ± 71	3306 ± 117

Tab. 4: Body weights (g), uterus wet weights (mg/kg BW), heart weight (mg/kg BW). ** $p < 0.01$ vs. IDD OVX; $^{++}$ $p < 0.01$ vs. IDD E_2; (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

To evaluate the influence of ISO on proliferation of the mammary gland, the protein expression of the Proliferating Cell Nuclear Antigen (PCNA) was determined. Western Blot analysis of PCNA showed a significant increase in the GRD fed animals compared to the IDD and IRD group at PND 50, but no differences were detected in PCNA expression at PND 80 in intact animals (Fig. 2).

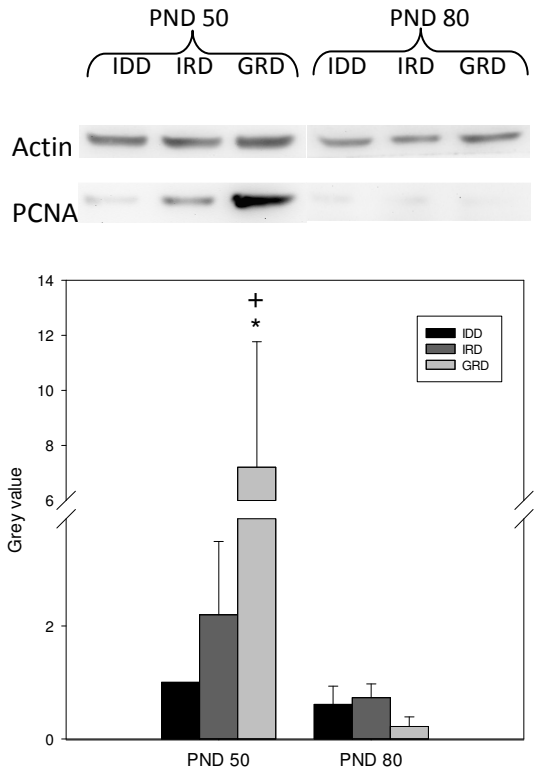

Fig. 2: Protein expression of PCNA in the mammary gland of intact 50 and 80 days old rats. Depicted is a representative Western Blot of PCNA in intact animals and the quantitative analysis of the Western blots. IDD PND 50 served as control and were set to one. The bars shown are mean +SD. Six rats were included to each group. * = sign. vs. IDD PND 50 ($p < 0.05$); $^{+}$ = sign. vs. IRD PND 50 ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

To investigate the influence of in utero and postnatal exposure to ISO on the sensitivity of the mammary gland towards estrogen exposure mammary gland tissue obtained from rats used in an uterotrophic assay was examined. Immunfluorescence analysis of the mammary gland of OVX animals showed no differences in the PCNA expression in the OVX animals of either diet, whereas treatment with E_2 led to an increased proliferation rate (Fig. 3 A). No differences could be seen in GEN treated groups.

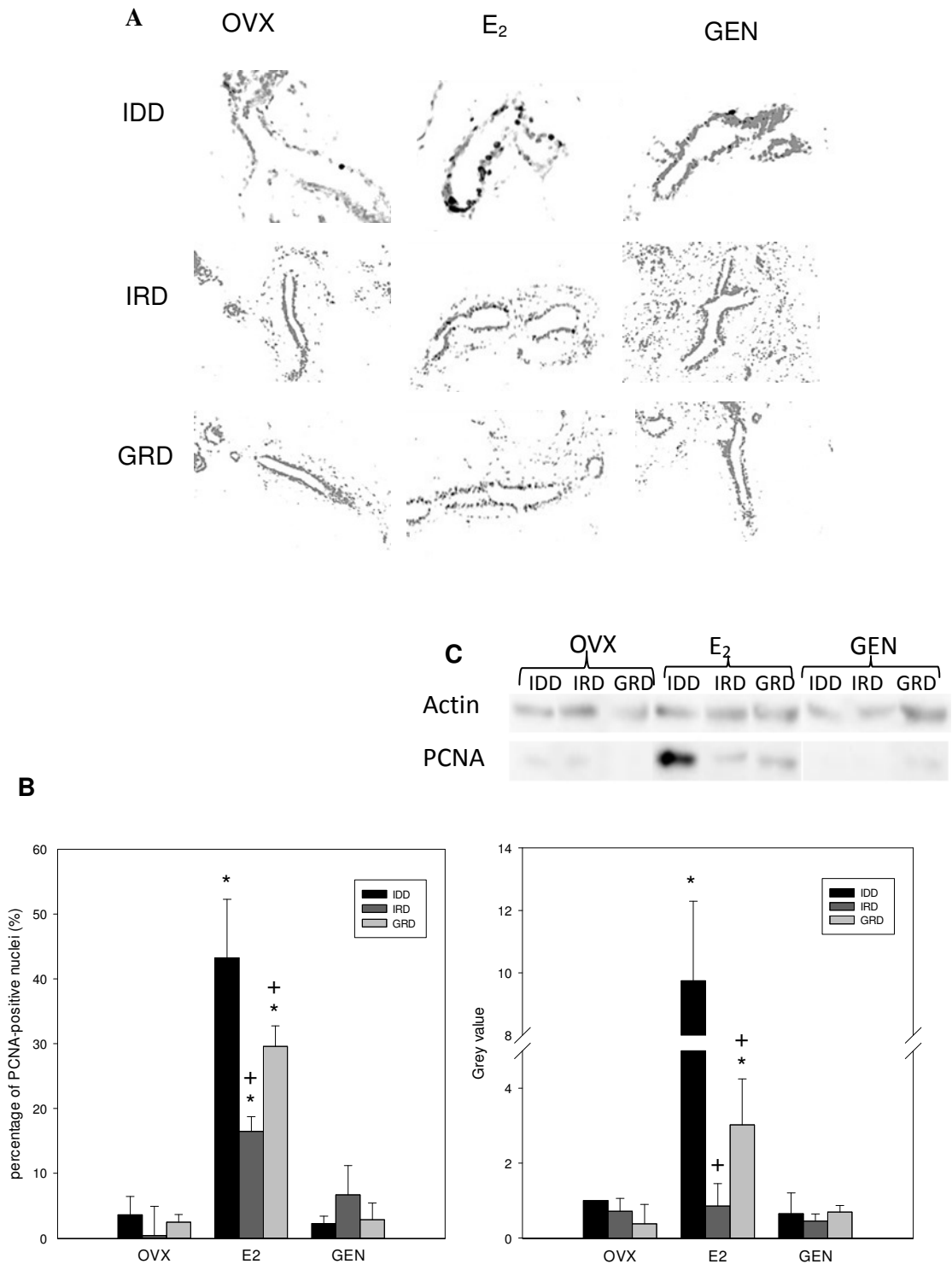

Fig. 3: Protein expression of PCNA in the mammary gland of 97 days old ovariectomized animals. **A** Representative pictures of Immunhistochemical stained sections of the mammary gland. Proliferating nuclei were stained with PCNA labelled with Cy3 (black). Stained grey with DAPI are all nuclei. **B** Quantitative analysis of PCNA staining. **C** Depicted is a representative Western Blot of PCNA in the mammary gland of OVX rats and the quantitative analysis of the Western blots. IDD OVX served as control and were set to one. The bars shown are mean +SD. Six rats were included to each group. * = sign. vs. IDD OVX ($p < 0.05$); $^{+}$ = sign. vs. IDD E_2 ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

Quantitative analysis showed a significant increase of PCNA expression in the E_2 treated groups compared to the OVX groups. Interestingly, after pre-exposure to IRD and GRD the E_2 stimulation of PCNA protein expression detectable in IDD was found to be significantly diminished (Fig. 3 B). The observation was verified with western blot analysis. As in the immunfluorescence analysis, no differences in OVX and GEN treated animals were detected, but a significant increase in PCNA expression in IDD fed animals treated with E_2 (Fig. 3 C). The IRD showed no increase in PCNA expression after E_2 treatment, while in the GRD group an increase in PCNA expression was detected.

A well established marker for estrogen action in the mammary gland is the progesterone receptor (PR) bearing an estrogen response element (ERE) in its promoter (Kraus *et al.*, 1993). Immunhistochemical analysis of PR expression in the mammary gland revealed only a slight expression in OVX and GEN treated groups. E_2 treatment results in a significant increase of PR staining in all groups (Fig. 4 A). Quantitative analysis of the immunhistochemical staining indicated that the increase was significant higher in the animals which received in utero and postnatal an IDD (Fig. 4 B). This observation was confirmed by semi-quantitative analysis of PR mRNA expression (Fig. 4 C). None of the GEN-treated groups showed differences in the mRNA expression of PR compared to the OVX groups.

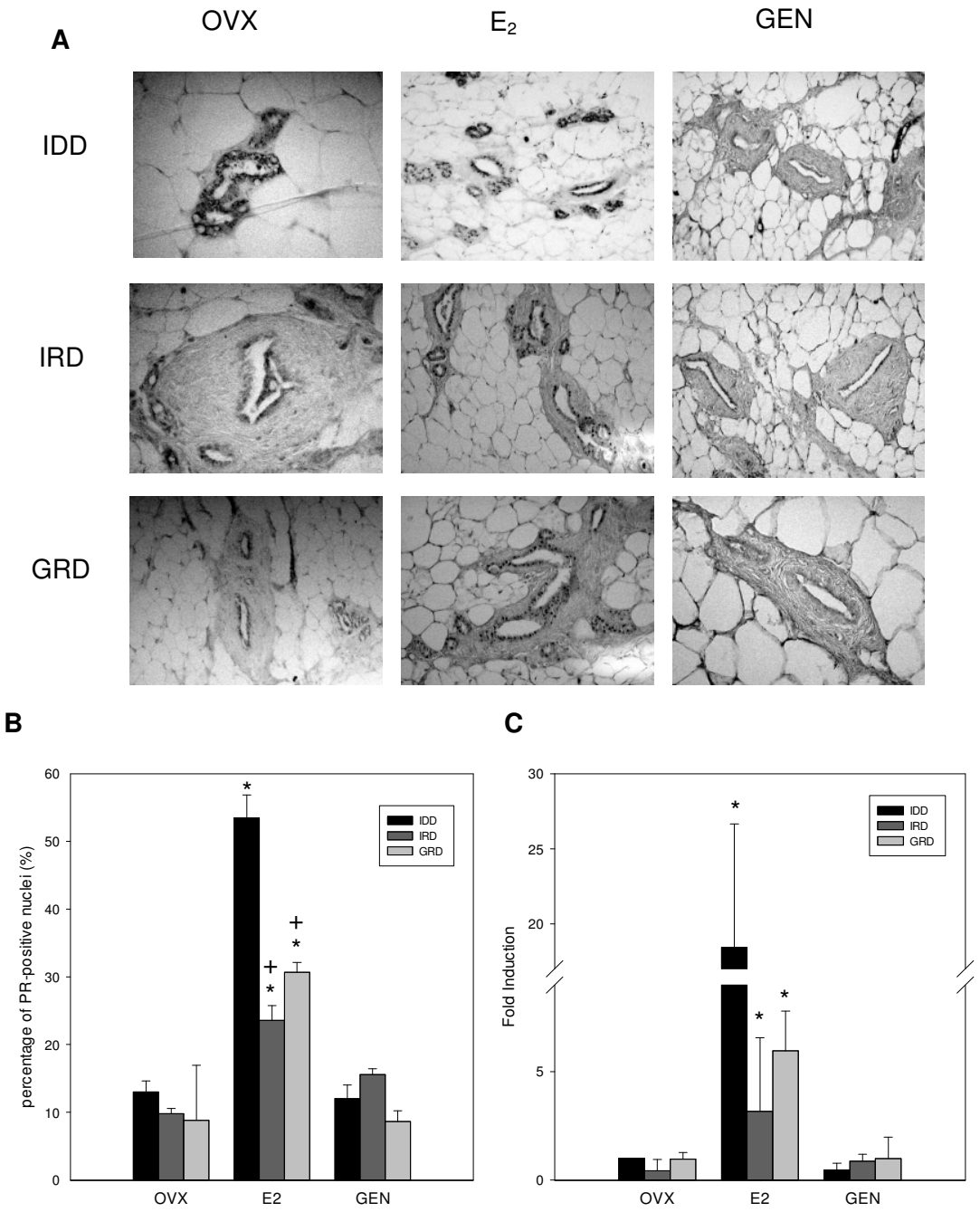

Fig. 4: Expression of the progesterone receptor (PR) in the mammary gland of 97 days old ovariectomized rats. **A** Protein expression of the PR. Depicted are representative pictures of immunhistochemical stained sections of the mammary gland. **B** Quantitative analysis of PR staining. **C** mRNA expression of the PR in the mammary gland. IDD OVX served as control and were set to one. The bars shown are mean +SD. Six rats were included to each group. * = sign. vs IDD OVX ($p < 0.05$); + = sign. vs. IDD E_2 ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

Beside PR, the expression of the estrogen receptor α (ERα) and the PS2 gene was measured in the mammary gland using real-time RT-PCR. For ERα it has been demonstrated that mRNA and protein expression show a good regulation in this tissue (Schams *et al.*, 2003). As shown in Figure 5 A, in utero and postnatal exposure to an IRD showed only a slight increase of ERα mRNA expression in OVX animals, while treatment with E_2 resulted in a significant increase in ERα expression in the animals fed in utero and postnatal an IDD compared to those fed an IRD or GRD. PS2 expression (Fig. 5 B) was increased in OVX IRD and GRD animals compared to OVX IDD. E_2 treatment resulted in a significant decrease of PS2 expression in the IDD group but in a strong stimulation of PS2 expression in the IRD and GRD group. In response to GEN treatment, no significant differences could be measured.

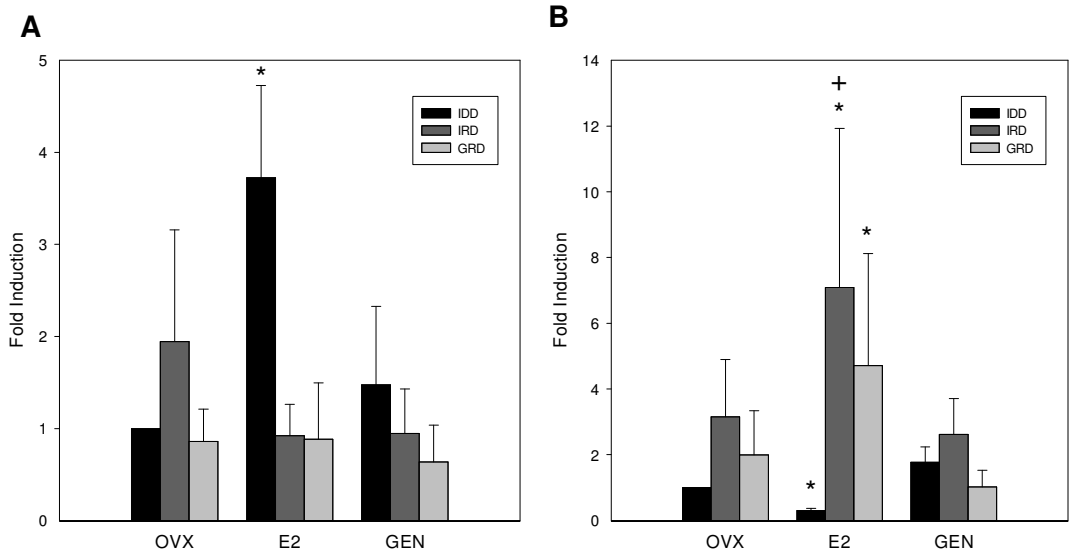

Fig. 5: mRNA expression of the estrogen receptor α (ERα) (**A**) and PS2 (**B**) in the mammary gland of 97 days old ovariectomized rats. IDD OVX served as control and were set to one. The bars shown are mean +SD. Six rats were included to each group. * = sign. vs. IDD OVX ($p < 0.05$); $^{+}$ = sign. vs. IDD E_2 ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

To reveal differences in the expression patterns of the estrogen receptors, the protein expression of estrogen receptor β (ERβ) were analysed by immunhistochemistry (Fig. 6). ERβ staining of the mammary gland showed only marginal differences of its expression in the OVX groups (Fig. 6 A). After treatment with E_2, an increase of expression could be detected in IRD and GRD animals but not in animals receiving an IDD. This observation was confirmed by densitometrical

analysis (Fig. 6 B). The highest expression of ERβ was measured after treatment of GRD animals with GEN.

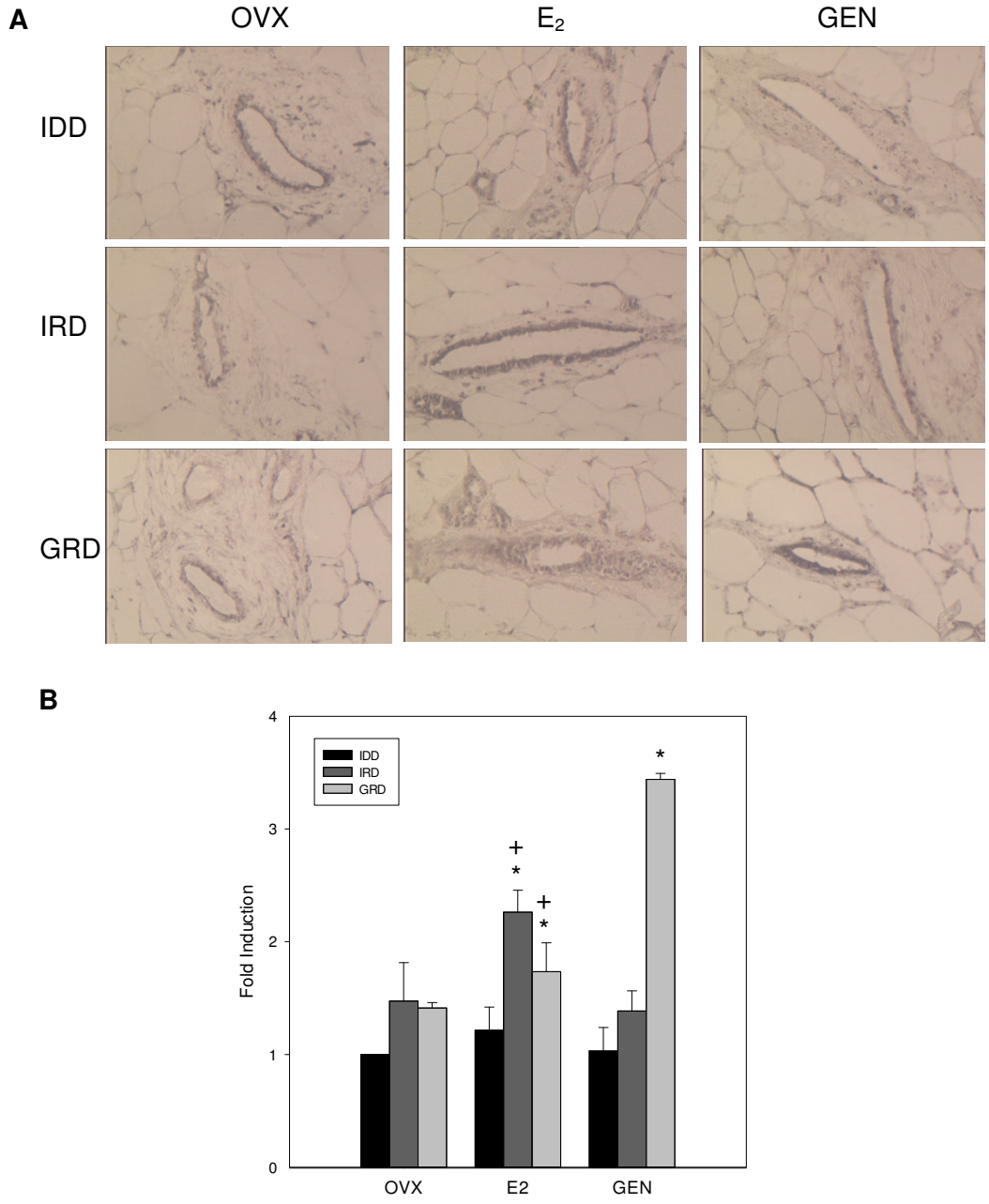

Fig. 6: Expression of the estrogen receptor β (ERβ) in the mammary gland of 97 days old ovariecotmized rats. **A** Protein expression of the ERβ. Depicted are representative pictures of immunhistochemical stained sections of the mammary gland. **B** Densitometric analysis of ERβ staining. IDD OVX served as control and were set to one. The bars shown are mean +SD. Six rats were included to each group. * = sign. vs. IDD OVX ($p < 0.05$); $^{+}$ = sign. vs. IDD E_2 ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

Discussion:

Epidemiological studies suggest that soy consumption is associated with a lower risk of developing breast cancer in the Asian population. It is hypothesized that the exposure to ISO has to start before adolescence sets in to exert protective effects. Studies investigating the effects of in utero and postnatal exposure to ISO are still inconsistent (Warri *et al.*, 2008). The present study was performed to analyse the impact of in utero and postnatal exposure to ISO on the mammary gland. In order to make sure that the offspring was exposed to ISO already in utero the dams received one of three different diets, respectively. The IRD was chosen because of its high ISO content, as the aim of the study was to investigate estrogenic effects of an ISO-rich diet on the mammary gland. The diets differ in protein and fat content, but in order that the IRD exert similar effects as the GRD, we can be sure that these differences did not influence the estrogenic response of the mammary gland (Tab. 1). The long-term exposure to IRD and GRD consequently resulted in increased serum concentrations of GEN (Tab. 3). In case of the IRD increased concentrations of DAI and its colonic microbiota-derived metabolite equol have additionally to be taken into account. Unfortunately we have not determined the serum equol concentration in our study, but its remarkable formation in rodents is well described. For instance, Poulsen et al. found almost equally high DAI and equol plasma concentrations in ovariectomized rats fed a diet supplemented with DAI (Poulsen *et al.*, 2009).

As we observed a strong influence of ISO and GEN exposure on mammary gland biology the question arises about a potential developmental origin of these effects. Previous studies suggested that the placental transfer of ISO is high, whereas the lactational transfer is low to negligible (Doerge, 2011; Doerge *et al.*, 2006). On the other hand, even if the lactational transfer seems to be low, quite significant alterations of the proteome can be achieved in mammary glands of prepubertal animals which were feed by lactating dams which were exposed to a diet containing 250 ppm of GEN (Wang *et al.*, 1996). Applying these findings to our study it has to be assumed that in utero exposure most likely contributes to the effects observed, however, a contribution to the effects through lactational exposure cannot be excluded.

As shown in Table 4 and as reported before (Hertrampf *et al.*, 2009a), no differences in the uterine wet weights could be detected in 50 and 80 days old intact animals, indicating a low estrogenic potential of the different diets.

In the first part of the present study the mammary gland was analysed at postnatal day (PND) 50 and at PND 80. As shown in Figure 2 no differences could be observed between the different dietary groups of 80 days old adult intact animals, whereas significant differences were observed at day 50 (Fig. 2), where PCNA expression was strongly stimulated in the GRD group. A recent review on xenobiotic exposure and breast cancer risk in animal models came to the conclusion that acceleration or delay of glandular maturation appears to be a crucial point regarding whether exposure to a xenobiotic leads to prevention or increases the risk to develop breast cancer (Jenkins *et al.*, 2012). A possible explanation for our effect may indeed be an acceleration of the differentiation program of the mammary gland in response to GEN treatment which has already been shown by other groups (Warri *et al.*, 2008). In fact, in a recent neonatal exposure study a transient, statistically not significant increase in Ki-67 immunoreactivity, occurring earlier than the increase in PCNA immunoreactivity in our study, was reported in response to GEN which decreased statistically significant later in life (Wang *et al.*, 1996).

To study if in utero and postnatal exposure to IRD or GRD alters the estrogen responsiveness of the mammary gland, in OVX animals serving as a model system of the situation in postmenopausal woman, an uterotrophic assay was performed. As shown in Table 4, E_2 exposure always resulted in an increase of uterine wet weights, but the uterine weights of IRD and GRD were significantly higher than in IDD animals. These data indicate that the estrogen responsiveness of the uterus was highly modulated by long-term ISO exposure. Interestingly, no influence on uterine proliferation could be observed between the dietary groups after E_2 treatment, whereas the water homeostasis was highly affected. Möller et al. speculated that the methylation pattern of genes associated with transcellular water transport in the uterus was changed after in utero and postnatal ISO exposure (Moller *et al.*, 2010).

In the mammary gland of OVX IRD, IDD and GRD, similar to intact 80 days old animals, there were no significant differences in PCNA expression. In order to stimulate cell-proliferation in mammary gland tissue, animals were treated with E_2 for 3 days subcutaneously (s. c). In line with published data (Hertrampf *et al.*, 2006; Rimoldi *et al.*, 2007; Rachon *et al.*, 2008) the protein expression of PCNA in the mammary gland increased significantly in response to the E_2 treatment in all dietary groups. In contrast, exposure to GEN had no effect in treatment groups. This confirms a previous study from our laboratory (Hertrampf *et al.*, 2006), where neither s. c. nor oral treatment with GEN affected the PCNA expression and only slight

effects on the PR expression in the mammary gland of adult OVX Wistar rats could be observed.

A key result of the study presented here is the observation that the E_2 induced stimulation of mammary gland PCNA expression was significantly lower following dietary exposure of animals in IRD and GRD groups if compared to IDD fed animals. This strengthens the hypothesis that pretreatment with ISO accounts for a lower sensitivity of mammary gland tissue towards estrogen treatment.

A possible explanation for this altered sensitivity may be the anatomy of the mammary gland. Several studies have investigated the effects of in utero, perinatal and prepubertal exposure towards ISO on the morphology of the mammary gland (Warri *et al.*, 2008). Whereas results obtained for in utero, perinatal and prenatal exposure scenarios are not consistent, it has been shown that pre-pubertal exposure to GEN increases mammary tissue differentiation by leading to a reduction in the number of terminal end buds (TEB) and an increase in the number of differentiated lobules (Hilakivi-Clarke *et al.*, 1999; Cotroneo *et al.*, 2002; Lamartiniere *et al.*, 2002; Cabanes *et al.*, 2004). It is possible that the lack of increased proliferation after treatment with E_2 in the IRD fed group is a result of a reduced number of TEB in the mammary gland.

Taken further, the present 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. 4) and ERα (Fig. 5 A) are very similar to those of the PCNA expression. Both receptors have been implicated in the etiology and the pathogenesis of breast cancer. In the normal mammary gland, progesterone is needed for lobulo-alveolar development and ductal branching, whilst estradiol regulates ductal elongation. Russo et al. (Russo *et al.*, 1999) reported that the content of ERα and PR in the lobular structures in the breast is directly proportional to the rate of cell proliferation. In normal resting mammary glands, the percentage of ERα-positive cells is generally low, and increases in proliferative benign disease, particularly when associated with atypia (Roger *et al.*, 2000). Additionally, the dual expression of the ER and the proliferating marker Ki-67 seemed to be the manifestation of an important early molecular change in the development of malignant breast neoplasia (Shoker *et al.*, 1999). Furthermore, an important function of the ERα in the epithelium is the induction of PR (Saji *et al.*, 2000), and it is known that co-expression of both is inversely associated with breast cancer risk among postmenopausal women (Lagiou *et al.*, 2009).

Remarkable is the observation regarding the regulation of ERβ by E_2 in the different nutritional groups. It is hypothesized that one function of ERβ is to counter that of ERα (Saji *et al.*, 2005). In vitro studies showed a reduction in estrogen stimulated proliferation after introduction of the ERβ expression vector into representative ERα-positive breast cancer cell lines MCF-7 and T47D (Omoto *et al.*, 2003; Strom *et al.*, 2004). In line with these observations is our finding that the reduced sensitivity of the mammary gland to respond to E_2 treatment by proliferation in GRD and IRD fed animals was associated with an elevated expression of ERβ (Fig. 6) and a decreased expression of ERα (Fig. 5 A). Also notable is the fact that the expression pattern of the PS2 gene is similar to that of ERβ (Fig. 5 B). There are speculations that high expression levels of PS2 may be protective against the development of breast cancer. Transgenic mice, which overexpress PS2 in their mammary gland do not develop tumors (Tomasetto *et al.*, 1989). Our observation that PS2 expression can be induced by ISO is in line with results of Hargreaves et al. (Hargreaves *et al.*, 1999) showing that treatment of premenopausal women with dietary soy supplements for two weeks results in an increased expression of PS2 in the breast. Interestingly, the control of the PS2 gene also depends upon epigenetic factors (Chatagnon *et al.*, 2010; Ribieras *et al.*, 1998) as the tissue-specific methylation of its proximal promoter/enhancer region correlates directly with its expression (Martin *et al.*, 1995; Martin *et al.*, 1997). The increased mRNA expression of PS2 in the IRD fed group could lead to the suggestion that ISO could exert their protective effects via epigenetic modulation. Indeed, recently a variety of studies have shown epigenetic changes induced by ISO (Guerrero-Bosagna *et al.*, 2008; Li *et al.*, 2009; Molinie & Georgel, 2009) having positive effects on breast or prostate cancer.

In summary, our results provide evidence that in utero and postnatal exposure to a diet rich in soy ISO in their natural chemical form as β-glucoside conjugates as well as to a diet solely enriched with GEN in the aglycone form may alter the gene expression of the mammary gland, which consequently results in a changed sensitivity of this tissue towards estrogens. This observation leads to the hypothesis, that in utero and postnatal ISO exposure may reduce the risk to develop breast cancer in a protective manner. To clarify the underlying mechanisms, i.e. if the altered susceptibility of the mammary gland is a result of epigenetic mechanisms during the development further studies are needed.

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CHAPTER THREE

Lifelong exposure to isoflavones influences the gene expression in the small intestine of female rats*

**submitted to Eur J Nutr*

Abstract

Nutritive uptake of isoflavones (ISO) is discussed as one factor responsible for the low incidence of gastrointestinal cancer in Eastern Asia. Our aim was to investigate the effects of lifelong ISO exposure on the tissue homeostasis and estrogen sensitivity in the small intestine of female rats.

Animals were exposed to either an ISO-free diet (IDD), ISO-rich diet (IRD) or an IDD supplemented solely with genistein (GRD). After sacrifice at postnatal days 25 (PND 25), 50 (PND 50) and 80 (PND 80), the expression of molecular markers for proliferation (PCNA), intestinal tumor protection (p63, PS2) and estrogen receptor α and β (ERα, ERβ) was analysed in the small intestine. Furthermore, IDD, IRD and GRD rats were ovariectomized (PND 80) and treated either with vehicle (OVX) or estradiol (E_2) for 3 days.

Lifelong exposure to IRD or GRD results in increased expressions of PCNA and PS2 at PND 50 and 80. In ovariectomized animals p63 expression was lower in IRD and GRD, whereas E_2 treatment resulted in a decrease of p63 in all groups. IRD and GRD OVX had lower ERα but higher ERβ expression. The expression of PS2 was increased in GRD OVX, but decreased in all dietary groups after treatment with E_2.

In summary our results provide evidence that lifelong exposure to ISO improves intestine homeostasis, and modulate the expression of molecular markers related to tumor protection and the responsiveness of this tissue towards E_2. Consequently, the susceptibility of this tissue to develop cancer is affected.

Introduction

The incidence of gastrointestinal cancer is much lower in Eastern Asia compared to Europe or USA (Adlercreutz & Mazur, 1997; Martinez, 2005). Apart from other factors epidemiological and migration studies suggests that the soy rich diet containing high amounts (20–50 mg isoflavones per day) of the isoflavones (ISO) genistein (GEN) and daidzein (DAI) reduces the cancer susceptibility in these countries (Budhathoki *et al.*, 2011; Adlercreutz, 1990; Lechner *et al.*, 2005). ISO are naturally occurring soy compounds, and are able to bind to the estrogen receptor (ER) and initiate estrogenic action.

Via binding to the ER estrogens are able to mediate the tissue homeostasis in the intestine, which is usually characterized by a steady state of proliferation and apoptosis (Lechner *et al.*, 2005; Wada-Hiraike *et al.*, 2006; Weige *et al.*, 2009). A shift in this homeostasis is an initial event in tumor progression (Koornstra *et al.*, 2003). That estrogens have an impact on bowel cancer has been described in 1996. Di Domenico showed that the growth of the colon cancer cell line Caco-2 increased after treatment with Estradiol (E_2) (Di Domenico *et al.*, 1996). It has been shown that long-term supplementation with synthetic E_2 lowers the risk of developing colorectal cancer (Nelson *et al.*, 2002; Chlebowski *et al.*, 2004). Many studies regarding the health effects of hormone replacement therapy (HRT) showed protective effects of estrogen treatment towards the intestine (Nelson *et al.*, 2002; Wada-Hiraike *et al.*, 2006).

The predominantly ER in the intestine is the ERβ (Kuiper *et al.*, 1997; Campbell-Thompson *et al.*, 2001; Konstantinopoulos *et al.*, 2003). Previous studies conducted with ERβ-selective ligands or ERβ-Knockout mice showed that activation of the ERβ is involved in cellular anti-inflammatory pathways and tissue homeostasis in the colon (Harris *et al.*, 2003; Wada-Hiraike *et al.*, 2006; Weige *et al.*, 2009). The importance of these effects of the ERβ additional becomes apparent by the observation that its expression is significantly reduced in colon cancer cell lines (Konstantinopoulos *et al.*, 2003; Jassam *et al.*, 2005; Martineti *et al.*, 2005). In this context ERβ-selective compounds seem to be useful in treating disease in the intestinal tract via activating the ERβ.

Due to the specifically high binding affinity of ISO to the ERβ, GEN may serve as a potential regulator of intestinal tissue homeostasis. The action of ISO depends on several factors. Numerous studies have led to the suggestion that the time of ISO exposure is important for their protective effects (Cabanes *et al.*, 2004; Padilla-

Banks *et al*., 2006). Additionally, the action of ISO differs dependent on the estrogen status. The group of Hwang showed that in the presence of premenopausal level of E_2 ISO act as estrogen antagonists and thus inhibits estrogenic action, whereas at lower E_2 doses which occur in postmenopausal women, ISO act as estrogen agonists (Hwang *et al*., 2006).

While many findings on these effects are available, the data regarding lifelong effects of ISO on homeostasis and estrogen sensitivity of the intestine are still limited. The purpose of this study was to evaluate the influence of lifelong ISO or GEN exposure on tissue homeostasis of the female intestine. Additionally, we aimed to analyse whether ISO influences the estrogen responsiveness of this tissue. As molecular read out the mRNA expression of markers for proliferation (PCNA), tumor protection (p63 and PS2) and of ERα and ERβ was determined in the small intestine.

Materials and Methods:

Experimental animals and study design

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.

Wistar rats were obtained from Janvier (Le Genest St Isle, France) and kept under controlled conditions of temperature (20℃ ± 1), relative humidity (50–80 %) and illumination (12 h dark, 12 h light). Female rats were mated and the dams were fed one of three diets during pregnancy and nursing. After weaning the female offspring (n = 30 each dietary group) were fed the appropriate diet. The animals had ad libitum access to the diets and water. Each dietary group was further divided into five subgroups (n = 6 each group). The first subgroup was sacrificed on postnatal day (PND) 25 (childhood), the second on PND 50 (puberty), and the third on PND 80 (adult). The other two subgroups were ovariectomized at day 80. An uterotrophic assay was performed at day 94: the animals received either 17β-estradiol (E_2; 7.8 µg/kg BW/day in 20 % DMSO/peanut oil) or purely the vehicle (OVX) subcutaneously for three days and were then sacrificed.

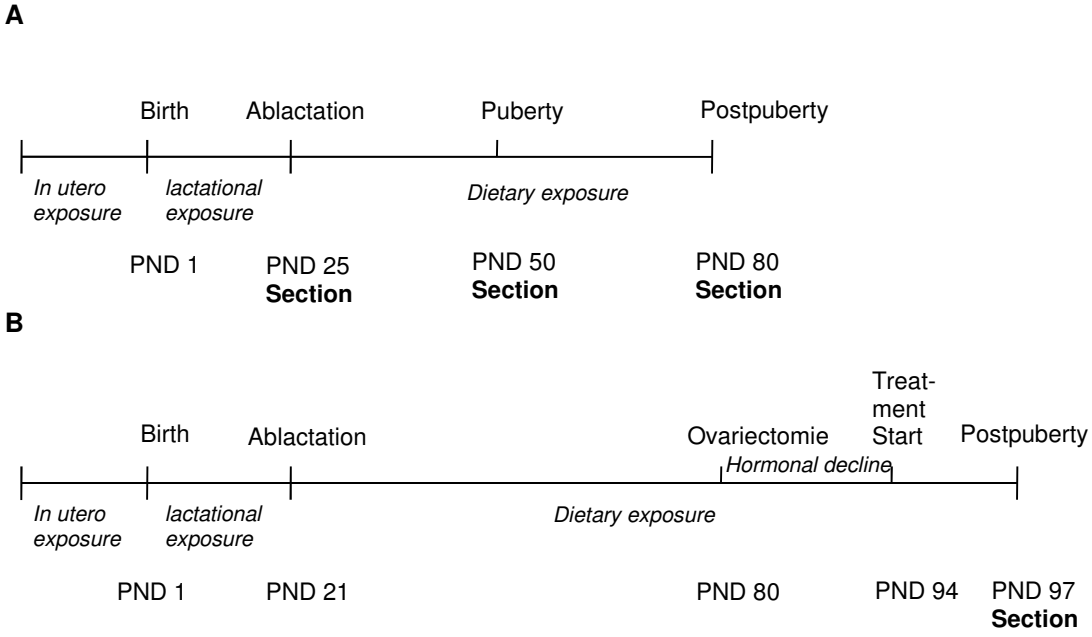

Fig. 7: Timeline and experimental setting of the study depicting modes of isoflavone (ISO) exposure (in utero, lactational, and dietary). **A** Intact animals received an ISO-free diet (IDD), an ISO-rich diet (IRD) or an IDD supplemented with Genistein (GRD). The small intestine was analysed at postnatal day

(PND) 25, 50, and 80. **B** Animals received an IDD, IRD, or GRD were ovariectomized on PND 80 and were treated with E_2, or the vehicle from PND 94 up to PND 97.

Diets

The rats had free access to one of three diets: an ISO-free diet (IDD; SSniff R/M-H, Ssniff GmbH, Soest, Germany), an ISO-rich diet (IRD; Harlan Teklad 8604 rodent diet, Harlan Winkelmann, Borchen, Germany), or an IDD supplemented with 700 µg GEN (GRD; 4´,5,7-trihydroxyisoflavone, LC Laboratories, Woburn, USA) per g diet. The IRD contains 248 µg GEN/g, 213 µg DAI/g and 59 µg glycitein/g diet (each calculated as aglykone).

Tissue preparation

Body weights of animals were determined. Afterwards, animals were decapitated and blood was collected. The small intestine and the uterus were removed and the uterus was weighted. Specimen of each was either snap frozen in liquid nitrogen for mRNA and protein preparation.

RNA preparation

Frozen tissue specimen of the intestine was powdered and homogenized in TRIzol®. Total RNA was isolated from cells using the TRIzol® (Invitrogen Life Technologies, Karlsruhe, Germany) standard protocol (Chomczynski & Sacchi, 1987) followed by cDNA synthesis with the Quantitect® Reverse Transcription Kit (Quiagen, Hilden, Germany).

Real-time RT PCR

To determine the mRNA expression of ERα, ERβ, p63, PCNA and PS2 quantitative real-time RT-PCR was performed in the MxPRO (Stratagene) with Platinum® Taq DNA Polymerase (Invitrogen, Karlsruhe, Germany). SybrGreen I® was used as detection dye. As housekeeping gene served the Cytochrome-C-oxidase subunit 1A (1A), and the expression of all genes was normalized to 1A. Specific primers were

designed with the primer3 software (Whitehead Institute for Biomedical Research, Cambridge, USA) based on the cDNA sequences available at the EMBL database: 1A: up: 5'-CGTCACAGCCCATGCATTCG-3 ', dw: 5'-CTGTTCATCCTGTTCCAGCTC-3 '; PS2: up: 5´-GGAAAGGGTTGCTGTTTTG-3´, dw: 5´-ACAGGTGTGTATGAAGCAGGTG-3´; ERα: up: 5´-GGAAGCACAAGCGTCAGAGAGAT-3´, dw: 5´-AGACCAGACCAATCATCAGGAT-3´; ERβ: up: 5´-CTACAGAGAGATGGTCAAAAGTGGA-3´, dw: 5´-GGGCAAGGAGACAGA AAGTAAGT-3´; P63: up: 5'-ATCGTTACTCTGGAAACCAG-3', dw: 5'- CATGTGAGTGCCCATCATAG-3'. The PCR program consisted of a first denaturation step at 95°C for 4 min, followed by 45 cycles of 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. The fluorescence was quantified during the 72°C elongation step and the product formation was confirmed by melting curve analysis (55–95°C). For calculation of relative rates of gene expression the $\Delta\Delta C_T$ method was used (Pfaffl, 2001). Gene expressions were compared to those of control animals fed with the ISO-free diet.

Statistical analysis

Statistical analysis was performed using the SPSS Statistical Analysis System, SAS, Version 12.0. All data are expressed as arithmetic means with standard deviations. First a global Kruskal-Wallis-H-Test was performed to analyse if there are significant differences between the groups. In case of differences, additionally a Mann-Whitney-U-Test was performed. At PND 25, 50, and 80 the nutrition groups were compared to each other, and E_2 were compared to OVX. Statistical significance was established at $p < 0.05$.

Results

The experimental setting of the study is depicted in Figure 7. ISO Serum concentrations were measured as reported before (Molzberger *et al.,* 2012). Consumption of an IDD revealed no detectable serum concentrations of ISO, whereas an IRD resulted in 1.1 µM GEN and 2 µM DAI. After GRD 2.5 µM GEN was measured in the serum, whereas no DAI was detectable.

To investigate the effects of the diets on estrogen mediated signaling pathways, we determined the ERα and ERβ mRNA expression at PND 25, 50 and 80. No significant differences in the gene expression of the ERα (Fig. 8 A) could be observed.

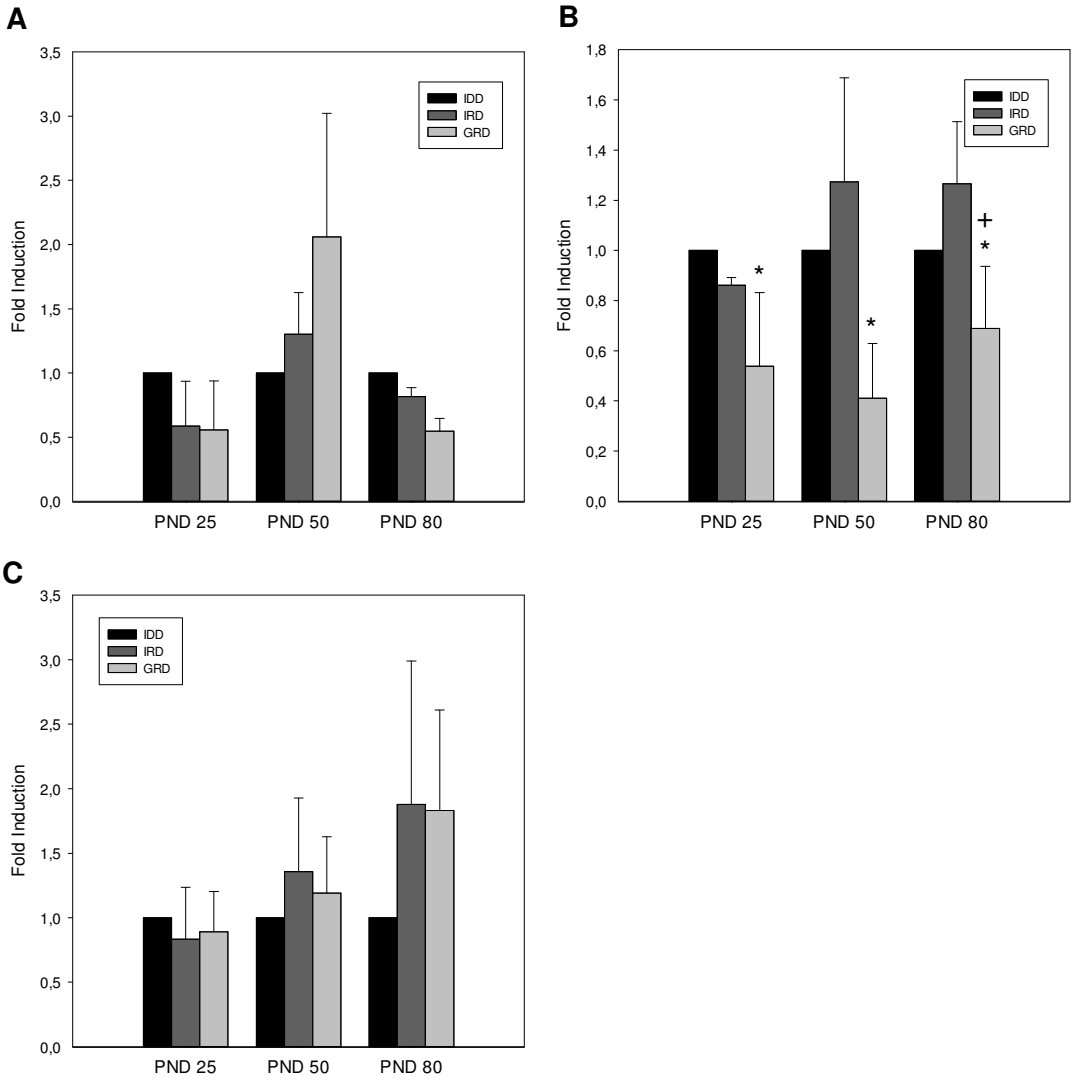

Fig. 8: mRNA Expression of ERα (**A**), ERβ (**B**), and p63 (**C**) in the small intestine of 25, 50, or 80 days old female wistar rats. IDD served as control and were set to one. The bars shown are mean +SD. Six rats were included to each group. * = sign. vs. IDD ($p < 0.05$); $^{+}$ = sign. vs. IRD ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

The mRNA expression of ERβ (Fig. 8 B) was significantly decreased in GRD animals compared with all ages. As an additionally molecular marker we also studied the gene expression of the oncogene transformation related protein p63 in the small intestine, which were not affected by the diets (Fig. 8 C).

To evaluate the influence of lifelong exposure to ISO on tissue homeostasis of the small intestine, the gene expression of PCNA and PS2 were determined in 25, 50 and 80 days old intact female rats (Fig. 9). The mRNA expression of PCNA was significantly down-regulated in IRD and GRD animals at PND 25 but significantly increased at PND 50 and 80 (Fig. 9 A). The expression of PS2, a gene believed to be involved in mucosal repair, was significantly increased at day 25 in the IRD but not in the IDD and GRD group (Fig. 9 B). At PND 50 and 80, the PS2 gene expression was significant higher in IRD and GRD animals compared to IDD.

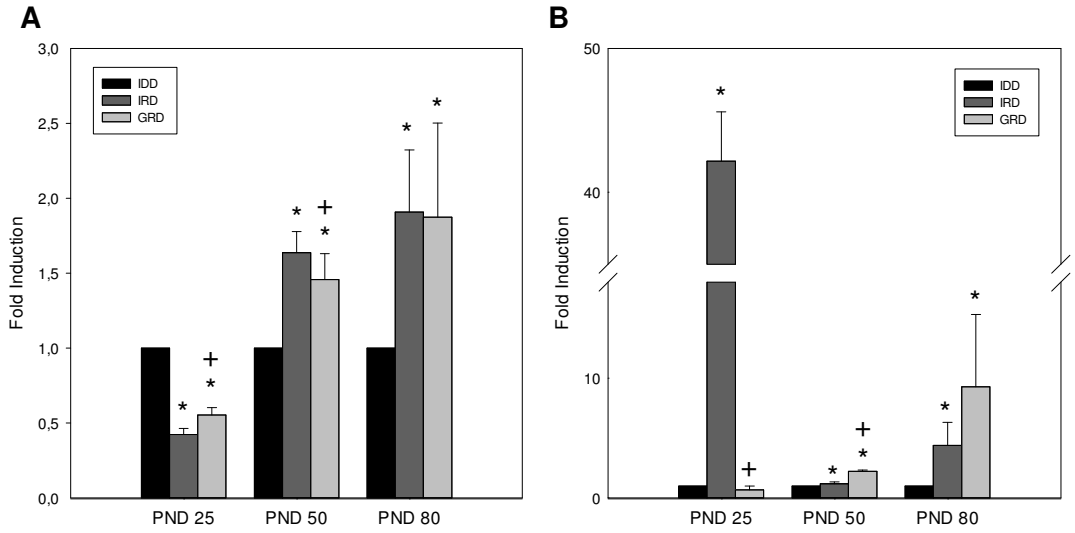

Fig. 9: mRNA Expression of PCNA (**A**) and PS2 (**B**) in the small intestine of 25, 50, or 80 days old female wistar rats. IDD served as control and were set to one. The bars shown are mean +SD. Six rats were included to each group. * = sign. vs. IDD ($p < 0.05$); $^{+}$ = sign. vs. IRD ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

After ovariectomie, we investigated the effect of a lifelong exposure to different diets on the E_2 sensitivity of the small intestine. As molecular markers, the gene expression of ERα, ERβ, p63, PCNA and PS2 were determined. The ERα mRNA expression (Fig. 10 A) was significantly reduced in OVX IRD and GRD compared to IDD animals. Treatment with E_2 did not affect ERα mRNA expression in the IDD

group, whereas in the IRD and GRD group the mRNA expression of ERα and ERβ was reduced (Fig. 10 B). Additionally, the mRNA expression of p63 was reduced in OVX animals of the IRD and GRD group compared to IDD animals. After treatment with E_2, a significant decrease could be measured in all dietary groups (Fig. 10 C).

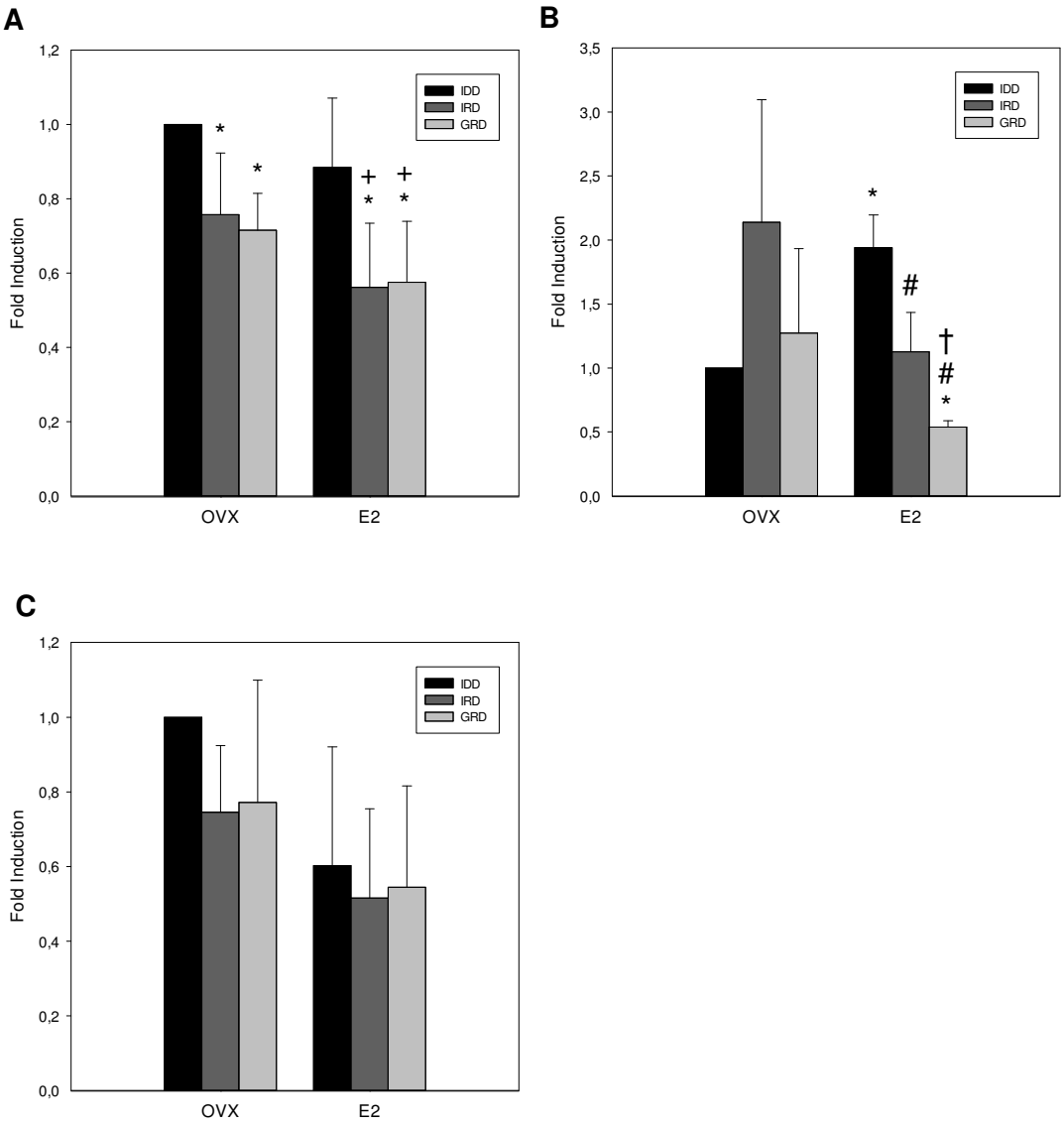

Fig. 10: mRNA Expression of ERα (**A**), ERβ (**B**), and p63 (**C**) in the small intestine of ovariectomized wistar rats after treatment with E_2 or the vehicle (OVX). IDD OVX served as control and were set to one. The bars shown are mean +SD. Six rats were included to each group. * = sign. vs. IDD OVX ($p < 0.05$); $^{+}$ = sign. vs. IRD OVX ($p < 0.05$); $^{\#}$ = sign. vs. IDD E_2 ($p < 0.05$); † = sign. vs. IRD E_2 ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

No differences in the PCNA expression in the small intestine could be observed between OVX animals of the respective nutrition groups, and also treatment with E_2 had no effect (Fig. 11 A). The PS2 expression (Fig. 11 B) was significantly decreased in OVX IRD animals but significantly increased in OVX GRD animals compared to the IDD group. Treatment with E_2 resulted in a significant down-regulation of PS2 in all dietary groups, with the lowest expression in IRD animals.

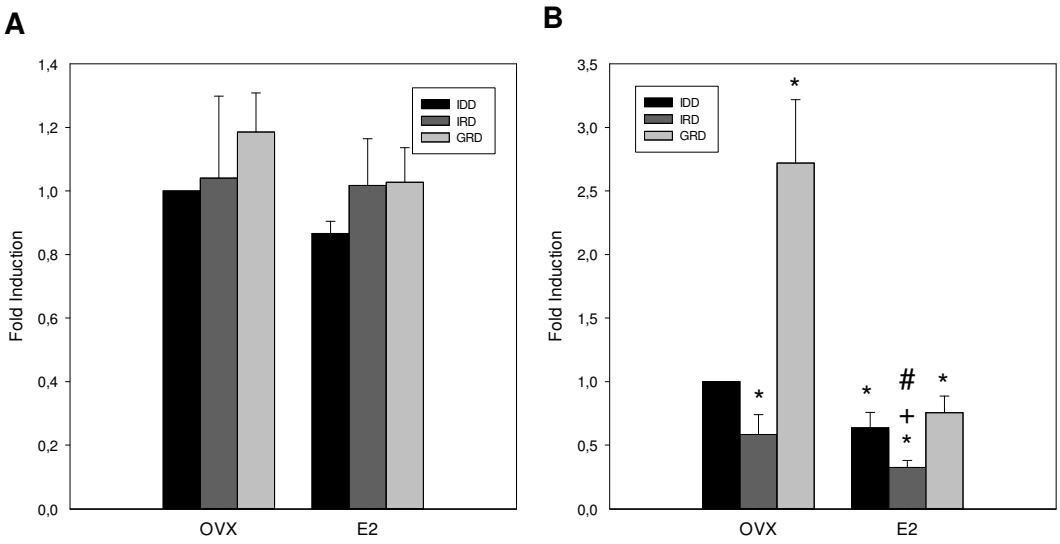

Fig. 11: mRNA Expression of PCNA (**A**) and PS2 (**B**) in the small intestine of ovariectomized wistar rats after treatment with E_2 or the vehicle (OVX). IDD OVX served as control and were set to one. The bars shown are mean +SD. Six rats were included to each group. * = sign. vs. IDD OVX ($p < 0.05$); $^{+}$ = sign. vs. IRD OVX ($p < 0.05$); $^{\#}$ = sign. vs. IDD E_2 ($p < 0.05$); † = sign. vs. IRD E_2 ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

Discussion

The ERβ is the predominantly expressed ER in the intestine, and it is known to affect the homeostasis of this tissue (Weige *et al.*, 2009). In studies with ERβKO mice it was shown that the ERβ is essential for maintenance of tissue homeostasis. In a study of our group an ERβ-specific agonist and GEN were able to inhibit proliferation and to induce apoptosis in the large and small intestine of ovariectomized rats (Schleipen *et al.*, 2011). This observation indicates that the activation of ERβ results in a modulation of homeostasis of intestine tissue which again may result in protection from tumor development.

Analysis within our study of the ERα expression at different PND showed no significant differences between the nutrition groups (Fig. 8 A). Here it has to be stated that in the intestine the expression of ERα is generally low, and did not differ between normal and malign intestinal cells (Campbell-Thompson *et al.*, 2001; Konstantinopoulos *et al.*, 2003).

In Figure 8 B it is clearly visible that GRD significantly reduced the expression of ERβ at PND 25, 50 and 80 but is not affected in the IDD and IRD group. The observed down regulation of ERβ mRNA expression may be due to the higher serum concentrations of GEN in the animals of the GRD group compared to IDD and IRD. This assumption is based on the knowledge that binding of a ligand to the ERβ results in a reduced ERβ mRNA expression and that GEN binds with high preference to ERβ (Barkhem *et al.*, 1998; Wood *et al.*, 2006).

In our study at PND 25, 50 and 80 no significant differences in p63 expression could be detected. P63 is a homologue of p53, and is overexpressed in human tumors (Flores *et al.*, 2005). A previous study of our laboratory showed that p63 expression decreases in response to GEN and the ERβ-specific ligand (Schleipen *et al.*, 2011). It seems to play an important role in regulating epithelial proliferation and differentiation, and to be involved in tumorigenesis. P63 is able to bind to the ligand binding domain of the ER (Littlewood *et al.*, 1995; Caserta *et al.*, 2006; Nguyen *et al.*, 2006). We hypothesized that expression of p63 is decreased after lifelong exposure to ISO or GEN solely, but no differences could be measured in its expression level.

As shown in Figure 9 A the proliferative activity, indicated by the expression of the proliferating cell nuclear antigen (PCNA), was significantly lower compared to the IRD and GRD groups at day 25, but increased at PND 50 and PND 80. The reduced

expression of PCNA at day 25 is in line with observations in piglets (Chen *et al.*, 2005) demonstrating that treatment with GEN (5 mg GEN/kg BW/day) for 10 days resulted in inhibition of proliferation. It is assumed that activation of the ERβ is responsible for this inhibitory effect (Helguero *et al.*, 2005; Martineti *et al.*, 2005). Our observation that proliferation is increased at day 50 and 80 could be due to the fact that ISO act as estrogenic compounds in the presence of low estrogen concentrations, whereas at higher estrogen levels they act anti-estrogenic (Hwang *et al.*, 2006). In line with this assumption PCNA expression was reduced at PND 25, because at this age the hormone concentrations are expected to be low. After gaining puberty around PND 35 the ovaries produce estrogens and therefore the ISO may act as anti-estrogens and so the PCNA expression raise at PND 50 and 80. This hypothesis is supported by a study with ovariectomized rats with low estrogenic background where the expression of PCNA is reduced after three weeks treatment with GEN (Schleipen *et al.*, 2011).

The PS2 gene is described to have an important role in regenerating tissue during wound healing in inflammatory bowel diseases and stimulation of mucosal repair (Playford *et al.*, 1996; Ribieras *et al.*, 1998). PS2 is regulated by estrogens via transcriptional mechanisms (Berry *et al.*, 1989). In our study IRD and GRD increase PS2 expression at PND 25, 50 and 80 (Fig. 9 B). This observation confirms a previous study in premenopausal women where supplementation with dietary soy (45 mg ISO/d) for two weeks resulted in an increased expression of PS2 in the breast (Hargreaves *et al.*, 1999). We see two possible szenarios explaining our results that the exposure to ISO results in an increase of PS2 expression. On the one hand, a high nutritive GEN intake could lead to intestinal damage, and therefore the PS2 expression rose to induce wound healing and the proliferation increased resulting from regenerating cells. On the other hand, it could be an indication for an increased resistance to intestinal damage and protection against malignant transformation. The latter would follow the findings that loss of PS2 may occur as an early event in the malignant transformation process of intestinal-type tumors (Wu *et al.*, 1998).

In Figure 10 A and B it is demonstrated that lifelong exposure to ISO also affects the response of ERα and ERβ towards E_2 treatment. Although the binding affinity of ISO is lower to the ERα than to the ERβ ISO are able to reduce the ERα expression. As the mRNA expression of the ER is down-regulated in response to binding of a ligand, it can be assumed that binding of ISO is what reduces ERα. Furthermore,

mRNA expression of ERβ is lower in the GRD than in the IRD group indicating a higher estrogenic potency of GEN solely.

The expression of p63 was strongly effected as well by the diets and E_2 (Fig. 10 C). It is clearly visible that IRD and GRD resulted in a decreased p63 expression in the intestine. The fact that treatment with E_2 resulted in a down regulation of p63 also in the IDD group and further reduced the p63 expression in IRD and GRD animals, is in line with recent observations demonstrating that treatment with ERβ-specific agonists led to significant down-regulation of p63 expression (Schleipen *et al.*, 2011).

Like shown in Figure 11 in this experimental design independent of the diet, administration of E_2 does not affect intestinal proliferation (Fig. 11 A). PS2 displays a nutrition dependent E_2 response in OVX animals. In our study the PS2 expression was highest in OVX rats who received a GRD throughout life which could be taken as an indicating for a tumor protective effect of GEN in this tissue (Fig. 11 B). A previous study from our laboratory points out that E_2 decreases the expression of PS2 in the intestine of female rats (Hofer *et al.*, 2010), which is confirmed by our own findings. E_2 treatment resulted in a significant reduction of PS2 in all nutrition groups. Additionally, these results are supported by a study with postmenopausal macaques where the PS2 expression is down-regulated at high doses of ISO (509 mg ISO (GEN + DAI) per day) in a high estrogenic environment (Wood *et al.*, 2006). On the opposite, treatment with HRT over 3 months resulted in a rise of PS2 expression in the postmenopausal female breast (Harding *et al.*, 2000). However, as the intestine does not belong to the classical estrogen sensitive tissues, it can be assumed that the treatment period of three days was too short to induce estrogenic actions.

Same argumentation can be applied to the PCNA expression of our study. Only a tendency of reduced proliferation could be observed possible due to the short treatment period regarding the inhibition of proliferation showed in the study of Schleipen (Schleipen *et al.*, 2011), while a lot of studies showed a protective effect of HRT against developing cancer in the intestine (Nelson *et al.*, 2002). The reduced expression of PS2 leads to the suggestion that on the one hand the protection against intestinal damage were lost after E_2 treatment. On the other hand, E_2 treatment could result in a decrease of intestinal damage. This is underlined by the reduced expression of p63 in these groups, indicating a reduced risk of developing cancer.

In summary our results provide evidence that a lifelong exposure to an ISO-rich diet may improve intestine homeostasis, and modulate the expression of molecular markers related to tumor protection and the responsiveness of this tissue towards E_2. Consequently, this may affect the susceptibility of this tissue to develop cancer.

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CHAPTER FOUR

Pre- to post-pubertal exposure to isoflavones modulates the sensitivity of the mammary gland towards estrogens in female rats

Abstract:

The aim of this study was to identify critical periods during development responsible for the tissue specific alterations of estradiol (E_2) sensitivity induced by isoflavones (ISO). Female rats were divided into three nutrition groups: first group received an ISO-free diet (IDD) throughout life, second group an ISO-containing diet (ISD) throughout life, and a third group was exposed to an ISD from postnatal day (PND) 30 to PND 60 which cover the time period shortly before onset of puberty up to end of puberty (pISD). The onset of puberty was ascertained, the menstrual cycle length was measured and from each nutrition group one subset of animals was sacrificed during puberty (PND 50) and during adulthood (PND 80). Onset of puberty occurred significantly earlier in pISD and ISD compared to IDD. Menstrual cycle length was shortest in pISD. While proliferative activity and PR expression in the mammary gland was not affected by IDD and ISD, a significant increase could be detected in pISD animals at PND 50. Rats were ovariectomized and subcutaneously treated either with E_2, GEN, or the vehicle (OVX), for three days (PND 77–80) to determine estrogen sensitivity. Analysis of Ki-67 and PCNA showed a reduced proliferative response to E_2 in pISD and ISD compared to IDD, while the induction of PR was higher in both IDD and pISD compared to ISD. Our results indicate a reduction of the proliferative response of the mammary gland towards estrogens due to ISO exposure solely during PND 30 and 60, while this timeframe seems too late to influence estrogen sensitivity.

Introduction:

There is increasing evidence that the reduced breast cancer risk in eastern Asian countries compared to Europe and the USA is related to high soy consumption (Ursin *et al.*, 1994; Adlercreutz, 2002; Cassidy, 2003; Badger *et al.*, 2005; Nichenametla *et al.*, 2006; Usui, 2006). Soy is the main source of isoflavones (ISO), which are polyphenolic non-steroidal compounds. After ingestion, an initial hydrolysis of the sugar moiety of the glycosides seems to be required for absorption, which is catalysed by the brush border glucosidase in the small intestine or by bacterial β-glucosidases or β-glucuronidases in the colon (Setchell *et al.*, 2002; Mortensen *et al.*, 2009). The enterobacteria metabolize daidzein (DAI) to dihydrodaidzein (DH-DAI), which can be further converted to equol or O-desmethylangolensin (Key *et al.*, 1999). Genistein (GEN) is reduced to dihydrogenistein (DH-GEN) by the gut microbiota as well, and is further metabolized to 6´-hydroxy-O-desmethylangolensin (6´-OH-ODMA). The gut microbiota is essential for the metabolism of ISO, and its composition is influenced by physiological, pathological and environmental factors, but also by gender, genetics, and ethnicity (Mortensen *et al.*, 2009).

Because of their structural similarity to endogenous estrogens, ISO are able to bind to the estrogen receptor α (ERα) and β (ERβ). Both receptor subtypes can be found in the mammary gland. Albeit the expression of the ERα is generally low in normal mammary gland, it increases in proliferative benign tissue (Roger *et al.*, 2000). The content of ERα in the breast is proportional to the rate of proliferation, and the co-expression of ER and the proliferation marker Ki-67 is described as the manifestation of an important early molecular change in the development of cancer (Shoker *et al.*, 1999).

One function of the ERβ seems to be the counteraction of ERα (Saji *et al.*, 2005; Chang *et al.*, 2006). There is evidence that activation of the ERβ reduces the estrogen stimulated proliferation in MCF-7 and T47D breast cancer cells (Omoto *et al.*, 2003; Strom *et al.*, 2004). As the binding affinity of ISO is 30-times higher to ERβ than to ERα it seems reasonable to assume that ISO are able to protect from developing breast cancer via activating ERβ. Still, the effects of ISO are discussed controversially. Studies in breast cancer cells and animal cancer models described a higher growth of the tumor in response to treatment with ISO (Wang *et al.*, 1996; Ju *et al.*, 2006).

The controversial discussion might be resolved when taking the timing of the ISO exposure into account. Numerous studies have shown that ISO intake has to start pre-pubertal to exert protective effects. There is evidence that ISO may influence the development of the mammary gland, and therefore it seems to be reasonable that ingestion of ISO has to start before pubertal changing of the breast occurs (Cabanes *et al.*, 2004; Padilla-Banks *et al.*, 2006). Morphological alterations of the mammary gland are one factor of influencing breast cancer risk. Further risk factors for developing breast cancer is the extent of lifelong estrogen exposition implicating early onset of menarche, late onset of menopause, high levels of free circulating estrogens, and short menstrual cycle length (Toniolo *et al.*, 1995; Lu *et al.*, 1996; Hulka & Moorman, 2001). The critical role for estrogens in the development of breast cancer is under investigation for over thirty years (Noble & Cutts, 1959; Wotiz *et al.*, 1978; Cavalieri & Rogan, 2006; Yager & Davidson, 2006; Santen *et al.*, 2009). Elevated levels of endogenous estradiol in postmenopausal women have been found to increase the risk of developing breast cancer. Additionally, a recently performed meta-analysis found a positive association between premenopausal estrogen concentrations and breast cancer risk (Walker *et al.*, 2011).

A previous study from our laboratory showed a significant decrease in estrogen sensitivity of the mammary gland after lifelong exposure to an ISO-rich diet or GEN-rich diet (Molzberger *et al.*, 2012). In that study, the exposure to ISO started already in utero, but there is evidence that ISO exposure starting pre-pubertal is sufficient to exert protective effects towards developing breast cancer (Lee *et al.*, 2009). To evaluate the relevance of the time of ISO exposure, we designed this further animal experiment, where lifelong exposure to an ISD is compared to an ISD starting just before puberty onset up to adulthood from postnattally day (PND) 30 to PND 60. Within the experiment puberty onset and menstrual cycle length were determined and the expression of Ki-67, PCNA, and PR was measured.

Materials and Methods:

Diets

The animals had free access to one of two diets containing different amount of ISO: an ISO-free diet (IDD; Sniff RM/H phytoestrogenfree, Ssniff GmbH, Soest, Germany), or an ISO-containing diet (ISD; Sniff RM/H, Ssniff GmbH, Soest, Germany). The ISO content of the diets are depicted in Tab. 5.

Experimental animals and study design

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.

Wistar rats were obtained from Janvier (Le Genest St Isle, France) and kept under controlled conditions of temperature (20°C ± 1), relative humidity (50–80 %) and illumination (12 h dark, 12 h light). The female rats were mated and the dams were fed one of the two diets during pregnancy and nursing (IDD: n = 14, ISD: n = 7). After weaning the female offspring were divided into three dietary groups (n = 30 each dietary group) and had ad libitum access to the diets and water.

The first dietary group received an IDD throughout life (IDD). The second received an ISD for part of their life (pISD) before onset of puberty up to young adulthood from postnatal day (PND) 30 to 60. The third dietary group received an ISD throughout life (ISD). Each dietary group was further divided into five subgroups (n = 6 each group). The first subgroup of each dietary group was sacrificed in their puberty life period on postnatal day 50 (puberty), the second in the adult phase of life on PND 80. The other three subgroups were ovariectomized (PND 60) and an uterotrophic assay was performed at day 77. The animals received 17β-estradiol (E_2; 4 µg/kg BW/day), genistein (GEN; 100 mg/kg BW/day), or purely the vehicle (OVX) subcutaneously for three days and were afterwards sacrificed. The estrogenic compounds were solved in 20 % DMSO/peanut oil.

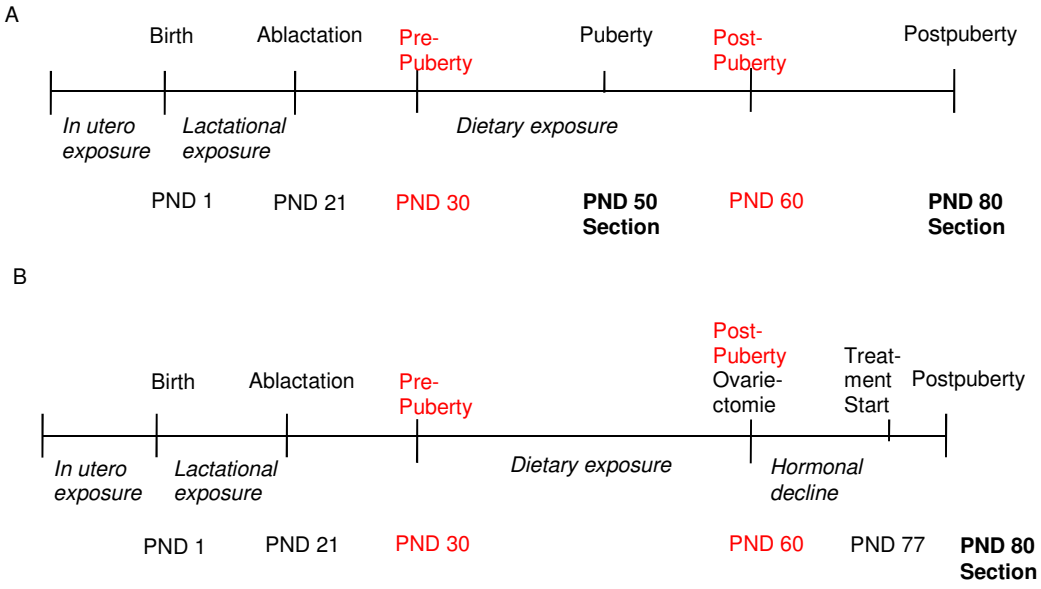

Fig. 12: Timeline and experimental setting of the study. **A** Intact animals received either lifelong and IDD or ISD, or an ISD starting pre-pubertal up to the end of puberty (PND 30–PND 60, pISD). **B** Animals received an IDD, pISD or ISD were ovariectomized on PND 60 and were treated with E_2, GEN, or the vehicle from PND 77 up to PND 80. Afterwards, these animals were sacrified and the proliferation and the estrogenic response of the mammary gland were determined

Quantification of ISO derivatives in the diet by HPLC/DAD analysis

The pelleted diet ISD was crushed using mortar and pestle. Approx. 250 mg of an accurately weighted sample of each homogenized diet powder were vortexed for 30 s in exactly 40 mL 65 % (v/v) methanol and extracted gently for 60 min at room temperature using an overhead rotation shaker. The suspensions were centrifuged at 8600 g for 5 min and filtered using 15 mm 0.45 um PTFE syringe filters. The filtrate was diluted by pipetting 1 ml of the solution into a 50 ml graduated flask and make up to the mark with 65 % (v/v) methanol.

The LC-DAD analyses were performed on a Shimadzu LC system equipped with a controller (CBM-20A), a degasser (DGU-20A3), two pumps (LC-20AD), an autosampler (SIL-20AC HT), a column oven (CTO-20AC) and a diode array detector (SPD-M20A). The LC system was controlled by the software LC solution 1.24. Separation of the ISO derivatives was performed on a Phenomenex Kinetex PFP column (3.0 mm internal diameter, 100 mm length, 2.6 µm) with an oven temperature of 35°C. Solvent A was 0.1 % (v/v) formic acid in bidest. water and

solvent B was acetonitrile (LC grade). Flow rate was 0.7 mL/min, the injection volume 10 µL. The LC gradient started with an initial period of 3 min at 10 % B, increasing linearly to 45 % B at 12 min, and finally to 100 % at 12.5–15.5 min, re-equilibrating the system in a 7.5 min postrun (10 % B). Eluent was monitored between 200 and 500 nm using diode array detection. Peaks were integrated at 250 nm. The identity of each compound was confirmed by the retention time and the UV-Vis spectra. The limit of quantification (LOQ) and lowest calibration point was 0.8 nmol for all target analytes.

Chemicals used for measurement of ISO-metabolites in the blood serum

DAI, GEN and glycitein were purchased from LC Laboratories (Woburn, MA, USA). Daidzin, genistin, glycitin, 6''-O-acetyl-daidzin, 6''-O-acetyl-genistin, 6''-O-acetyl-glycitin, 6''-O-malonyl-daidzin, 6''-O-malonyl-genistin and 6''-O-malonyl-glycitin were purchased from Wako (Neuss, Germany). DH-DAI was purchased from Toronto Research Chemicals (North York, Canada). DH-GEN and equol were purchased from APIN Chemicals LTD (Abingdon, UK). ODMA and 6'-OH-ODMA were purchased from Plantech UK (Reading, UK). [3,4,8-$^{13}C_3$]daidzein was provided by Nigel Botting (University of St. Andrews, UK).

Measurement of ISO-metabolites in the blood serum

Rat serum samples (90 µl) were thawed and 5 µl of internal standard (2 µM [3,4,8-$^{13}C_3$]daidzein stock solution in DMSO) was added. 500 µl of an ammonium acetate buffer (0.1 M, pH 5.0) containing 1,4-dithiothreitol (100 mM), 1200 U β-glucuronidase (bovine liver, Typ B-3) and 120 U arylsulfatase (Helix pomatia, Typ H-1) was added, and the mixture was incubated for 2 h at 37°C. The sample was acidified with 1200 µl of 50 mM H_3PO_4 solution (ice-cooled) and purified via SPE extraction. Therefore the SPE cartridges (Strata-X AW, 60 mg, 3 ml, Phenomenex, Torrance, USA) were conditioned with 2 ml of methanol and equilibrated with 2 ml of water. After loading with the sample solutions the cartridges were first washed with 2 ml of 2 % (v/v) formic acid solution. The second wash step was performed with 2 ml of 50 % (v/v) methanolic solution. The analytes were eluted with 1 ml of 2 % (v/v) ammonia (25 %) in methanol and evaporated to dryness under a nitrogen stream. The residues were redissolved in 100 µl of 30 % (v/v) methanolic solution and an aliquot of each sample was analysed by LC/MS as described below. For calibration

blank plasma was worked up as described above and analytes were added before analysis in an end concentration range between 5 and 10,000 nM.

The modest recovery rate of 6'-OH-ODMA was acceptable because of the fact that the measured levels of this metabolite were very low, and therefore not of biological significance.

The HPLC-MS analyses were performed on an ABSciex QTrap 5500 mass spectrometer equipped with a Shimadzu LC system, which consisted of a controller (CBM-20A), a degasser (DGU-20A5), two pumps (LC-30AD), an autosampler (SIL-30AC) and a column oven (CTO-20AC). The LC-MS system was controlled by the software Analyst 1.5.2. The Turbo Spray ESI Source was operated in the negative mode. The source parameters were as follows: Curtain gas (CUR) 40 psi, ion spray voltage (IS) -4500 V, ion source gas-1 (GS 1) 80 psi, ion source gas-2 (GS 2) 70 psi, ion source gas-2 temperature (TEM) 600°C. Two multi-reaction monitoring (MRM) transitions (a quantifier and a qualifier ion transition for each compound) were used.

Separation of the analytes was performed on a Waters Acquity HSS T3 (2.1 mm internal diameter, 100 mm length, 1.8 µm) with an oven temperature of 40°C. Solvent A was a 20 mM ammonium formate buffer (pH 3) and solvent B was an acetonitrile/methanol mixture (1 / 2.5, v/v) (LC grade). Flow rate was 0.5 ml/min, the injection volume 10 µl. The elution profile was as follows: 0–2.6 min isocratic with 3 % B, 2.6–16.7 min from 3 % to 56 % B, 16.7–17.3 min from 56 % to 95 % B, 17.3–19.9 min isocratic with 95 % B, 19.9–20.5 min from 95 % to 3 % B and 20.5–24.7 min isocratic with initial conditions. The limits of quantification (LOQ) for each analyte in serum were as follows: 50 fmol for DAI, GEN, DH-DAI, DH-GEN, ODMA and 6'-OH-ODMA. The LOQ for equol was 1 pmol. We defined the limit of detection (LOD) as one third of the respective LOQ.

Determining puberty onset and menstrual cycle length

To investigate the puberty onset the day of vaginal opening was determined. Additionally, menstrual cycle length was measured. Therefore a vaginal smear test was performed daily for one week starting the day after puberty onset.

Tissue preparation

Animals were decapitated after weighing and blood was collected. The mammary gland and the uterus were removed and the uteri were weighed. Specimen of each was snap frozen in liquid nitrogen for mRNA preparation.

RNA preparation

Frozen mammary gland tissues were powdered, pooled and homogenized in TRIzol®. Total RNA was isolated from cells using the TRIzol® (Invitrogen Life Technologies, Karlsruhe, Germany) standard protocol (Chomczynski & Sacchi, 1987) followed by cDNA synthesis with the Quantitect® Reverse Transcription Kit (Quiagen, Hilden, Germany).

Real-time RT-PCR

To determine the mRNA expression of Ki-67, PCNA and PR quantitative real-time RT-PCR was performed in the MxPRO (Stratagene) with Platinum® Taq DNA Polymerase (Invitrogen, Karlsruhe, Germany). SybrGreen I® was used as detection dye. The Cytochrome-C-oxidase subunit 1A (1A) was used as housekeeping gene, and the expression of all genes were normalized to 1A. Specific primers were designed with the primer3 software (Whitehead Institute for Biomedical Research, Cambridge, USA) based on the cDNA sequences available at the EMBL database: 1A: up: 5'-CGTCACAGCCCATGCATTCG-3 ', dw: 5'-CTGTTCATCCTGTTCCAGCTC-3 '; PR: up: 5'-CATGTCAGTGGACAGATGCT-3' ', dw. 5'-ACTTCAGACATCATTTCCGG-3 '; PCNA: up: 5´GAGCAACTTGGAATCCCAGAACAGG-3´,dw: 5´ CCAAGCTCCCCACTCGCAGAAAACT-3´; Ki-67: up: 5´-AACCAGGACTTTGTGCTCTGTAA-3´, dw: 5´-CTCTTTTGGCTTCCATTTCTTC-3´. The PCR program consisted of a first a denaturation step at 95°C for 4 min, followed by 45 cycles of 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. The fluorescence was quantified during the 72°C elongation step and the product formation was confirmed by melting curve analysis (55–95°C). For calculation of relative rates of gene expression the $\Delta\Delta C_T$ method was used (Pfaffl, 2001). Gene expressions are shown in relation to control animals fed with the ISO-free diet.

Statistical analysis

Statistical analysis was performed using the SPSS Statistical Analysis System, SAS, Version 12.0. All data are expressed as arithmetic means with their standard deviations. First a global Kruskal-Wallis-H-Test was performed to analyse if there are significant differences between the groups. In case of differences, a Mann-Whitney-U-Test was additionally performed to identify the groups with statistical significant variance. At PND 50 and 80 nutrition groups were compared to each other, and the different treatment groups were compared to the OVX control groups. Statistical significance was established at $p < 0.05$.

Results:

The experimental design of this study is depicted in Fig. 12. The IDD contains no detectable amounts of ISO (< 10 µg/g). The ISD contains 113.54 µg GEN/g, 147.41 µg DAI/g and 36.06 µg GLY/g (each calculated as aglycone) as depicted in Table 5. Food intake was monitored during the study but no significant differences could be shown between the nutrition groups (data not shown).

ISO derivative	**MW [g/mol]**	**mean value µg/g ISD**	**mean value µM/g ISD**	**calculated as aglyc.**
Daidzein	254.24	11.54	0.045	11.54
Genistein	270.24	12.91	0.048	12.91
Glycitein	284.27	1.66	0.006	1.66
Daidzin	416.38	86.22	0.207	52.65
Genistin	432.38	104.84	0.242	65.52
Glycitin	446.4	33.38	0.075	21.26
6''-*O*-Acetyl-Daidzin	458.41	19.83	0.043	11.00
6''-*O*-Acetyl-Genistin	474.41	20.63	0.043	11.75
6''-*O*-Acetyl-Glycitin	488.44	5.36	0.011	3.12
6''-*O*-Malonyl-Daidzin	502.42	75.79	0.151	38.35
6''-*O*-Malonyl-Genistin	518.42	109.78	0.218	57.23
6''-*O*-Malonyl-Glycitin	532.45	18.76	0.035	10.02

Sum of daidzein derivatives calculated as daidzein aglycone in µg/g ISD 113.54

Sum of genistein derivatives calculated as genistein aglycone in µg/g ISD 147.41

Sum of glycitein derivatives calculated as glycitein aglycone in µg/g ISD 36.06

Tab. 5: Content of ISO derivatives in the ISO-standard diet (ISD).

In Table 6, body weights and uterus wet weights are listed. No significant influence could be observed in subject to nutrition or treatment related to body weights, whereas treatment with E_2 led to a significant increase in uterus wet weights in all nutrition groups compared with their OVX groups. Treatment with GEN had no significant effect on uterus wet weights in any group.

		Body W. (g)	**Uterus (mg/kg BW)**
PND 50	IDD	194 ± 2	1986 ± 277
	pISD	182 ± 3	1348 ± 99
	ISD	195 ± 9	1300 ± 237
PND 80	IDD	265 ± 4	982 ± 76
	pISD	260 ± 9	1548 ± 176
	ISD	245 ± 8	1124 ± 100
OVX	IDD	300 ± 7	266 ± 13
	pISD	301 ± 12	259 ± 19
	ISD	328 ± 14	273 ± 24
E_2	IDD	286 ± 6	1170 ± 98**
	pISD	269 ± 8^{++}	1264 ± 103**$^{++}$
	ISD	301 ± 5^{++}	1464 ± 175**$^{\#\#}$
GEN	IDD	297 ± 9	343 ± 16**
	pISD	268 ± 7^{++}	380 ± 30**$^{++}$
	ISD	324 ± 9^{++}	340 ± 24*

Tab. 6: Body weights (g) and uterus wet weights (mg/kg BW). ** $p < 0.01$ vs. IDD OVX; $^{++}$ $p < 0.01$ vs. pISD OVX; $^{\#\#}$ $p < 0.01$ vs. ISD OVX; (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

Table 7 shows the concentrations of DAI, GEN and the metabolites DH-DAI, DH-GEN, ODMA, 6´-OH-ODMA and equol in the blood serum of 50 (PND 50) and 80 (PND 80) days old female rats. No ISO-metabolites could be detected in IDD animals at PND 50 and 80. Measurement of GEN, DAI and DH-DAI in the blood serum revealed significant higher concentrations in the ISD compared to the pISD group, whereas no differences were found in case of DH-GEN, ODMA and equol. On day 50 and 80, the serum concentrations of GEN, DAI and their metabolites were significantly higher after ISD compared to the other diets.

		DAI	GEN	Equol	DH-DAI	DH-GEN	ODMA	6´-OH-ODMA
PND 50	IDD	n. d.	n. d.	n. d.	n. d.	n. d.	0.009±0.00	n. d.
	pISD	0.044 ± 0.06	0.016±0.03	1.644±0.40**	0.036±0.04**	0.068±0.08**	0.119±0.06**	n. d.
	ISD	0.909±0.27**$^{++}$	0.901±0.37**$^{++}$	2.611±1.10**	0.213±0.10**$^{++}$	0.062±0.04*	0.155±0.08**	n. d.
PND 80	IDD	0.011±0.01	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	pISD	0.039±0.01	0.005±0.01	n. d.	0.028±0.02	n. d.	0.008±0.00	n. d.
	ISD	0.547±0.28**$^{++}$	0.566±0.42**$^{++}$	3.039±1.64**$^{++}$	0.099±0.06**$^{++}$	0.033±0.04*$^{+}$	0.190±0.08**$^{++}$	n. d.

Tab. 7: Serum-concentrations (µM) of DAI, GEN and their metabolites equol, DH-DAI, DH-GEN, ODMA and 6´-OH-ODMA in 50 (PND 50) and 80 (PND 80) days old female rats. N. d. = not detectable. * = sign. vs. IDD ($p < 0.05$); ** = sign. vs. IDD ($p < 0.01$); $^{+}$ = sign. vs. pISD ($p < 0.05$); $^{++}$ = sign. vs. pISD ($p < 0.01$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

The influence of pre- to postpubertal and lifelong exposure to ISO on puberty onset and menstrual cycle length were determined. It could be shown that ISO exposure at any time led to an earlier onset of puberty (Fig. 13 A). Menstrual cycle length was shorter in animals where the exposure to ISO started prepubertal compared to those who were exposed lifelong or not at all (Fig. 13 B).

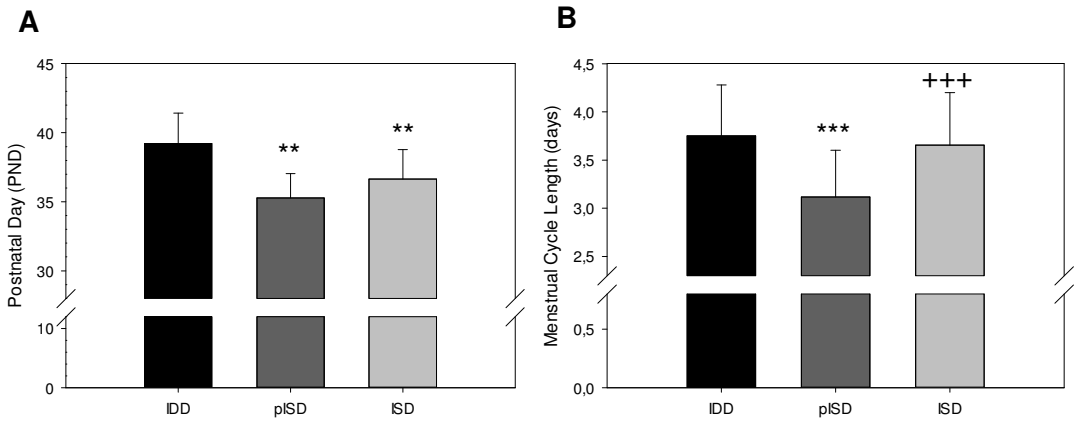

Fig. 13: A Puberty onset indicated by the day of vaginal opening (postnatal day, PND). **B** Length of menstrual cycle in days. lDD served as control and were set to one. The bars shown are mean +SD. * = sign. vs. lDD ($p < 0.05$); $^{+}$ = sign. vs. pISD ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

To evaluate the influence of ISO exposure during different periods of life on the proliferation of the mammary gland, the expression of the proliferation markers Ki-67 and PCNA were analysed. At PND 50, an elevated mRNA expression of Ki-67 and PCNA could be measured in pISD and ISD animals compared to IDD, whereas the expression in the ISD group were significant lower as in the pISD group (Fig. 14 A/B). Measurement of Ki-67 and PCNA at PND 80 revealed a significant decrease in rats fed an ISD compared to those received and IDD or an pISD. In addition to the proliferation markers, the estrogen sensitivity was determined using the progesterone receptor (PR). A significant increase in PR mRNA expression could be shown in the pISD group at PND 50 and PND 80 (Fig. 14 C).

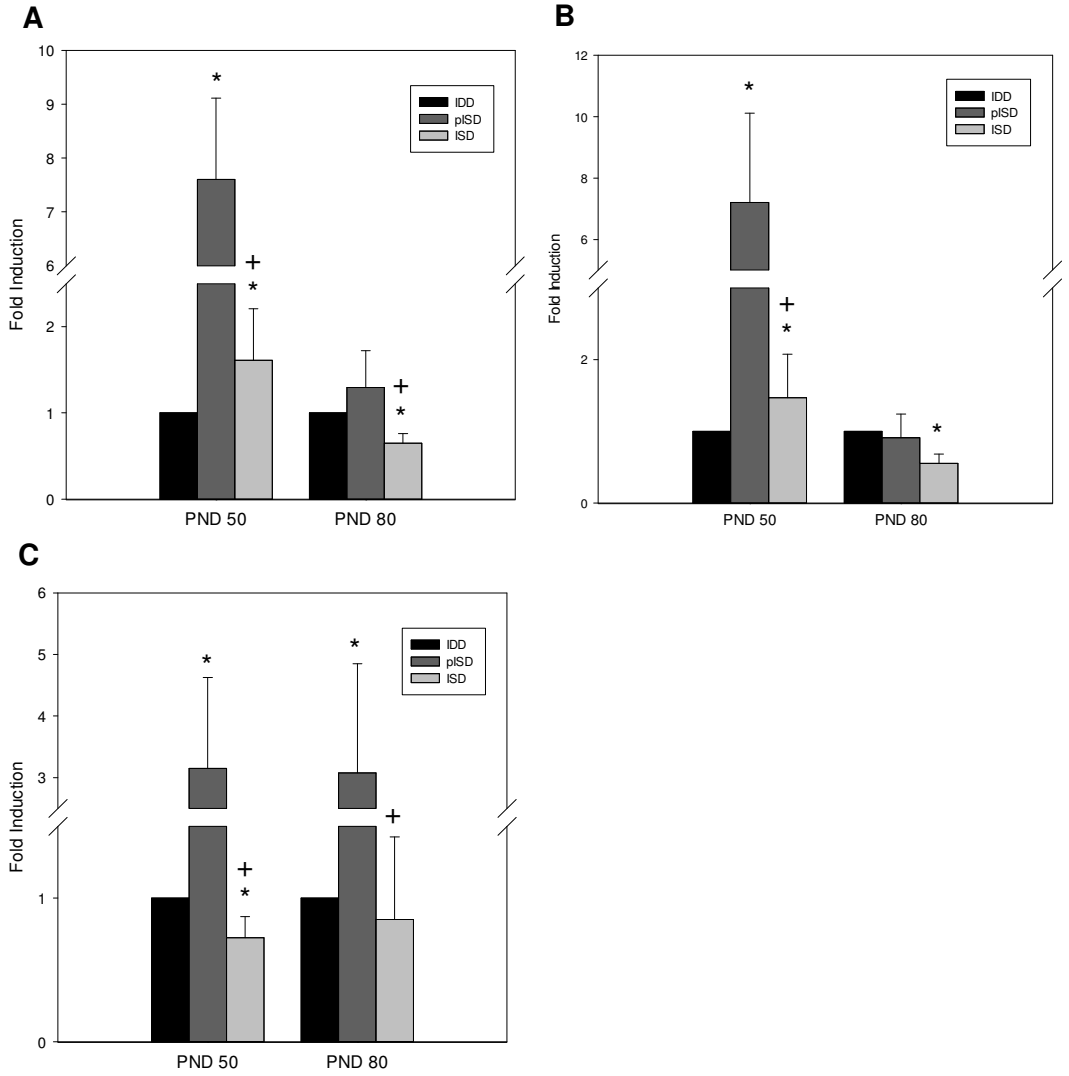

Fig. 14: mRNA expression of PCNA (**A**), Ki-67 (**B**) and PR (**C**) in the mammary gland of 50 (PND 50) and 80 (PND 80) days old female Wistar rats. IDD served as control and were set to one. The bars shown are mean +SD. Six rats were included to each group. * = sign. vs. IDD ($p < 0.05$); $^{+}$ = sign. vs. pISD ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

To investigate the influence of ISO exposure during different development stages on the estrogen responsivity of the mammary gland, adult female rats were ovariectomized and an uterotrophic assay was performed. In mammary gland tissue of these rats, proliferation (Ki-67, PCNA) and estrogen sensitivity (PR) were determined. No differences in Ki-67 and PCNA mRNA expression could be observed in OVX animals. In response to E_2 treatment, the proliferation was increased in all nutrition groups, whereas pre-exposure to ISO (pISD, ISD) significantly diminished the E_2 stimulated proliferation (Fig. 15 A/B).

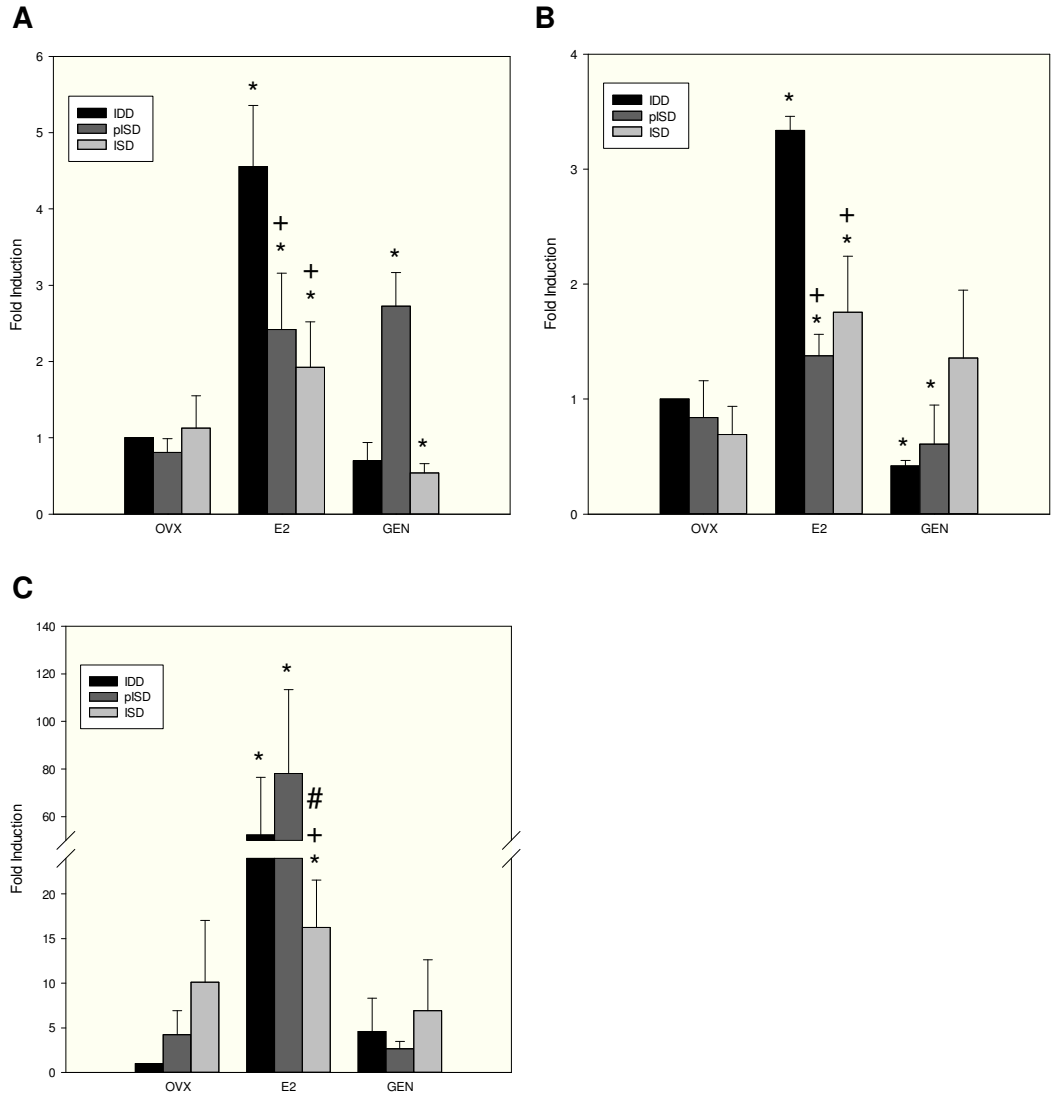

Fig. 15: mRNA expression of PCNA (**A**), Ki-67 (**B**) and PR (**C**) in the mammary gland 80 days old female Wistar rats after ovariectomie followed by three days of treatment with the vehicle (OVX), estradiol (E_2), or genistein (GEN). IDD OVX served as control and were set to one. The bars shown are mean +SD. Six rats were included to each group. * = sign. vs. IDD OVX ($p < 0.05$); $^{+}$ = sign. vs. IDD E_2 ($p < 0.05$); $^{\#}$ = sign. vs. pISD E_2 ($p < 0.05$); (Kruskal-Wallis H-Test followed by a Mann-Whitney U-Test)

The progesterone receptor was determined as estrogenic marker on mRNA level showing that treatment with E_2 resulted in an elevated mRNA expression of PR in all groups, whereas IDD and pISD fed animals showed an additional increase compared to rats received an ISD (Fig. 15 C).

Discussion:

Our observation that animals receiving ISO either lifelong or during puberty have a significant earlier onset of puberty than animals receiving no ISO confirms the animal study conducted by the group of Whitten (Whitten & Naftolin, 1992). They reported that chronic exposure (PND 22–PND 60) to dietary concentrations (0.1 % of the diet) of the phytoestrogen coumestrol led to an accelerated vaginal opening. Our results correlates as well with several studies which show that menstrual cycle length is elongated in response to ISO exposure (Lu *et al*., 1996; Kumar *et al*., 2002). In our animal experiment, the menstrual cycle length was shortest for animals where the ISO exposure started prepubertal. As both puberty onset and menstrual cycle length being estrogen sensitive markers, these observations lead to the assumption that estrogen sensitivity increases if the ingestion of ISO starts prepubertal but not already in utero.

Uterus wet weights remained unaffected by the different diets indicating a low estrogenic potential of the ISD.

Regarding the ISO serum concentrations on PND 50, significant differences could be observed between pISD and ISD, although animals of both groups received the same diet at this time. Exposure to ISO starting prepubertal resulted in significant lower concentrations of GEN and DAI than lifelong ISO exposure, whereas their metabolites show no differences, which could lead to the assumption that ISO metabolism is altered in pISD animals. The ISO metabolism depends amongst others on enterobacteria (Mortensen *et al*., 2009). The different ISO content in the blood serum could be explained by altered gut microflora in animals receiving ISO pre- to postpubertal compared to animals receiving ISO lifelong. The gut microflora begins to develop within a week of birth and continues to change from infancy into adulthood. As it is known that ISO are able to cross the placenta and that they occur to a little extent in mothers milk, it could be assumed that animals where the exposure started in utero developed a different gut microflora compared to animals where ISO exposure started prepubertal. These differences in enterobacteria could be responsible for the altered metabolism of ISO in pISD compared to ISD animals.

In a previous experiment of our group proliferation of the mammary gland was elevated after lifelong exposure to ISO or GEN solely at PND 50 (Molzberger *et al*., 2012). This observation is confirmed by the results of the present study where pISD and ISD led to an increase of proliferation on PND 50 (Fig. 14 A/B). A possible explanation for this effect is a change in the differentiation of the mammary gland in

response to ISO. This explanation goes hand in hand with observations by Fritz et al. who showed that early exposure (GD 0–PND 21) to physiological doses of the ISO GEN (0, 25, 250 mg/kg diet) via diet induces cell differentiation. This reprogramming of the mammary gland resulted in a reduced susceptibility of this tissue to develop cancer (Fritz *et al.*, 1998).

To investigate whether the ISO exposure during different periods of life influences the estrogen responsivity of the mammary an uterotrophic assay was performed. This assay served as a model system of the situation in postmenopausal women receiving HRT (Odum *et al.*, 1997). Treatment with E_2 resulted in an increase of the uterus wet weights, whereas nutrition exerts no effects (Tab. 6). In a previous experiment of our laboratory, an additional increase in the uterine wet weights could be observed in animals which received lifelong an ISO-rich or GEN-rich diet compared to an IDD group (Moller *et al.*, 2010). The ISD of the present study contained 2-times lower concentrations of ISO as the ISO-rich diet, indicating a dose-response effect of ISO on uterine wet weights. Additionally, a lower E_2 concentration was chosen in the present study, as it has been shown that E_2 concentrations at doses between 1 and 10 µg/kg BW (s. c.) exert physiological effects on the rat uterus (Odum *et al.*, 1997).

Analysis of the proliferation marker Ki-67 and PCNA on mRNA level revealed that the E_2-induced proliferation was reduced after pre-exposure to ISO (Fig. 15 A/B). This observation indicates that exposure to ISO starting prepubertal is sufficient to reduce the estrogen induced proliferative response of the mammary gland. Numerous studies described that ISO exert protective effects on the mammary gland if the exposure starts prepubertal (Wu *et al.*, 1996; Shu *et al.*, 2001; Lamartiniere, 2002; Wu *et al.*, 2002). This could be explained by the fact that GEN is able to alter the morphology of the mammary gland if the ingestion starts before pubertal breast development occurs. It has for example already been shown that prepubertal exposure to GEN increases the differentiation of the mammary gland by leading to an increase in the number of differentiated lobules and a reduction in the number of terminal end buds. The increased differentiation of the mammary gland results in a reduced risk to develop cancer in this tissue (Cotroneo *et al.*, 2002; Lamartiniere, 2002; Cabanes *et al.*, 2004).

In contrast to proliferation, PR expression was induced in IDD as well as in pISD animals, suggesting an increased estrogen sensitivity in animals which received ISO only pre- to postpuberty (Fig. 15 C). This is in line with the observations in the PND

50 and 80 animals, which also showed an increased PR expression after pre- to postpubertal ISO exposure (Fig. 14 C). In addition to the earlier onset of puberty and the shorter menstrual cycle length, ISO exposure starting shortly before the onset of puberty seems to increase the estrogen sensitivity of the mammary gland. This led to the hypothesis that the imprinting of the estrogen sensitivity occurs at an earlier stage of life, before PND 30. As proliferation of the mammary gland is reduced after pre- to postpubertal ISO exposure, it could be assumed that the proliferative response of the mammary gland is formed at a later developmental stage, during PND 30 and 60. In line with other studies, it could be hypothesized that the reduced proliferative response to estrogens results from an altered pubertal breast development after ISO exposure. Our assumption is confirmed by a study of Clarke who showed that proliferating cells rarely express the PR, indicating that there are two distinct populations in normal mammary glands (Clarke *et al.*, 1997).

As reported before, numerous studies suggested a protective role of ISO exposure towards the development of breast cancer, if the ingestion starts prepubertal, because of the ability of ISO to alter the morphology of the mammary gland during pubertal breast growth (Limer & Speirs, 2004; Warri *et al.*, 2008; Messina & Hilakivi-Clarke, 2009). Further studies revealed that ISO are also able to induce epigenetic changes (Guerrero-Bosagna *et al.*, 2008; Li *et al.*, 2009; Molinie & Georgel, 2009). The group of Dolinoy showed that in utero exposure to Bisphenol A (50 mg/kg diet) resulted in epigenetic abnormalities, which could be reversed through maternal dietary supplementation with GEN (250 mg/kg diet) (Dolinoy *et al.*, 2007). There is evidence that the early post-conceptional period displays a critical window, as it could be shown that in mammals the genomic methylation profile is reprogrammed during gametogenesis and in early embryogenesis (Barker, 2004; Gluckman *et al.*, 2007; Reik, 2007). This lead to the assumption that the increased estrogen sensitivity in the pISD compared to the ISD group could be due to epigenetic changes in the ISD which are mediated by early life ISO exposure.

In summary, our results provide evidence that the imprinting of the estrogen sensitivity occurs in an earlier stage of life than the imprinting of the proliferative response of the mammary gland towards estrogens. In the present study, the estrogen induced proliferation was reduced due to ISO exposure solely during PND 30 and 60, while this timeframe seems too late to influence estrogen sensitivity.

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CHAPTER FIVE

General discussion

Differences in the incidence of hormone dependent cancer between Eastern Asia and Europe or the US have been described many years ago (Ursin *et al.*, 1994; Adlercreutz & Mazur, 1997; Martinez, 2005). Studies indicate that the traditional East Asian diet which is rich in soy may be responsible for cancer preventive effects.

Aim of the present thesis was to analyse whether dietary exposure to ISO during different windows of development alters the estrogen susceptibility of the mammary gland and the tissue homeostasis of the small intestine in female rats.

The first question addressed was whether lifelong exposure to dietary soy ISO or lifelong exposure solely to GEN influences the sensitivity of the mammary gland towards estradiol (E_2). In the corresponding experiment three different kinds of diets were chosen: an ISO-free diet (IDD), an ISO-rich diet (IRD) and a GEN-rich diet (GRD). The IDD can be compared to the European nutrition containing less than 1 mg ISO per day, resulting in plasma concentrations of 10 nM ISO (Adlercreutz *et al.*, 1993). The IRD reflects the Eastern Asian diet traditionally being rich in soy (20–50 mg ISO/day), and resulting in a total serum ISO level (sum of GEN, DAI and equol) of 0.87 µM (Adlercreutz, 2002). The IRD used in the present study contained 248 µg GEN/ g, 213 µg DAI/g and 59 µg GLY/g diet (each calculated as aglycones). This results in an average daily ISO intake of 33 mg/kg BW of an adult female rat and ISO serum concentrations of 1.1 µM GEN and 2 µM DAI. Compared to the human situation, this concentration of the IRD is relatively high.

During the last years the range of soy supplement products increases, although the effects of isolated intake of ISO are uncertain. In the present study, the GRD should, in regards to the daily ISO intake and the ISO plasma level, reflect the consumption of relatively high amounts of solely GEN (700 µg/g diet) in the form of soy supplements. It is described that consumption of soy supplements results in an average ISO intake of 20–80 mg/day, and plasma ISO levels of 1–3 µM are achieved (Gardner *et al.*, 2009). Given the chosen diet, the adult female rats consuming the GRD accordingly reached a daily intake of 42 mg GEN/kg BW, resulting in serum concentrations of 2.5 µM.

The results have shown that mammary gland proliferation was increased after IRD and GRD on PND 50. This suggests that puberty was still ongoing, and goes along with the assumption of an improved breast development after lifelong ISO exposure (Fritz *et al.*, 1998). As expected, no proliferation could be detected at PND 80. Both results show that the points of time were appropriate to analyse the influence of ISO on the mammary gland in regards to the investigated hypothesis.

After ovariectomie and treatment with E_2, the protective effects of ISO exposure became even more apparent. Lifelong exposure to IRD or GRD resulted in a reduced sensitivity of the mammary gland towards estrogens which was indicated by reduced expressions of PCNA and PR, increased expressions of PS2 and a decrease in the ratio of ERα:ERβ compared to IDD fed animals. The fact that an expected increase of proliferation could be detected after treatment with E_2 confirmed the general effectiveness of the treatment and thus the assay setup to analyse estrogenic effects in the mammary gland.

Comparison of the results of the IRD and GRD groups revealed that both diets led to a similar reduction of estrogen sensitivity, whereas the IRD seemed more effective, although it contains less ISO than the GRD (IRD: sum of GEN, DAI, and GLY: 520 µg/g diet; GRD: 700 µg GEN/g diet). This can be explained by the fact that the IRD included the ISO DAI, which is converted to equol in rodents. Equol has more estrogenic potency than GEN and DAI, and could therefore be responsible for the stronger effects of the IRD (Lamartiniere *et al.*, 2002b; Rafii *et al.*, 2007). Taken together, lifelong consumption of a diet containing the whole spectrum of soy ISO results in a further reduction of the mammary gland sensitivity than a diet containing high amounts of solely GEN.

The second question addressed the effects of an IRD or GRD on the small intestine of female rats. The experiment utilized the same animals as from the first part of the thesis. In contrast to mammary gland, which is only rudimentary present before onset of puberty, the small intestine was additionally analysed on PND 25. In the small intestine, the elevated expression of PS2 and PCNA indicates an increased resistance of intestinal damage, in line with the hypothesis that loss of PS2 seems as an early indicator in developing intestinal cancer (Wu *et al.*, 1998).

The E_2 treatment led to reduced expressions of p63 and PS2 and a tendency of reduced proliferation in the present experiment. As p63 and PS2 expressions were lowest after lifelong exposure to IRD or GRD it can be taken as an indication of improved intestinal protection. The fact that only a tendency of reduced proliferation was shown can be explained by the treatment period of three days which is very short for non-classical estrogen tissues like the small intestine. Previous experiments by other groups with long-term treatment with E_2 have shown stronger protective effects (Chlebowski *et al.*, 2004; Wada-Hiraike *et al.*, 2006).

Overall, lifelong dietary exposure to ISO may improve not only the development of the mammary gland but also the small intestine of female rats. Thus, these

observations as well confirmed the hypothesis that lifelong exposure to ISO and solely GEN protects against hormone-dependent cancers such as breast or bowel cancer.

The third and final question addressed aimed to analyse whether not lifelong but only pre- to postpubertal exposure to ISO is sufficient to reduce the sensitivity of the mammary gland towards E_2. For a better comparability of the diets, the IRD was replaced by an ISO-containing diet (ISD), which had the same composition as the IDD, but included soy ISO. The ISD contained less ISO than the IRD (ISD: 147.41 µg GEN/g diet, 113.54 µg DAI/g diet, 36.06 µg GLY/g diet; IRD: 247.59 µg GEN/g diet, 213.09 µg DAI/g diet, 58.89 µg GLY/g diet, each calculated as aglycones). This results in a daily ISO consumption for an adult female rat of 33 mg/kg BW (for IRD) respectively of 19 mg/kg BW (for ISD). Given the high turn-over rate in rats compared to humans, a daily ISO intake of 17 mg/kg BW of rodents is comparable with an average ISO intake of 2.5 mg/kg BW in Eastern Asian populations, thus having the ISD appropriately reflecting the ISO intake of Asians.

For the induction of estrogen-dependent proliferation in the mammary gland, an E_2 dose of 4 µg/kg BW was chosen. While this is a lower dose than in the first breeding experiment (7.8 µg/kg BW), it falls into the range between 1 and 10 µg/kg BW which exerts physiological estrogenic effects in the rat uterus (Odum *et al.*, 1997). Both estrogen concentrations resulted in a significant increase of estrogen-induced proliferation in the mammary gland.

The results of this breeding experiment revealed that pre- to postpubertal exposure to dietary ISO results in an earlier onset of puberty and a shorter menstrual cycle. Furthermore, these animals showed an increased PR expression in the mammary gland at PND 50 and 80. In response to treatment with E_2 the PR expression was highest in pISD compared to IDD and ISD while the proliferation was reduced. This firstly suggests that pre- to postpubertal exposure is not effectual in reducing the estrogen sensitivity of the mammary gland. Secondly it could be assumed that the imprinting of estrogen sensitivity and proliferative response of the mammary gland occurs not contemporaneous but at different developmental stages.

In summary, the results from both experiments provide evidence that lifelong exposure to dietary ISO cause reduced estrogen sensitivity and proliferative response of the mammary gland, which may lead to a reduced breast cancer risk. This reduction is more significant after the intake of the full spectrum of soy ISO than after GEN alone. Furthermore, the estrogen sensitivity of the mammary gland

seems to be formed at an earlier developmental stage compared to the estrogen-dependent proliferation. Additionally, the tissue homeostasis of the small intestine was improved in the animals of the first breeding experiment, indicating a protection from developing cancer in the intestine.

Given the indication from the present thesis that lifelong exposure to dietary ISO reduces the estrogen sensitivity of the mammary gland of female rats, whereas pre- to postpubertal exposure did not, future experiments may analyse different windows of development to more precisely define the time frame of protective ISO exposure.

Future research should as well take into consideration which setup – animal or human studies – can achieve best results given the limitations of the circumstances. Extrapolations from in vivo studies to the human situation are quite difficult and should only be made cautiously. For example, this study used a conversation factor to improve comparability as the metabolism of nutrients occurs faster in rats than in humans (Setchell *et al.*, 2011). Going further, some studies indicated that equol contributes to the protective effects of ISO, and while rats are able to convert DAI to the more estrogenic metabolit equol, this ability exerts on only 30–50 % of the human population.(Shor *et al.*, 2012). Furthermore, nutritional composition differs between rats and humans, which may influence the metabolism of ISO. For example, it has been suggested that high intake of dietary fat impairs the production of ISO metabolites (Rowland *et al.*, 2000).

On the other hand, in human studies, it is not possible to include or even to determine and control all variables having an impact on the study readout, such as nutrition or life habits, making it difficult to compare the study subjects with each other. Furthermore, because of differences between the individuals, a large number of study subjects are needed to give evidence. Taken together, both study designs have advantages and disadvantages, resulting in the conclusion that a combination could be the method of choice.

The present thesis investigated the impact of ISO on the most common cancers in females: breast and bowel cancer. Looking further, the question arises about the impact of ISO exposure on cancers in men. Most frequently in men are prostate and bowel cancer and differences in the incidence of bowel cancer in men and women have been discussed (Jimenez *et al.*, 2011). The risk for bowel cancer seems to be dependent on the estrogen level as shown in studies where postmenopausal women receiving a hormone replacement therapy (HRT) have a lower cancer risk than women receiving no HRT (reviewed in (Di Leo *et al.*, 2001; Nelson *et al.*, 2002;

Kennelly *et al.*, 2008)). Yet, the influence of ISO on bowel cancer in men is discussed controversial. The Fukuoka Colorectal Cancer Study showed an inverse relationship between nutritional intake of ISO and colorectal cancer risk in men and postmenopausal women, but not in premenopausal women (Budhathoki *et al.*, 2011). In contrast, a meta-analysis revealed that soy intake was associated with a reduction of colon cancer risk in women, but not in men (Yan *et al.*, 2010).

Furthermore, the incidence of prostate cancer is much lower for men from Eastern Asia compared to European men (Adlercreutz, 1995; Fritz *et al.*, 2002; Kurahashi *et al.*, 2007), and a lot of animal models showed a reduction in prostate cancer in response to GEN (Mentor-Marcel *et al.*, 2001; Lamartiniere *et al.*, 2002a; Wang *et al.*, 2002). The underlying mechanisms are still unclear, but it has been described that GEN is able to improve prostate differentiation which may lead to reduced cancer development (Fritz *et al.*, 2002). A further study showed a reduction in the weight of prostate and seminal vesicle after oral treatment with GEN (100 mg/kg BW) for 14 days. Additionally, in this study beneficial effect of GEN treatment in a model of chronic colitis could be observed (Seibel *et al.*, 2009).

The controversial discussion shows that while the results from this study strengthen the hypothesis on ISO impact for females, it cannot be easily transferred to men. Further studies are needed to identify the impact of ISO exposure on men.

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