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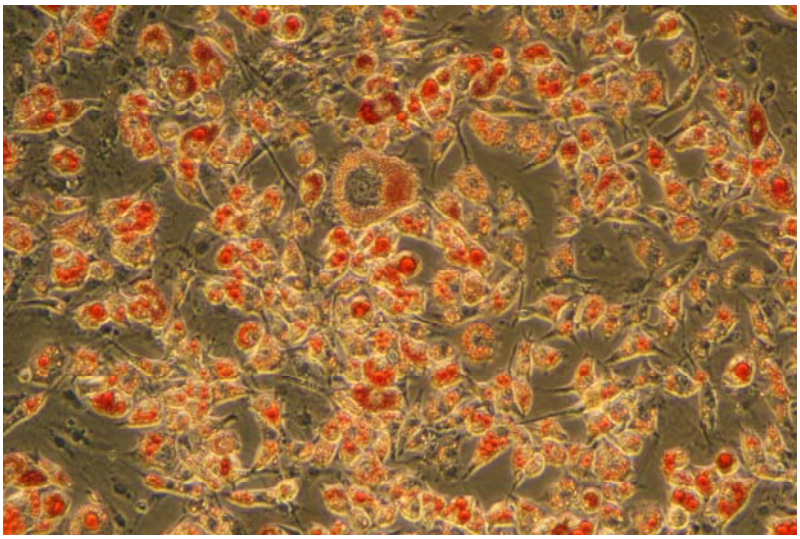
DTU Food
National Food Institute

Mechanistic evaluation of endocrine disrupting chemicals

PhD thesis by

Camilla Taxvig



Division of Toxicology and Risk Assessment
National Food Institute, Technical University of Denmark
2011

DATA SHEET

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Preface and Acknowledgements

This PhD project was carried out at the National Food Institute, Division of Toxicology and Risk Assessment, at the Technical University of Denmark under the supervision of Christine Nellemann.

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I have been fortunate to be able to go abroad during my PhD study and visit another laboratory to learn new methods and experience working both in another lab but also outside Denmark. I would in that regard like to thank Bruce Blumberg an all the people in his lab at the University of California, Irvine, Department of Development and Cell Biology, for both giving me the opportunity to come and work in his lab, but also to everybody in the lab for your help and for making me feel welcome. It was a great experience for me, which I am thankful for.

I also acknowledge the funding by The Danish Environmental Protection Agency, who financially supported part of my PhD. by the project "Metabolism of compounds before testing for effects in the T-Screen assay" (grant no. 111-00012).

And last but not least, I would like to thank my family, for support, patience and love, and the willingness to lend an ear, whenever I had a talk I needed to practice.

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Appendix 1 – Papers included as part of the PhD project

Appendix 2 – Additional published papers not part of this thesis

Summary

BACKGROUND:

This PhD project is part of the research area concerning effects of endocrine disrupters at the National Food Institute at DTU in Denmark. Endocrine disrupting chemicals (EDCs) have proved to be important for improper development of the male reproductive organs and subsequent for the potential inability to reproduce. Most recently, it was found that combinations of chemicals - each at a concentration where the single compound gave no effect - led to significant effects in experimental animals. There is a need for more knowledge about the mechanisms behind the observed effects, to be able to detect effects and predict mixture effects. In addition, a new hypothesis have emerge concerning a potential role of exposure to endocrine disrupting chemicals, and the development of obesity and obesity related diseases.

AIM:

This PhD project aimed to gain more information regarding the mechanisms behind the effects of EDCs. The focus of the current project has been on the use of *in vitro* assays to investigate:

- The endocrine disrupting potential of phytoestrogens (PEs) and mixtures of PEs
- How PEs and mixtures of PEs affect pathways involved in the development of obesity
- The use of *in vitro* metabolising systems in conection with *in vitro* testing of EDCs

METHODS:

Twelve dietary relevant phytoestrogens (PEs) either alone or in mixtures were analysed in a battery of *in vitro* bioassays designed to look for effects on: a) steroid hormone production in human adrenal corticocarcinoma cells (H295R steroid synthesis assay), b) aromatase activity in human JEG-3 choriocarcinoma cells, c) estrogenic activity using the human MCF-7 cell proliferation assay, d) interaction with the androgen receptor (AR) in a reporter gene assay, e) effects on adipogenesis in the 3T3-L1 preadipocyte cell line, and f) effect on PPAR α and γ using a transactivation assay.

For the *in vitro* metabolism studies, ten selected EDCs: five azole fungicides, three parabens, and two phthalates, were tested *in vitro* in the T-screen assay to determine possible changes in the ability of the EDCs to bind to and activate the thyroid receptor (TR) after biotransformation. The two *in vitro* metabolising systems applied were human liver S9 mix and PCB-induced rat microsomes. The ability of the two selected *in vitro* metabolising systems to metabolise the ten test

compounds, as well as the evaluation of the endogenous metabolic capacity of the GH3 cells, applied in the T-Screen assay, were examined using LC-MS analysis

RESULTS:

The results showed that all the tested PEs and PE-mixtures increased estradiol production in the H295R cells. The mixture containing all tested PEs, as well as the isoflavonoids also decreased testosterone production in H295R cells, indicating an induced aromatase activity. Furthermore, many of the tested PE-mixtures significantly stimulated MCF-7 human breast adenocarcinoma cell growth, and induced aromatase activity in JEG-3 choriocarcinoma cells.

In the PPAR transactivation assay, the different PE-mixtures had stronger effect on PPARγ than on PPARα, with some mixtures showing PPARγ agonistic effects while others had more PPARγ antagonistic effects. Furthermore, the tested single PEs, as well as mixtures of PEs had an inhibitory effect on lipid accumulation *in vitro,* although at higher concentrations than nutritionally relevant.

In the *in vitro* metabolism studies no marked difference in the effects in the T-screen assay was observed between the parent compounds and the tested metabolic extracts. The GH3 cells themselves significantly metabolised the two tested phthalates, dimethyl phthalate (DMP) and diethyl phthalate (DEP). The two *in vitro* metabolizing systems tested gave an almost complete metabolic transformation of the tested parabens and phthalates, with a recovery rate of the parent compounds of less than 1%. However, a difference was found between the human S9 and rat microsome assay systems when looking at the metabolism of the azole fungicides. The PCB-induced rat microsomes gave a statistically significant difference between the amount of parent compound before and after treatment with the microsomes for four out of the five azole fungicides tested. When using the human liver S9, no significant metabolic transformation of the azole fungicides was detected.

CONCLUSIONS AND PERSPECTIVES:

Overall, the results from the studies presented in this thesis support the evidence suggesting that nutrition relevant concentrations of PEs, both alone and in mixtures, induce various endocrine disrupting effects. The main effect seems to be an estrogenic effect mediated both at the receptor level as seen in the MCF-7 cell proliferation assay, but also at the level of steroid synthesis, as seen in the H295R cell assay. Additionally, many PEs, as well as mixtures of PEs have an inhibitory

effect on lipid accumulation *in vitro*, an effect that could involve the estrogen receptor, and also a result that could suggest a beneficial effect of PEs with regard to obesity. However, the role of the different players involved in adipogenesis and lipolysis is still not understood. Therefore, based on the current results, the influence of PEs on adipogenesis and their effects on the different pathways involved in the development of obesity and obesity related diseases remains unclear, and needs further investigation.

Finally, the results and qualitative data from the *in vitro* metabolising studies show that an *in vitro* metabolising system using liver S9 mixtures or hepatic rat microsomes could be a convenient method for the incorporation of metabolic aspects into *in vitro* testing for endocrine disrupting effects.

Dansk resumé

BAGGRUND:

Dette Ph.d.-projekt er en del af den forskning indenfor hormonforstyrrende stoffer, der foregår på DTU, Fødevareinstituttet i Danmark. Hormonforstyrrende stoffer har gennem de senere år vist sig at have betydning for udviklingen af misdannelser af drengebørns kønsorganer og for deres senere eventuelle manglende evne til at reproducere sig. Senest har det vist sig, at kombinationer af kemikalier - hver ved en koncentration, hvor enkeltstoffet ikke gav nogen effekt - medførte markante effekter i dyreforsøg. Der er brug for mere viden om mekanismerne bag de observerede effekter, for bedre at kunne detektere og forudsige dem, bl.a. også, når det gælder effekter af kombinationer af flere stoffer. Derudover er der indenfor området hormonforstyrrende stoffer kommet et nyt emne på banen, nemlig hvorvidt hormonforstyrrende stoffer kan have indvirkning på fedmeparametre og dermed på udviklingen af fedme og fedme relaterede sygdomme.

FORMÅL:

Formålet med dette Ph.d.-projekt har været at udbygge vores viden omkring mekanismerne bag effekterne af hormonforstyrrende stoffer. Fokus i dette projekt har været på brug af *in vitro* assays til at undersøge:

- De hormonforstyrrende egenskaber af phytoøstrogener og blandinger af phytoøstrogener
- Hvordan phytoøstrogener og blandinger af phytoøstrogener påvirker signalveje involveret i udviklingen af fedme
- Brugen af *in vitro* metaboliserings systemer i forbindelse med *in vitro* testning af hormonforstyrrende stoffer

METODER:

Tolv fødevare relevante phytoøstrogener enten alene eller i forskellige blandinger, blev analyseret i et batteri af *in vitro* bioassays (celleforsøg) designet til at undersøge efter effekter på: a) steroid hormon produktion i humane binyre corticocarcinoma celler (H295R steroid syntese assay'et), b) aromatase aktivitet i humane JEG-3 choriocarcinoma celler, c) østrogen aktivitet ved brug af det humane MCF-7 celle proliferations assay, d) interaktion med androgen receptoren (AR) i et reportergen assay, e) effekter på dannelsen af 3T3-L1 fedtceller samt f) påvirkningen af PPARα og γ receptorerne ved anvendelse af et transaktiverings assay.

Til *in vitro* metabolismestudierne blev ti udvalgte hormonforstyrrende stoffer: 5 azol fungicider, 3 parabener og 2 phthalater, testet i T-screen assay'et, for at undersøge eventuelle ændringer i de ti stoffers evne at binde til og aktiverer thyreoidea hormon receptoren (TR) efter omdannelse/ metabolisering. De to *in vitro* metaboliseringssystemer, der blev anvendt, var human lever S9-miks samt PCB-inducerede rotte mikrosomer. De to metaboliseringssystemers evne til at nedbryde de ti hormonforstyrrende stoffer, samt evalueringen af den endogene metaboliske kapacitet af den GH3 cellelinje, der anvendes i T-Screen assay'et, blev undersøgt ved af LC-MS analyse.

RESULTATER:

Resultaterne fra de undersøgte phytoøstrogener alene og i forskellige blandinger viste en øget østrogen produktion i H295R cellerne. Blandingen indeholdende alle 12 phytoøstrogener samt isoflavonoiderne gav også et signifikant fald i testosteron produktion, hvilket kunne tyde på en øgning af aromatase aktiviteten. Ydermere sås, at mange af de testede blandinger gav en signifikant stimulering af MCF-7 cellevæksten, samt en øget aromatase aktivitet i JEG-3 cellerne.

Forsøgene, der undersøgte PPAR receptor aktivering, viste, at de forskellige blandinger af phytoøstrogener havde større effekt på PPAR gamma end på PPAR alpha. Endvidere havde både de testede enkeltstoffer samt phytoøstrogenblandingerne en hæmmende virkning på lipid-akkumuleringen i 3T3-L1 celleforsøgene, dog ved højere koncentrationer end dem man finder i fødevarer.

I *in vitro* metabolismestudierne sås ingen signifikant forskel på effekten af moderstofferne og effekten af de metabolske ekstrakter testet i T-screen assay'et. GH3 cellerne, som blev anvendt i T-screen assay'et, var selv i stand til at metabolisere de to testede phthalater dimethyl phthalat (DMP) og diethylphthalat (DEP), hvorimod der ikke sås nogen signifikant omsætning af de andre teststoffer.

De to anvendte *in vitro* metaboliseringssystemer gav en næsten fuldstændig metabolisering af de testede parabener og phthalater, med en genfindelsesprocent af moderstoffet på mindre end 1 %. Der blev fundet en forskel mellem metaboliseringen med det humane liver S9 og de PCB-inducerede rotte mikrosomer, når det handlede om omdannelsen af azol fungiciderne. De PCB-inducerede rotte mikrosomer gav en statistisk signifikant forskel mellem mængden af moderstof målt før og efter behandling med mikrosomerne, for fire ud af de fem testede azol fungicider.

Anvendelse af den humane liver S9-miks gav ingen signifikant omdannelse af de testede azol fungicider.

KONKLUSIONER OG PERSPEKTIVER:

Samlet set støtter resultaterne fra studierne præsenteret i denne afhandling vidnesbyrdet om, at phytoøstrogener både alene og i blandinger kan have forskellige hormonforstyrrende effekter. Den overordnede effekt ser ud til at være en østrogen effekt, medieret både via receptor aktivering, som det fremgår af MCF-7 celleforsøgene, men også via effekter på steroidsyntesen, som det ses af forsøgene med H295R cellerne. Endvidere har flere phytoøstrogener og blandinger af phytoøstrogener vist sig at have en hæmmende virkning på dannelsen af lipider og på fedtcelle differentiering i *in vitro* tests. Disse effekter kan muligvis involvere østrogeneceptoren og er desuden et resultat, der kunne tyde på en gavnlig effekt af phytoøstrogener med hensyn til udviklingen af fedme. Baseret på de aktuelle resultater, samt de mage forskellige mekanismer, der er involveret i dannelsen og nedbrydningen af fedt og fedtceller, så er phytoøstrogenernes rolle eller indvirkning på disse mekanismer dog fortsat uklar og yderligere studier er nødvendige, for at kunne sige noget mere konkret om phytoøstrogenernes eventuelle gavnlige indvirkning på f.eks. udviklingen af fedme.

Endelig viser resultaterne samt de kvalitative data fra *in vitro* metabolismestudierne, at et *in vitro* system med brug af lever S9 eller rottelever mikrosomer kunne være en anvendelig metode i forbindelse med indkorporering af metabolisme i *in vitro* testning af hormonforstyrrende stoffer.

List of papers included as part of this PhD thesis

The present thesis is based on the work contained in the following papers, referred to in the text by their roman numerals:

I.: Camilla Taxvig, Anders Elleby, Katrine Sonne-Hansen, Eva C. Bonefeld-Jørgensen, Anne Marie Vinggaard, Anne Lykkesfeldt, and Christine Nellemann. Effects of nutrition relevant mixtures of phytoestrogens on steroidogenesis, aromatase, estrogen and androgen activity. *Nutrition and Cancer* 2010; 62(1):122-31

II.: Camilla Taxvig, Pelle Thonning Olesen, and Christine Nellemann. Use of external metabolizing systems when testing for endocrine disruption in the T-screen assay. *Toxicology and Applied Pharmacology* 2011 250(3): 263–269

III.: Camilla Taxvig , Ina Olmer Specht, Julie Boberg and Christine Nellemann. Effects of mixtures of phytoestrogens on adipocyte differentiation, and PPARα and PPARγ activation *in vitro*. *Submitted to Toxicological Sciences*

Abbreviations

3β/17β-HSD	3β/17β-hydroxysteroid dehydrogenase
4-AOD	4-androsten-4-ol-3,17-dione
AGD	Anogenital distance
AhR	Aryl hydrocarbon receptor
AR	Aandrogen receptor
ARE	Androgen response element
BPA	Bisphenol A
CA	Concentration addition
DEHP	Di(2-ethylhexyl) phthalate
DES	Diethylstilbestrol
DiBP	Diisobutyl phthalate
EDCs	Endocrine disrupting chemicals
ER	Estrogen receptor
GD	Gestation day
GH	Growth hormone
HPG	Hypothalamic-pituitary-gonadal
HSDs	Hydroxysteroid deshydrogenases
IA	Independent action
LOAELs	Lowest observed adverse effect levels
NOAELs	No observed adverse effect levels
NOAEC	No observed adverse effect concentration
NRs	Nuclear receptors
OECD	Organisation for economic co-operation and development
PCB	Polychlorinated biphenyls
PCO	Polycystic ovary syndrome
PEs	Phytoestrogens
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptor
SHBG	Sex hormone–binding globulin
SRM	Selective receptor modulator
SULT	Sulfotransferase enzyme
TBT	Tributyltin
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
T3	3,3',5-triiodo-thyronine
TH	Thyroid hormone
THR	Thyroid hormone receptor
TR	Thyroid receptor

1 Introduction

The overall purpose of this PhD project was broad, and aimed to gain more knowledge regarding the mechanisms of endocrine disruption chemicals (EDCs) using *in vitro* systems. Knowledge of the mechanisms of action of EDCs can help improve risk assessment, and mechanistic knowledge can be a key to understanding whether the effects are relevant to humans.

This PhD project focused on studying compounds as well as mixtures of compounds from different groups or classes, which have previously been shown to have different endocrine disrupting properties. The groups of compounds investigated included phytoestrogens (PEs), pesticides, as well as a few parabens and phthalates. The main focus, however, has been on the PEs and different mixtures of PEs.

The PhD project included two studies on mixtures of PEs. The first study examined the effects of mixtures of PEs on steroid hormone production, aromatase activity, estrogenic activity and interaction with the androgen receptor using a battery of *in vitro* assays. In the second study, mixtures of PEs as well as a few single PEs were examined for their effect on different obesity parameters *in vitro*. Specifically, the PEs were tested for their ability to affect the differentiation of preadipocytes into mature adipocytes as well as their ability to activate the peroxisome proliferator-activated receptors (PPARs).

Recently, studies conducted at the National Food Institute have shown that exposure to diisobutyl phthalate (DiBP) and butyl paraben during gestation resulted in significantly decreased plasma levels of insulin and leptin in rat foetuses. These results show that substances with known effects on steroid synthesis or estrogenic endpoints could have effects on other endocrine systems. In combination with the increasing number of published data this is supporting a new hypothesis correlating exposure to EDCs to the development of obesity. This new area of EDC research was one of the focus points in the current PhD project, with the overall aim of eventually gaining a better understanding of the mechanisms involved in the hypothesised link between EDCs and obesity.

Another area of interest in the current PhD project was the use of *in vitro* metabolising systems in connection with *in vitro* testing for endocrine disruption. Lack of knowlege on metabolism of test

substances is one of the reasons why *in vitro* research in risk assessment does not stand stronger in the regulation of chemicals. This is an area that has been almost absent in the initial tests of endocrine disruptors. In this PhD project, the metabolism or breakdown of 10 known endocrine disrupters was evaluated using different *in vitro* metabolising systems (e.g. liver S9 mix). Additionally, both the parent compounds as well as metabolic extracts were tested in an established *in vitro* assay (the T-screen assay) to compare potential effects between the effect of parent compound and metabolites in the selected *in vitro* assay. The intrinsic ability of the particular cells, applied in the selected *in vitro* assay, to metabolise the test compounds was also evaluated.

The present PhD thesis is based on the work described in papers I to III, however my contribution to the work presented in the papers is different for the three papers.
Regarding paper I: "Effects of nutrition relevant mixtures of phytoestrogens on steroidogenesis, aromatase, estrogen and androgen activity", the different studies described in the paper were the result of a collaboration with multiple national partners. I was not involved in the initial planning and the design of the studies, but got involved after the studies had been performed and have written the paper describing the results from these different *in vitro* studies.

Concerning paper II and III, I was involved in most of the work, from planning the studies to conducting the different laboratory work and analysing the data. As part of my PhD study I visited Professor Bruce Blumberg's laboratory at the University of California, where I was taught the 3T3-L1 adipocyte assay presented in paper III. However, a laboratory technician student and a master student performed the PPAR transactivation assay experiments presented in paper III, and a chemical engineer, in our division at the National Food Institute, conducted the LC-MS analyses presented in paper II.

2 Background

EDCs are a structurally diverse class of synthetic and natural compounds. The EDCs do not adversely affect organisms in the traditional sense by directly damaging cellular or physiological processes, but instead interfere with the endocrine systems, either by mimicking hormones, by blocking their effects, or by interfering with their synthesis or excretion (1, 2).

EDCs act through a variety of receptor-mediated and non-receptor-mediated mechanisms to modulate different components of the endocrine system. Receptor-mediated mechanisms have received the most attention and EDCs are typically identified as agonists or antagonists of endogenous hormone receptors. However, EDCs can act through multiple mechanisms of action such as hormone synthesis, transport, and metabolism (Figure1) (3).

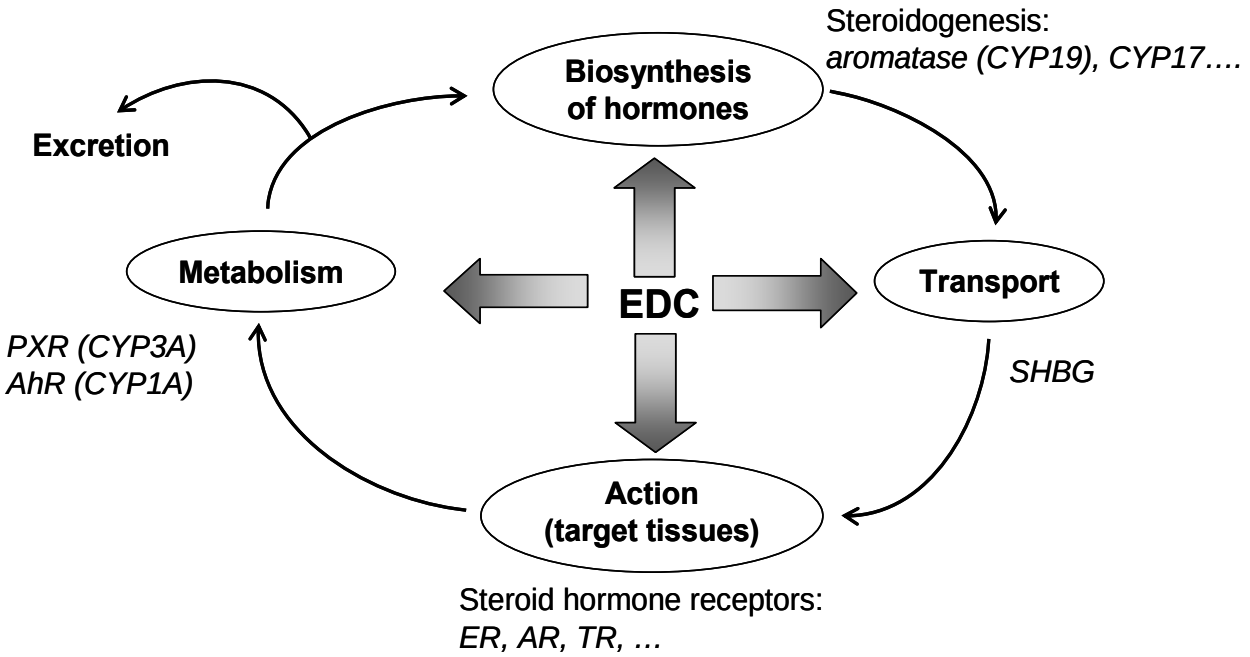

Figure 1- Endocrine disrupting chemicals (EDC) can exert their effect through a number of different mechanisms: They can mimic the biological activity of a hormone by binding to a cellular receptor (acting as an agonist or antagonist). They can bind to transport proteins in the blood, as a result altering the amounts of natural hormones that are present in the circulation. They can interfere with the metabolic processes in the body, affecting the synthesis, or breakdown rates of the natural hormones (Modified from Devillers, 2009 (2)).

Although there is considerable information on the early molecular events involved in EDC response, we still need more knowledge concerning the relationship between those molecular events and adverse health effects. For many of the associations that have been found between exposure to EDCs and a variety of biological effects, the mechanisms of action are poorly understood. This

makes it difficult to distinguish between direct and indirect effects and between primary versus secondary effects of exposure to EDCs. It also means that caution is necessary when extrapolating from *in vitro* data to *in vivo* effects, and when extrapolating from experimental data to human or wildlife situations. Concerns regarding exposure to EDCs have primarily been related to adverse effects observed in wildlife, but increased incidences of malformations of the reproductive organs in newborn boys, early onset of puberty, as well as increased incidence of certain endocrine-related human diseases are also generating concern (3-5).

EDCs include a variety of both natural compounds like phytoestrogens as well as man-made substances including pharmaceuticals and pesticides such as prochloraz and tebuconazole, as well as a number of industrial chemicals like polychlorinated biphenyls, and phthalates. Because EDCs consist of so many diverse compounds from many different sources, an exact level of EDC exposure is hard to give. The levels of exposure to EDCs also vary between people, it can differ over time, and different periods in life can be more sensitive to such exposure. The time period considered to be particular sensitive to EDC exposure is foetal development, and foetuses and infants as well as pregnant women are of special concern when it comes to risk assessment of EDCs.

2.1 Mechanisms of Endocrine Disruption

2.1.1 Receptor-mediated mechanisms

The first characterised mechanism of action for endocrine disruptors was the ability to act directly as ligands for steroid hormone nuclear receptors (NRs), in particular estrogen, androgen, and thyroid hormone receptors. NRs are a class of proteins found within the interior of cells that are responsible for sensing the presence of hormones. In response, these receptors act together with other proteins to regulate the expression of specific genes (6).

It has been observed that some effects of binding of hormone to the receptor occur within seconds or minutes, which is inconsistent with the genomic mechanism of nuclear receptor action, (genomic pathway: hormone binding to cytosolic receptors, subsequent modulation of gene expression followed by protein synthesis) and it is therefore believed that there are variants of nuclear receptors that are membrane associated instead of being located in the cytosol or nucleus (7, 8). The existence of a specific plasma membrane estrogen receptor has been known for many years, but in general the area of membrane associated NRs is still poorly understood (7, 9).

When a compound is bound to a receptor, it can have various effects. A substance that binds to a receptor and triggers a response is called an agonist. Agonists often mimic the action of the naturally occurring ligand, however, often with different potencies. In contrast to an agonist, an antagonist blocks the action of the agonist or natural ligand.

Thus, receptors can be activated or inactivated by either endogenous (e.g., hormones and neuro-transmitters) or exogenous (e.g., drugs) agonists and antagonists, resulting in stimulaton or inhibition of biological responses, respectively. The potency of an agonist is inversely related to its EC_{50} value. The EC_{50} can be measured for a given agonist by determining the concentration of agonist needed to elicit half of the maximum biological response of the agonist. The EC_{50} value is useful for comparing the potencies of drugs with similar efficacies producing physiologically similar effects. The smaller the EC_{50} value, the greater the potency of the agonist, and the lower the concentration required to elicit the maximum biological response.

Studies have demonstrated that ligands can concurrently behave as agonist and antagonists at the same receptor, depending on effector-pathways or tissue type (10-12). Selective receptor modulators (SRMs) are receptor ligands that exhibit agonistic or antagonistic ability in a cell- and tissue dependent manner. An example of a SRM is tamoxifen, which is a selective estrogen receptor modulator that can activate or inhibit estrogen receptor action, acting as an agonist in some tissues and an antagonist in other tissues (13).

The properties of SRMs are due in part to unique ligand-induced conformational changes in the hormone receptor that influence their abilities to interact with other proteins, such as co-activators and co-repressors. The relative balance of co-activator and co-repressor expression within a given target cell determines the relative agonist vs. antagonist activity of the SRMs. However, other factors, like cellular environment also play a critical role in determining the properties of the SRMs. Cellular signalling, for example, influences the activity and sub-cellular localization of co-activators and co-repressors as well as nuclear receptors and this contributes to gene-, cell-, and tissue-specific responses to SRM ligands (13).

2.1.2 Non-receptor-mediated mechanisms

Interference with steroidogenic enzymes and hormone transport

The ability of xenobiotics to disrupt steroidogenesis and the mechanisms by which these compounds interfere with the function of steroidogenic enzymes are very complex. Nevertheless,

key enzymes involved in steroid hormone synthesis and metabolism are considered as important targets for EDCs. The cytochrome P450 enzymes responsible for the highly specific reactions in the steroid biosynthesis pathway are some of the molecular targets of interest, given their key role in the formation of various highly potent endogenous steroid hormones (Figure 2) (14).

The cytochrome P450 enzymes consist of several specific cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs), and steroid reductases (15). The enzyme that has received the most attention with regard to endocrine disruptors is aromatase (CYP19) that converts androgens to estrogens (Figure 2). However, all the steroidogenic enzymes are potential targets for disruption and we, as well as others have previously shown that CYP17 could be involved in the endocrine disrupting effect of azole fungicides (16, 17).

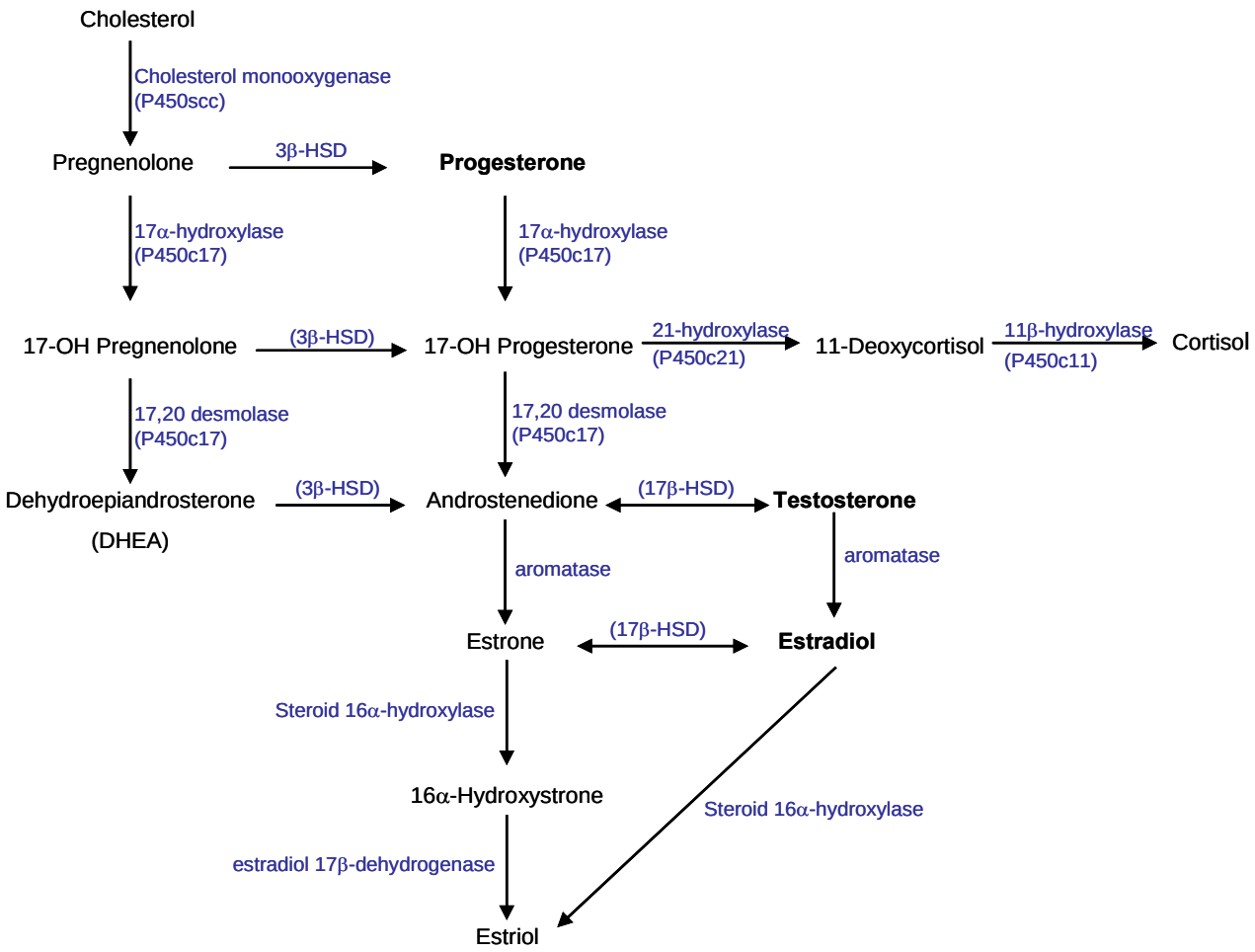

Figure 2 – The Steroid hormone synthesis pathway. Enzymes are highlighted in blue.

Another target for endocrine disrupters is binding proteins (Figure 1). In most vertebrate species, sex steroid hormones circulate in the plasma predominantly bound to a specific high-affinity sex hormone–binding globulin (SHBG) and low-affinity proteins such as corticosteroid-binding proteins and albumin (18). In the blood, typically 97% to 99% of total estrogens and androgens are carried bound to these proteins. Although the functions of the SHBG are not fully understood, SHBG is believed to be involved in regulating circulating endogenous sex steroids as well as cellular signal transduction to nuclear steroid receptors in sex steroid–sensitive tissues (19). Interestingly, binding proteins have been reported to bind several endocrine disruptors, including bisphenol A (BPA), 4-nonylphenol, and genistein (20, 21) and to alter the biological activity of natural and synthetic estrogens (22, 23). The consequence is that modulation of SHBG constitutes an indirect pathway for regulating steroid hormone action.

Transgenerational consequences of EDC exposure

Recent studies have demonstrated the ability of EDCs to have epigenetic effects (24-26). The term epigenetics is defined as the molecular phenomena that regulate gene expression without alterations in the DNA sequence (27). When these epigenetic changes occur during certain stages of development, they are permanent and can be inherited by the offspring. Epigenetics provides a framework to explain the source of variations between individual organisms (28) and also explains what makes cells, tissues, and organs different despite the identical nature of the genetic information in every cell in the body. The most studied epigenetic modification is DNA methylation of CpG nucleotides that are essential for mammalian development (29). In most studies, increased DNA methylation is associated with gene silencing, and decreased methylation is associated with gene activation. These epigenetic mechanisms can help explain the transgenerational effects observed for some hormonally active chemicals. Examples of this include exposure to diethylstilbestrol (DES) during pregnancy, which resulted in vaginal adenocarcinoma in female offspring in humans (30) and mice (31). Rats treated with the estrogenic pesticide methoxychlor or the antiandrogenic fungicide vinclozolin during pregnancy produced male offspring that had decreased sperm capacity and fertility, and the compromised fertility was passed on through the adult male germ line for four generations (26). The authors demonstrated altered patterns of DNA methylation in germ cells of generation two and three. Other environmental contaminants, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls (PCBs), and phthalate

esters have also been found to affect the reproductive system or induce tumour development by altering DNA methylation, and steroid hormone metabolism and signalling (32-34).

2.2 Methods for investigating EDCs *in vitro*

Both *in vivo* and *in vitro* tests are presently being developed and proposed for the testing of endocrine disrupting activity of different compounds. The *in vivo* or animal tests are necessary because it is not possible to model the complex responses of a whole body to endocrine disrupters (EDs), including aspects like uptake, distribution and metabolism of compounds. However, despite limitations like the ones just mentioned, *in vitro* studies have many advantages. These include their relative low cost, the fact that they are less time consuming than *in vivo* studies, the possibility to test many compounds at several different concentrations and repeat the test several times, and their usefulness in getting mechanistic information. The fact that an *in vitro* test is generally studying one mechanism, isolated, without interference from the total connecting environment, like you have in a whole body organism, can be both an advantage and a limitation. Therefore, a single *in vitro* assay can never stand alone, but will require information from other studies to give a realistic or good picture of what is going on in an *in vivo* situation.
Given the wide range of potential working mechanisms and sites of action of EDCs, different *in vitro* bioassays have been developed for the testing of EDCs:

Examples of assays for testing endocrine disruption *in vitro*:

- Binding assays (e.g., ERα)
- Reporter gene assays (e.g., ER, AR, AhR)
- Proliferations assay (e.g., E-screen and T-screen)
- Induction/inhibition of enzymes (e.g., aromatase)
- Production of steroid hormones (e.g., H295R steroidogenesis assay)

With regard to endocrine disruption, only few of the available *in vitro* tests have currently completed the process of validation and have been adapted as OECD (Organisation for Economic Co-operation and Development) test guidelines. Therefore, they can not be used for regulatory purposes. However, they are still very important and useful tools in research and in understanding the mechanisms behind the effects of the different EDC compounds.

In the studies included in this PhD project, different *in vitro* assays have been used in the investigation of the selected test compounds. In the methods section, a short description will be given of each assay employed in this PhD project.

2.3 The different groups of EDCs studied in this PhD project

In this PhD project the different studies conducted have together investigated the endocrine disrupting potential of four groups of compounds: Phytoestrogens, azole fungicides, parabens and phthalates. The current section will give a short general description of these four groups of endocrine disrupting compounds.

2.3.1 Phytoestrogens (PEs)

PEs are a diverse group of naturally occurring plant compounds that have the ability to cause estrogenic or/and antiestrogenic effects because of their structural similarity with oestradiol (35).
The broad classes of PEs includes the isoflavonoids (genistein, daidzein, formonetin, biochanin A, and equol) found predominantly in soy beans and red clover, the lignans (enterolactone, enterodiol, pinoresinol, lariciresinol, secoisolariciresinol, matairesinol) found particularly in flax seed and fruits, and the coumestans (e.g., coumestrol) found in red clover and a variety of other plants. Several of the PEs are ingested as precursors and then converted by the microflora in the mammalian gut. Lignans for instance, are metabolised by intestinal bacteria to form the mammalian lignans known as enterodiol and enterolactone (36). The major isoflavonoids daidzin and genistin are metabolised in mammals to form the active compounds daidzein and genistein. Daidzein is further metabolised to the compound equol (37). The extent of the metabolism of PEs by the intestinal bacteria appears to be highly variable among individuals and for example, only one-third to one-half of the population can metabolise the soy isoflavone daidzein to equol (38, 39).
Many of the metabolites have been shown to have more pronounced effects compared to the parent compound (37, 40). Several *in vivo* studies indicate that PEs may elicit cancer-preventing effects in laboratory animals exposed to a cancer-inducing agent (37, 41, 42). Furthermore, epidemiological data suggest that a diet rich in PEs decreases the risk of breast cancer, among others based on the fact that the breast cancer incidence is much lower in Asian populations in comparison with Western populations (43). However, other studies, both animal and *in vitro*, have shown that the effects of PEs may increase cancer risk (44-47).

PEs are weak estrogens, but may also function as estrogen antagonists by blocking the binding of the much more potent endogenous estrogen to its receptor. In addition, it has been shown that PEs can affect the synthesis or metabolism of steroid hormones, for example by affecting aromatase activity. Aromatase activity is important for the balance between the endogenous testosterone and 17ß-estradiol, and changes in this balance may be beneficial or adverse in relation to proliferation of e.g. breast cancer tumor cells as 17ß-oestradiol is considered the main determinant of proliferation of estrogen-dependent breast cancer cells (48). The major aromatase dependent estradiol synthesis occurs in the ovary in premenopausal women and in peripheral tissues in postmenopausal women (such as the adrenals) (49). Many papers refer to the inhibitory effects of PEs on the aromatase activity (40, 50-52), but it cannot be generalised that these compounds elicit inhibitory effects, and it has been found that some PEs in fact induce aromatase activity (53-55). The available literature provides conflicting findings as to the effects of PEs, which probably results from the multiple mechanisms of action of these compounds. The complexity of the influence of PEs at the cellular and molecular level is further increased by the fact that their effects are dependent on the dose, the class to which they belong, the presence or absence of endogenous estrogens, their different affinity for the estrogen receptor (ER) α and ERß, and the type of tissue or cell considered (52).

2.3.2 Azole fungicides

Pesticides are of great concern for potential adverse human health effects. The extensive use of pesticides leads to exposure risk of the general population and several studies have shown the presence of the pesticides and their residues in the environment, in food and in human tissues worldwide (56-58). Epidemiological research has indicated a causal connection between human exposure to pesticides and endocrine disrupting effects such as poor sperm quality (59) and increased incidence of cryptorchidism (60).

One class of pesticides that has been studied extensively is the socalled azole fungicides. Based on their chemical structure, azole compounds are classified as triazoles and imidazoles; however, their antifungal activity is due to the same molecular mechanism.

The azole fungicides are used agriculturally to control rust and mildew on fruit, vegetables, cereals and seeds, and in pharmaceutical applications for the treatment of local and systemic fungal infections, but also as drugs in tumour chemotherapy. Their antifungal activities are based on the disruption of fungal cell membranes by inhibiting fungal lanosterol-14α-demethylase (cyp51) (61). Cyp51 is evolutionarily conserved between plants, fungi, and animals and is critical for cholesterol synthesis and therefore steroid biosynthesis in animals (62). Besides Cyp51, triazoles also modulate

the gene expression and enzyme activity of multiple cytochrome P450 (CYP) and other metabolic enzymes in mammalian liver and other tissues (63-65), and the aromatase enzyme (Cyp19) is one of the cytochrome P450 monooxygenase enzymes that is inhibited by azoles (66-68). However, our own research on the imidazole fungicide prochloraz, as well as other studies on azole fungicides, indicates that azole fungicides have the ability to act through multiple mechanisms and to induce various endocrine disrupting effects (69, 70)

2.3.3 Parabens

Parabens are a group of alkyl esters of p-hydroxybenzoic acid and typically include methyl paraben, ethyl paraben, propyl paraben, butyl paraben, isobutyl paraben, isopropyl paraben, and benzyl paraben. The parabens or their salts are widely used as preservatives in cosmetics, toiletries, and pharmaceuticals. Additionally, methyl- and ethylparaben are permitted in certain foods in limited amounts. However, concern has been raised for the potential endocrine disrupting effects of parabens. They have been studied in a number of *in vitro* and *in vivo* systems, and many of the parabens have been shown to have weak estrogenic activity, and some, including butyl paraben, also caused reduction in testosterone levels and in sperm production in rats (71-75). However, the ability of parabens to activate the estrogen receptor may not be the main mechanism of action. Further endocrine disrupting activity has been demonstrated including the ability of parabens to antagonize AR-mediated events in androgen-responsive cells and to act as sulfotransferase enzyme (SULT) inhibitors, as well as the ability to impair mitochondrial function in rat hepatocytes (76).
Possibly, the influence of parabens (together with other chemicals) on human tissues is wider than what has previously been anticipated and research is needed to establish how wide the implications may actually be (77).

2.3.4 Phthalates

Phthalates are esters of phthalic acid that have been used worldwide as plasticizers to impart flexibility, softness, and elasticity to otherwise rigid polymers such as PVC. Phthalates are found in industrial paints and solvents but also in toys, personal-care products, food packaging materials, and medical devices such as intravenous tubing and blood transfusion bags.
Because phthalates are not covalently bound to the polymer matrix, they are highly susceptible to leaching. As a result, phthalates contaminate food, particularly meat and milk products, and are found nearly everywhere in interior environments. In addition, important routes of human exposure include dermal uptake from personal-care products and from plastic medical devices that come into

direct contact with biological fluids. Exposure to phthalates can occur in the developing foetus through the placenta-blood barrier and in postnatal stages during breast feeding or from toys and baby-care products (78).

Studies in rodents exposed to certain phthalates, have shown that high doses caused changes in hormone levels and birth defects (79), and experimental studies with low doses of di(2-ethylhexyl) phthalate (DEHP) have demonstrated reproductive toxicity in male rodents (80, 81). Maternal urine levels of metabolites of DEHP have been reported to be associated with a higher risk of incomplete testicular descent for male human infants as well as being inversely correlated with the anogenital distance (AGD), an endpoint often measured in animal studies, where it has been found to be a sensitive marker for antiandrogenic influence during gestation (4, 82, 83).

More recently, several studies have demonstrated a correlation between phthalates and metabolic disorders. In short- and long-term rodent studies, dose-related deregulation of levels of serum insulin, blood glucose, liver glycogen, and cortisol were observed (84, 85). In humans, the log-transformed concentrations of several phthalate metabolites positively correlated with abdominal obesity and insulin resistance in adult males (86), and mono-ethyl-hexyl-phthalate MEHP), a metabolite of DEHP, has been found to interact with all three PPARs, which are known to play a role in lipid and carbohydrate metabolism (87).

2.4 Evidence of a potential link between EDCs and development of obesity

It is now well documented that numerous environmental chemicals can interfere with complex endocrine signalling pathways and cause adverse effects (88). The centre of attention for EDCs has earlier been on reproductive parameters and potential carcinogenic effects, but it is now broadened by the knowledge that multiple organ systems can be affected by EDCs. One of the new emerging effects or mode of action for EDCs is that exposure to EDCs may play a role in obesity.

Much interest has focused on the need to incorporate healthy foods in our diets and more physical activity into our lifestyle. However, the prevalence of obesity is still rising and the exact cause of obesity is unclear. Today it is known that obesity is not simply a product of a too high caloric intake compared to the amount of calories burned. Although, it is a fact that fat cannot be accumulated without a higher caloric intake than expenditure, recent studies suggests the existence of chemicals that alter regulation of energy balance to favour weight gain and obesity (89). Nevertheless, the

relative contributions of environmental factors to the development of both obesity and obesity related diabetes are far from understood.

A variety of factors controls the accumulation of fat and mobilisation of lipids from adipose storage depots. These include the hormonal regulation of appetite, glucose level, central control of basal metabolic rate, regulation of metabolic set points, and the number, size and metabolic activity of adipocytes. Furthermore, adipose tissues themselves function as endocrine organs and participate in the body's feedback systems that help to fine tune appetite and satiety. Therefore, obesity is, among others thought to result from a prolonged disturbance in the regulation of energy metabolism in favor of triglyceride storage and adipocyte hypertrophy, as well as genetic factors known to impact hunger and satiety (89, 90).

Although the evidence is still limited, accumulating data are pointing to the potential role of EDCs, either directly or indirectly in the pathogenesis of adipogenesis and diabetes, as well as in the development of obesity itself. Some of the research supporting this hypothesis describes excess weight gain in animals treated developmentally with certain EDCs, including diethylstilbestrol (DES) and BPA (91-93) as well as data from epidemiologic studies showing a possible association between serum dioxin levels and the onset and incidence rate of diabetes (94). Numerous chemicals/pharmaceuticals, used for instance in oncology, cardiology, immunology, and psychiatry, have weight gain as a side effect (89, 95). Furthermore, many synthetic chemicals are known to increase weight gain in animals, because they have especially been used by the livestock industry to promote animal fattening and growth (96, 97).

Additionally, in one of our own studies, we found that DiBP, butylparaben, and rosiglitazone reduced plasma leptin and/or insulin levels in 21-day old male and female foetuses after exposure of pregnant rats from gestation day (GD) 7–21 (98).

However, to confirm the hypothesis about EDCs and their effect on the development of obesity, an appropriate molecular target affecting regulation of adipose physiology must be found. Altered parameters of adipocyte biology that result in depot remodeling or gain of adipose mass leading to obesity would be a good confirmation of a link between EDCs and obesity. Furthermore, a corresponding dose-response relationship between perturbations and realistic environmental exposures is needed, as well as epidemiological data that establish a connection for a significant fraction of the population (99).

Overall, the published studies so far, indicate that exposures to various EDCs may play a role in obesity and the complications connected to this, such as type 2 diabetes. However, since many

classes of chemicals maybe involved, the mode of action and the mechanisms behind are likely many and complex, acting through more than one pathway, and further research is required to elucidate all potential interactions between environmental substances and metabolic dysregulation (95).

2.4.1 The major modes and mechanisms of actions involved in potential EDC induced obesity

There are numerous potential targets for EDCs that can result in obesity. The mechanisms involved are very complex and not fully elucidated. Figure 3 shows schematically a simplification of the potential mechanisms involved in EDC induced obesity, as well as the different factors involved in these mechanisms. The suggested targets include ERs, the aryl hydrocarbon receptor (AhR) and PPARγ (100).

The most described mechanisms involve activation of PPARγ, dysregulation of sex steroids, and interference with the programming of metabolic set points.

At the basic level, the progression to obesity occurs when the adipocyte number and lipid content increases. A number of transcriptional regulators are critical with regards to the regulation of genes controlling intracellular lipid flux, and adipocyte proliferation and differentiation. Among these are nuclear hormone receptors, especially the PPARα, -β and –γ (Figure 9). The PPARs form heterodimers with the 9-cis retinoic acid receptor (RXR) and play a preeminent role, serving as metabolic ligand sensors for a variety of lipophilic hormones, dietary fatty acids and their metabolites (89, 90).

Activation of the RXR-PPARα heterodimer promotes peroxisome proliferation and stimulates β-oxidation of fatty acids (Figure 9). Therefore, xenobiotics that target PPARα usually act as lipid-lowering agents (90). In contrast, activation of the RXR-PPARγ heterodimer favors lipid biosynthesis and storage and is essential for the differentiation of pre-adipocytes (101). PPARγ agonists, such as the thiazolidinediones, rosiglitazone and pioglitazone, are potent insulin sensitizing pharmaceuticals used to improve glycemic control and serum triglycerides in diabetics. However, prolonged use of these drugs is associated with persistent weight gain (102).

In addition to nutrition sensing NRs, such as PPARs, NRs for sex steroid hormones are also known to influence the development of adipose tissue. The hormones help to integrate metabolic functions among major organs that are essential for metabolically intensive activities, like foetal development.

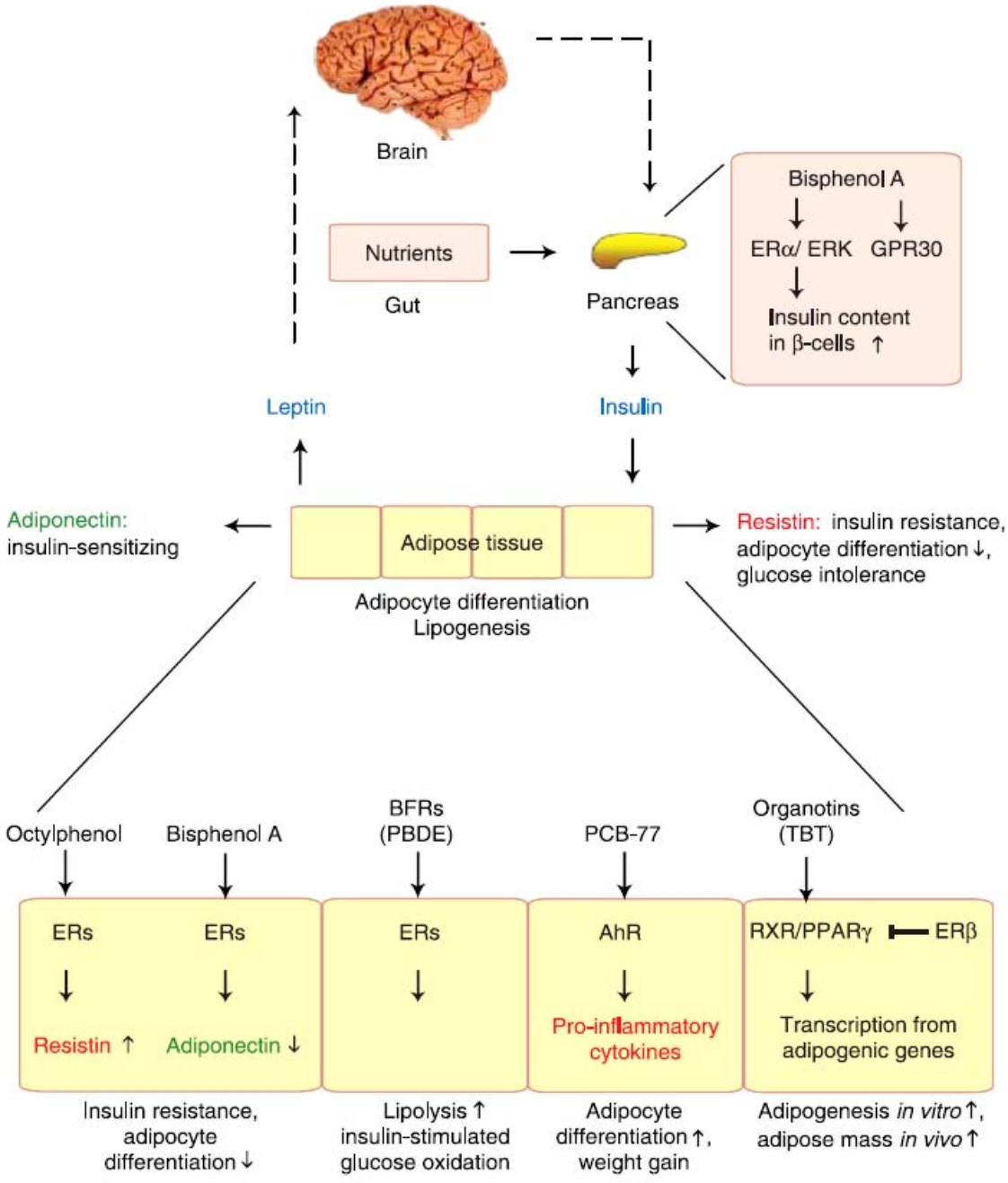

Figure 3 Summary of effects of EDCs on the metabolic system. Schematic showing simplified complex interactions between released adipokines, receptors, and EDCs in adipose tissue and pancreas. ERs, estrogen receptors, GPR30, G protein-coupled estrogen receptor 1, PBDE, polybrominated diphenyl ether, TBT, tributyltin, RXR, retinoid X receptor, PPAR, peroxisome proliferator-activated receptor, POPs, persistent organic pollutants, BFRs, brominated flame retardants and MetS, metabolic syndrome. (Borrewod from Swedenborg , et al., 2009;43:1-10 (100))

Knockout of sex steroid pathway components, for instance the FSH receptor, the aromatase, the estrogen receptor (ER), and the androgen receptor (AR), show that sex steroids are required to regulate adipocyte hypertrophy and hyperplasia, and that they influence the sex-specific remodeling of specific adipose depots (89, 90).

Together with peptide hormones such as growth hormone (GH), sex steroids mobilize lipid stores and help to counteract the actions of insulin and cortisol that promote lipid accumulation in adults. Therefore, imbalances in these hormones are often observed in genetic, clinical and physiological conditions, including Cushing's syndrome, polycystic ovary syndrome (PCO), GH-deficiency, menopause, alcoholism and depression, conditions that are correlated with dyslipidemia and obesity. Consistent with this, anti-androgenic therapies for prostrate cancer and PCO syndrome produce weight gain, whereas estrogenic hormone replacement therapy is generally protective against the changes in body fat remodeling seen with age and menopause in women (103). Notably dietary compounds such as isoflavonoids modulate ER signaling and have been shown to have similar positive effects on adipose tissues. As an example, nutritional levels of genistein and daidzein have been shown to reverse fat accumulation in post-menopausal women and ovariectomized rodent models (104-107).

Homeostasis is a central feature of all endocrine systems. However, this homeostasis or balance needs to be set up or programmed before the system will work correctly, and for many of the endocrine systems this programming takes place largely during foetal/neonatal development (108). Regarding the interference with the programming of metabolic set points, hypothalamic output plays an important role, because of its involvement in implementing adaptive responses that establish metabolic set points and regulate overall metabolic efficiency. The control over these adaptive processes lies in the hypothalamus-pituitary-thyroid axis that determines systemic thyroid hormone output. Thyroid hormones exerts widespread effects on metabolism and sets the basal metabolic rate (89).

Another critical factor of the hypothalamic output is the regulation of glucocorticoid hormone signaling that mediates stress responses and metabolic functions in peripheral tissues. Glucocorticoids play an important role in promoting adipocyte differentiation, and disruption of glucocorticoid signaling during critical periods in life (e.g., perinatal) can affect long-term metabolic programming and the response to physiological challenges (109, 110).

2.5 *In vitro* metabolism

In vitro studies are a good tool for getting mechanistic information regarding the effects of a compound. However, lack of metabolism of test substances is one of the limitations of many *in vitro* assays for endocrine disruption, and also one of the reasons why *in vitro* research do not stand stronger in the regulation of chemicals.

Metabolism occurs especially in the liver, but also in extra hepatic tissues (111, 112), and can be divided into phase I and phase II metabolism. Phase I metabolism involves the oxidation, reduction or hydrolysis of the parent compound into a more polar substance. The key enzymes for phase I oxidation are the isoforms of the CYP family of enzymes (113). However, CYP enzymes can often be induced or inhibited by exogenous chemicals that may exert endocrine effects through this mechanism. Phase I metabolism has been shown to metabolise xenobiotics to both more and less active or toxic compounds (114-116). Phase II metabolism often involves the further conjugation of the metabolite with polar molecules, such as sulphate, amino acids, glutathione or glucuronic acid, generating metabolites that are more soluble and thus more easily eliminated (112).

It is well established that information on the metabolism of a substance is important in the evaluation of its toxic potential. With regard to endocrine disruptors, one also needs to consider that metabolism of the xenobiotic can alter, not only its own potency, but also the toxic potential of other substances. Hormones, and in particular steroid hormones, are formed or metabolised by enzymes that participate in the metabolism of xenobiotics. A chemical that is metabolised by these enzymes may, by mere interaction with the enzyme, act as a competitor for the hormone or hormone precursor and therefore disturb hormone levels and hormonal equilibrium.

A number of *in vitro* systems are available for studying metabolism. They include both cellular (tissue slices, isolated and cultured hepatocytes, liver cell lines) and subcellular (microsomes, recombinant enzymes) systems (117).

Depending on the study, different factors can influence the choice of *in vitro* metabolising system. However, it is well known that there are many differences in the metabolic capacity between species and also differences within the same species associated with age, gender, nutrition, genetic predisposition, environmental factors and others. In regards to risk assessment for EDCs, it would be most relevant to use metabolising systems based on materials from humans. However, in every day research using materials from animals compared to humans would most likely be easier both in terms of amount of material assessable, and currently also financially more favourable. Therefore, it

is relevant to get as much data as possible in order to make valid comparisons and extrapolations between results from different species.

2.6 Assessing mixtures

Humans are exposed to a mixture of various chemicals some of which may have endocrine disrupting activity, and it is conceivable that the overall effect of exposure to many compounds simultaneously will result in more severe effects than observed for each individual chemical. Risk assessment of toxicological effects is currently based on determination of the effects of single compounds, i.e. the lowest observed adverse effect levels (LOAELs) and the no observed adverse effect levels (NOAELs) or no observed adverse effect concentration (NOAEC). However, even though the exposure scenario may be complex, the need to consider mixture effects when assessing the risk associated with not just endocrine disrupters but all types of chemicals has been widely recognised (118, 119). Increasing numbers of studies specifically involving chemicals with common modes of action, but also with mixtures composed of chemicals with dissimilar modes of action, show that many chemicals work together to produce combination effects that are larger than the effects of each mixture component by itself (120).

The prediction of mixture effects is complicated, but different models are available. Mixtures of compounds displaying a linear concentration-response relationship may be predicted using effect summation. The assumption behind this is that the total effect of the mixture is equal to the summation of the effect of the single compounds at a given concentration. However, most compounds display non-linear concentration-response curves and for these compounds two models are currently used: The concentration (or dose) addition' (CA) and 'independent action' (IA) models. Both models assume that the compounds in the mixtures do not interact (physically, chemically, or biologically), meaning that one compound does not influence the toxicity of the other compounds (120).

The CA model has generally proved applicable to mixtures of compounds known to have similar target sites, similar mode of action and that only differs in their individual potency. The mixture effects are predicted by summing up the concentrations of the individual chemicals adjusted for their relative potency (121). It is assumed that all compounds in the mixture behave as if they were a dilution of one another. This means that any concentration of any compound must be considered,

also compounds present in concentrations below their NOAEC, because it adds to the mixture concentration, and combination effects can result as long as enough compounds are present to sum up to a sufficiently high total concentration (119).

The IA model is generally said to be applicable for mixtures of compounds having different target sites or modes of action. The IA model uses the individual effects of the mixture components to calculate the expected mixture effect. This means that compounds present at doses below their individual effect thresholds or NOAECs will not contribute to the mixture effect. NOAEC is the highest test concentration that does not provoke any statistically significant effect, and it is a measure highly sensitive to experimental design issues. If the NOAEC is a true zero effect concentration, then it will not contribute to the mixture effect. However, if the NOAEC displays a small but significant (although maybe not statistically) effect, this effect could in a mixture with enough components sum up to have a significant effect, and then the IA model would underestimate the effect (120).

However, the theoretical use of IA and CA is based on previous knowledge of chemical mode of action, and studies suggest that toxic effects of mixtures in an intact organism are far too complicated to be predicted from knowledge of mode of action alone. Secondary modes of action, such as uptake kinetics, transportation, metabolism and excretion of the chemicals are processes that are not considered by the models, but can have large impacts on the combined effects (121).

In terms of risk assessment it is currently debated which reference model should be used. Recent data suggest that the IA model does not provide a considerably better prediction of a mixture effect than the CA model when measuring on mono-species test systems, and studies have shown that the CA model can be applicable both for similar as well as dissimilar acting endocrine disrupters (122, 123). Additionally, several *in vitro* studies have demonstrated that endocrine disrupters can generate additive mixture effects both on the ER (124-126) and the AR (127, 128), even when they are present at levels under their NOAEC (123, 129).

Currently, work is being done with the aim of creating useful guidelines for the implementation of assessment of mixture effects in risk assessment of EDCs (130), but so far there is good evidence that EDCs, at least them with similar effects, produce combination effects that can be predicted using the CA model (120).

3 Aims of the studies

As mentioned in the introduction of this thesis, the overall purpose of the PhD project was broad and aimed to gain more knowledge regarding the mechanisms of EDCs.
Using a panel of *in vitro* assays different groups of EDCs including both single compounds as well as mixtures were investigated in three different studies, each with a different aim.

1) Effects of nutrition relevant mixtures of phytoestrogens on steroidogenesis, aromatase, estrogen and androgen activity.

   This study examined the effects of different mixtures of PEs on steroid hormone production, aromatase activity, estrogenic activity and interaction with the androgen receptor, using a panel of four *in vitro* assays. The overall aim was to evaluate the endocrine disrupting potential of various PEs and mixtures of PEs naturally occurring in Western/Scandinavian food.

2) Use of external metabolising systems when testing for endocrine disruption in the T-screen assay

   In this study the use of *in vitro* metabolising systems in connection with *in vitro* testing for endocrine disruption was addressed.
   The aim of the study was threefold a) to test different *in vitro* systems for biotransformation of ten known EDCs, b) to determine possible changes in the endocrine disrupting ability of the EDCs after biotransformation using the T-Screen assay as a model, and finally c) to investigate the endogenous metabolic capacity of the applied cell line (GH3 cells).
   The overall purpose of the study was to find a potential method for the incorporation of metabolism into *in vitro* testing for endocrine disruptors.

3) Effects of mixtures of phytoestrogens on adipocyte differentiation, and PPARα and PPARγ activation *in vitro*

   In this study the same kind of mixtures of PEs as tested in study 1) as well as a few single PEs were examined for their effects on different obesity parameters *in vitro*. Specifically, the PEs were tested for their ability to affect the differentiation of preadipocytes into mature adipocytes, as well as their ability to activate the PPARα and γ receptors.

The overall aim of this study was to gain a better understanding of the mechanisms involved in the suspected link between exposure to EDCs, here phytoestrogens, and development of obesity and obesity-related diseases.

4 Methods

This section gives a general description of the *in vitro* methods used in the studies presented in paper I to III (Appendix 1). A more detailed protocol for the different *in vitro* assays is found in the respective papers (Appendix 1).

Table 1: Overview of the *in vitro* assays applied in the different studies

***In vitro* assay**	**Test compounds**	**Purpose**	**Paper**
AR reporter gene assay	PEs and mixtures of PEs (genistein, daidzein, equol, formonetin, biochanin A, enterolactone, enterodiol, pinoresinol, lariciresinol, secoisolariciresinol, matairesinol and coumestrol)	Detect interaction with the AR (AR agonist or antagonist)	I
MCF-7 cell proliferation assay	PEs and mixtures of PEs (as in the AR assay)	Detect estrogenic or anti-estrogenic effects	I
H295R steroidogenesis assay	PEs and mixtures of PEs (as in the AR assay)	Detect effects on steroid hormone synthesis	I
Aromatase assay	PEs and mixtures of PEs (as in the AR assay)	Detect inhibition of the aromatase enzyme	I
T-Screen assay	5 azole fungicides: prochloraz, ketoconazole, miconazole, tebuconazole and epoxiconazole; 3 parabens: butyl-, ethyl- and methyl paraben, and 2 phthalates: dimethyl phthalate (DMP) and diethyl phthalate (DEP).	Detection of TR agonistic or antagonistic abilities	II
PPAR transactivation assay	PEs and mixtures of PEs (as in the AR assay)	Detect PPAR α and/or γ agonist or antagonist effects	III
3T3-L1 adipogenesis assay	PEs and mixtures of PEs (as in the AR assay)	Detect effects on differentiation of preadipocytes into adipocytes	III
In vitro metabolising systems	5 azole fungicides: prochloraz, ketoconazole, miconazole, tebuconazole and epoxiconazole; 3 parabens: butyl-, ethyl- and methyl paraben, and 2 phthalates: dimethyl phthalate (DMP) and diethyl phthalate (DEP).	*In vitro* biotransformation of test compounds before further *in vitro* testing	II

4.1 AR reporter gene assay

In a reporter gene assay, the general principle is that ligand-bound nuclear hormone receptors bind specific response element sequences on the DNA and initiate transcription of a downstream gene (131).

The cell-based AR reporter gene assay investigates the ability of EDCs to interact directly with the AR or to interfere with the interaction of natural or synthetic androgens with the AR. The AR reporter gene assay uses the fact that the AR is a transcription factor that induces transcription of target genes after binding to an androgen response element (ARE) in their promoter. In the applied assay the ARE is linked to the gene of the easily measurable protein, the firefly luciferase, making the promoter activity and luciferase expression AR regulated.

In general, reporter gene assays provide a quick and accurate tool to determine and quantify hormone-induced transcriptional activity. However, the results of a reporter gene assay are influenced by factors like the use of cell line, promoter regions, and response element constructs, which one should take into account when comparing results from different studies (132).

4.2 MCF-7 cell proliferation assay

The MCF-7 cell proliferation assay is based on the MCF-7 human breast cancer cell line, which growth is estrogen dependent. Test chemicals with estrogen activity bind to the ER and activate the transcription of estrogen-responsive genes, which leads to proliferation of MCF-7 cells.

Proliferation of the cells is therefore an indication of the presence of estrogen or an estrogen like compound, and the cell proliferation assay can be used for determining both estrogenic as well as antiestrogenic activity (132, 133). However, besides expression the ERs, the MCF-7 cells also express CYP enzymes, including the aromatase, as well as other receptors, including the androgen, progesterone, glucocorticoid and retinoid receptors. Substances that can bind to these exogenous receptors can antagonise estrogen-induced cell proliferation. As a result, care should be taken when interpreting results from studies of compounds with unknown or multiple mechanisms of action (132). Because the MCF-7 cells also express the aromatase enzyme, effects on aromatase can be assessed by using testosterone as substrate. However, in this thesis, the purpose of using the MCF-7 cell proliferation assay was only for the detection of estrogenic effects.

4.3 H295R Steroidogenesis assay

The steroidogenesis assay is an *in vitro* model for the investigation of effects on steroid hormone synthesis. The assay is based on the H295R human adrenocortical carcinoma cell line, which expresses genes that encode for all the key enzymes for steroidogenesis (Figure 2) (134). This is a unique property because *in vivo* expression of these genes is tissue and developmental stage-

specific with typically no single tissue or developmental stage expressing all of the genes involved in steroidogenesis (135). The H295R cells represent a unique *in vitro* system in that they have the ability to produce all the steroid hormones found in the adult adrenal cortex and the gonads allowing testing for effects on both corticosteroid synthesis and the production of sex steroid hormones such as androgens and estrogens. However, in the current PhD project, the assay was only used to detect effects on the production of testosterone, estradiol and progesterone. Nevertheless, depending on the setup of the assay protocol, experiments can be conducted to study the effects of chemicals on specific enzymes and intermediate hormones.

The goal of using the assay is to provide an answer with regard to the potential of a chemical to induce or inhibit the production of the steroid hormones. The assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamic-pituitary-gonadal (HPG) axis (135).

4.4 Aromatase Assay

As previously mentioned, the aromatase enzyme is responsible for the conversion of testosterone to estrogen. Aromatase activity is expressed in several tissues and cells including the placenta, granulosa cells, Sertoli and Leydig cells, adipose tissue, and several sites in the brain (136).

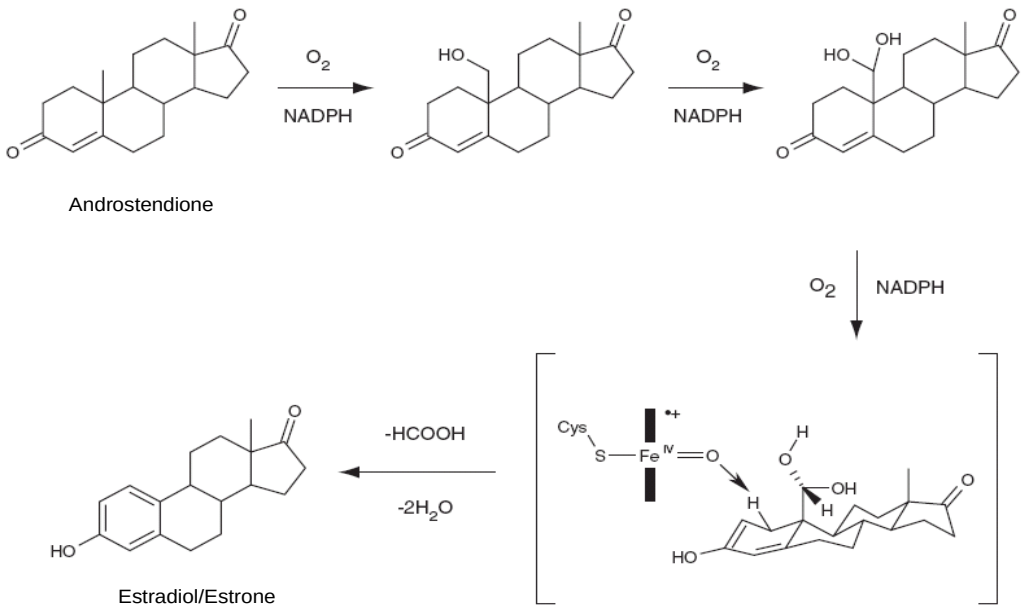

Figure 4 – Aromatasse enzyme reaction. The conversion of androgens to estrogens requires the consumption of three molecules of NADPH and O_2 for each molecule of estrogen formed, accompanied with the release of water. [The figure is modified from (137)]

There are many available aromatase assays. The aromatase assay applied, in the study presented in this thesis, used human placental JEG-3 choriocarcinoma cells and tritium labeled [1β-^{3}H] androst-4-ene-3,07-dione. Figure 4 illustrates the principle of the applied aromatase assay.

The aromatase activity is quantified by the loss of the 1β-^{3}H of the substrate into the aqueous phase of the reaction mixture during aromatization. The tritium atom in the β position of the substrate is incorporated into water during the aromatization reaction, and aromatase activity can be determined by measuring 1β-^{3}H from the tritiated water (136).

4.5 T-Screen assay

In addition to the well-documented estrogen and androgen pathways, there are other hormonal systems susceptible to disruption, including the thyroid axis. Thyroid hormone receptors (THRs) bind to short repeated sequences of DNA called thyroid hormone responsive elements (TREs), but in contrast to steroid hormone receptors, THRs bind DNA in the absence of hormone, and in general, binding of THR alone to DNA leads to repression of transcription, whereas binding of the THR with hormone activates transcription (2).

In vertebrates, thyroid hormones (THs) are essential for postembryonic development, such as establishing the central nervous system in mammals and metamorphosis in amphibians. The regulation of TH delivery to tissues and cells during development and in the adult represents a very complex web of feedback systems, providing redundant and compensatory regulatory responses to maintain TH signalling in the face of specific deficiencies in circulating levels of THs (138).

The interest in the thyroid receptor (TR) and the THs (T3 and T4) has arisen because they play a role in the control of development (e.g., metamorphosis in amphibians and brain development in mammals), and chemicals may alter their synthesis, transport and catabolism (139-144). Increasing numbers of studies are showing that xenobiotics may act at the THRs, and more and more chemicals are suspected of being able to interfere with the TR function (145). Compounds such as polybrominated diphenyl ethers (146) and PCB's have been shown to have the potential to interfere with the TR function (147) and some of these chemicals seem to suppress transcription that is linked to TR activation (148).

The T-screen assay is an *in vitro* assay, which can be used for the detection of agonistic and antagonistic properties of compounds at the level of the TR. The assay is a proliferation assay based on the growth of GH3 cells, which is a rat pituitary tumor cell line. The cell line growth is

dependent on the TH 3,3',5-triiodo-thyronine (T3) and the cells have a high expression of TRs. The growth stimulatory effect of T3 is mediated by specific, high-affinity TRs that upon binding of THs bind to TREs in the cell nucleus ultimately leading to gene expression (149). Thus the T-screen assay can be used for *in vitro* detection of agonistic and antagonistic properties of compounds at the level of the TR, but it will not catch effects on TH levels resulting e.g., from impairment in TH synthesis pathway.

4.6 3T3-L1 adipogenesis assay

Different *in vitro* systems exist for studying adipocyte differentiation. One of the most frequently employed cell lines is the 3T3-L1 cell line. The 3T3-L1 is a mouse embryonic fibroblast pre-adipocyte cell line. The 3T3-L1 cells are derived from the original 3T3-Swiss albino cell line, and the 3T3-L1 adipogenesis assay is a well established *in vitro* model of adipocytes for studying signaling pathways involved in adipocyte differentiation, and obesity or obesity related parameters *in vitro*. The preadipocyte 3T3-L1 cells have a fibroblast-like morphology during the growth phase. However, under appropriate conditions, the cells differentiate into an adipocyte-like phenotype where the preadipocyte converts to a spherical shape, accumulates lipid droplets, and progressively acquires the morphological and biochemical characteristics of a mature adipocyte (150). The cell line is considered a model for white adipose tissue, although the numerous fat droplets formed when the cells are fully differentiated is characteristic of brown fat cells, where as white adipocytes only contain a single lipid droplet (151). The effects of the test compounds on adipocyte differentiation can be measured by scoring the amount of lipid droplets formed in the 3T3-L1 adipocytes using Oil Red O staining.

4.7 PPAR transactivation assay

Adipogenesis and the many different mechanisms involved in the regulation of adipocyte differentiation is complex. However, one of the transcription factors shown to play a key role in adipocyte differentiation is the PPAR family (152-156). The PPAR α/γ transactivation assays are reporter gene assays used for the detection of the ability of a compound to activate the PPAR α and/or γ receptor and thereby a sort of measurement of a compound's ability to work as an agonist or antagonists of the different PPAR receptors. The effect of a compound on PPAR transactivation is assessed by adding different concentrations of test compound to the cells (NIH-3T3 cells), which

have been transiently transfected with a plasmids expressing, in our assays, the ligand binding domain of the mouse PPARα or PPARγ coupled to Gal4, and a plasmid containing an UAS (Upstream Activation Sequence) linked luciferase reporter construct. (The applied GAL4-UAS system is a well known system consisting of two parts: the GAL4 gene, encoding the yeast transcription activator protein Gal4, and the UAS, a short section of the promoter region, to which Gal4 specifically binds to activate gene transcription).

4.8 *In vitro* metabolising systems

In this PhD project, different *in vitro* systems for biotransformation of known endocrine disrupting chemicals were tested. In total, three different *in vitro* metabolising systems were tested using cryopreserved human hepatocytes, human liver S9 mix, and PCB-induced rat microsomes, respectively. Generally, for all the test systems, the test compounds were metabolised by incubating the compounds with either cryopreserved hepatocytes, liver S9 mix or PCB-induced rat microsomes, respectively. To evaluate the ability of the selected test system to metabolise the different test compounds, samples were analysed using HPLC. The extent of metabolic transformation of the different compounds were assessed by measuring the amount of the parent compounds before and after treatment with the different metabolising agents, expressed as the recovery rate (percentage) of the parent compounds.

5 Results

The results of the different *in vitro* assays with the respective compounds and mixtures are presented in paper I-III. This section will give a summary of the results obtained during this PhD thesis. The only results not presented in the papers are the results of the *in vitro* metabolising study using the human cryopreserved hepatocytes.

5.1 Endocrine disrupting effects *in vitro* of individual phytoestrogens and mixtures - Paper I and III

Figure 5 presents an overview of the different PEs and PE-mixtures that were tested in paper I and III: Mix 1 composed of the isoflavonoid metabolites genistein, daidzein and equol; Mix 2 composed of the two isoflavonoids formononetin and biochanin A; Mix 3 composed of the lignan metabolites enterolactone and enterodiol; Mix 4 containing the four lignans secoisolariciresinol, matairesinol, lariciresinol and pinoresinol, and a total mixture including all 12 selected PEs.

Overall, the individual PEs in each of the five mixtures studied in papers I and III are the same. However, in paper I the proportions of the single PEs in the mixtures were in equimolar concentrations. That is, the design of the mixtures was not based on effective doses, as different *in vitro* assays were used, and each chemical could have very different effect in each assay.

In paper III, the proportions of the individual PEs in the mixtures were not equimolar concentrations, but were based on the amount and number of the different classes (e.g., isoflavonoids lignans and coumestans) of PEs naturally found in Scandinavian food (157).

The studies presented in paper I and III examined the endocrine disrupting potential of the different PEs and PE-mixtures testing their effects on steroid hormone production, aromatase activity, estrogenic activity, interaction with the AR, as well as whether they could affect the ability of preadipocyte 3T3-L1 cells to differentiate into adipocytes, and also their ability to activate PPAR α and/or PPARγ.

The experiments with the steroid synthesis assay showed that at the highest concentration (10^{-5}M) all compounds/mixtures tested caused an increase in the estradiol production (Fig. 2, paper I). Furthermore, increasing concentrations of mix 1 (genistein, daidzein and equol), mix 2 (formononetin and biochanin A), the total-mixture as well as genistein alone caused dose-dependent reduction in the testosterone production although not statistically significant for mix 1 (Fig. 3, paper

I). In contrast to this, coumestrol and mix 3 caused a slight but significant increase in testosterone production (Table 2).

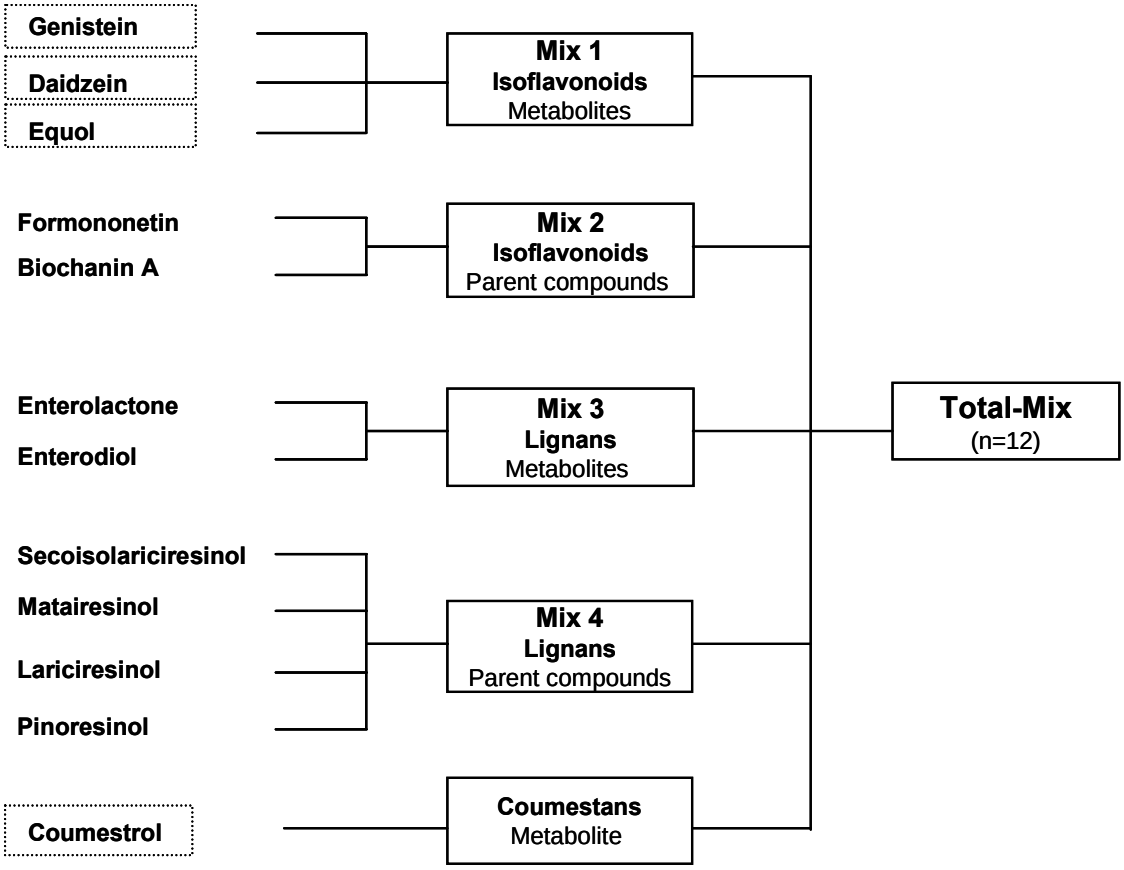

Figure 5 - Overview of the different phytoestrogens and mixtures studied in papers I and III
The selected PEs consist of both parent compounds (biochanin A, formononetin, secoisolariciresinol, metairesinol, lariciresinol, pinoresinol, and coumestrol) and metabolites (genistein, daidzein, equol, enterodiol and enterolactone). The different mixtures and the total mixture are presented in black boxes, and the compounds, which were (also) tested individually, are represented in light gray (dotted) boxes.

To elucidate the mechanism behind the increased levels of estradiol, the tested compounds at the highest concentration (10^{-5} M) were studied in combination with a specific aromatase inhibitor, 4-androsten-4-ol-3,17-dione (4-AOD). The results of these experiments revealed that the increased levels of estradiol following PE exposure was reduced to background levels following treatment with 100nM or 1µM of 4-AOD. Of the three single compounds tested, genistein was the compound found to have the most pronounced effect on the steroid hormone production in the H295R cells.
In the MCF-7 proliferation assay, performed at the Danish Cancer Society, estrogenic activity was observed for the isoflavone metabolites in mix 1 with the effect at the highest concentrations being

comparable to the maximal growth stimulation obtained with estradiol. In comparison the precursor plant isoflavones in mix 2 were less potent showing only a growth stimulatory effect at the second highest concentration tested (the highest concentration of mix 2 was cytotoxic). No effects were observed for the lignans in mix 3 and 4 (Table 2).

The mixture of all the PEs (total-mix) showed an estrogenic activity comparable to the maximal effect obtained with estradiol.

Table 2 – Summery of the endocrine disrupting effects observed in the *in vitro* studies presented in paper I

	Androgen activity	**Estrogen activity (MCF-7 cell assay)**	**Steroid hormone production (H295R cell assay)**		**Aromatase activity (JEG-3 cells)**
			Estradiol	Testosterone	
Mix 1	-	↑	↑	-	↑↓#
Mix 2	-	(↑)*	↑	↓	↑
Mix 3	-	-	↑	↑	↑
Mix 4	-	-	↑	-	↑
Totalmix	-	↑	↑	↓	↑
Coumestrol	-	↑	↑	↑	↑¤
Genistein	-	↑	↑	↓	-
Daidzein	-	↑	↑	-	-
Equol	-	↑	↑	-	-

- : no effect.
#: inhibition at the lowest concentration, but increased activity was observed at higher concentrations.
¤: only at the second highest concentration (1µM)
*: only at the second highest concentration (1µM), cytoticity was found at the highest concentration tested (10µM).

The experiments with the single compounds from mix 1 were conducted in order to evaluate whether some of the single compounds could account for the observed mixture effects or whether combination effects were present. The isoflavone metabolites in mix 1 all resulted in stimulation of MCF-7 cell growth with genistein being the most potent one (potency: genistein > equol > daidzein). The stimulatory effect of genistein alone was comparable to the effects observed with mix 1 and might contribute greatly to the effect of the PEs in mix 1. Coumestrol alone was also found to have an estrogenic activity with the stimulatory effect at 100 nM (the third highest concentration) being comparable to that of 100 pM estradiol (Table 2).

None of the tested PEs showed any antiestrogenic effect on MCF-7 cell growth, which was tested by the presence of 17β-estradiol.

To establish the mechanism behind the growth stimulatory effects observed for the MCF-7 cells, expression of the estrogen inducible progesterone receptor (PR) A and B proteins, as well as the ERα protein was assessed using Western blot analysis. The PR gene is an estrogen-regulated gene and the expression of the two forms of PR protein A and B is often used as a marker for the presence of functional ERα (29). Because the MCF-7 cells express extremely low level of ERβ (30), the expression of ERβ was not measured.

The addition of estradiol to the MCF-7 cells gave an increase in PR expression and a decrease in ERα expression (Fig. 5 in paper I). Addition of Mix 1, as well as coumestrol resulted in increased expression of PR up to the level observed for 100 pM estradiol (Fig. 5 in paper I), indicating that the PEs can act as potent estrogens via ERα. Furthermore, the antiestrogen ICI 182,780 could completely inhibit the stimulation of the PR expression (Fig. 5 in paper I), further supporting an ERα-mediated effect of the PEs.

Table 3 – Overview of the effects found on PPAR activation and lipid accumulation

Compounds	PPARα activation		PPARγ activation		Effect on lipid accumulation
	Agonism	Antagonism	Agonism	Antagonisme	
Mix 1	-	+	+	+	↓
Mix 2 #	-	-	+	+	↓
Mix 3	-	+	-	+	↓
Mix 4	-	-	-	+	-
Totalmix	-	-	+	+	-
Coumestrol	-	+	-	+	↓
Genistein	ND + *	ND	ND + *	ND	↓
Daidzein	ND + *	ND	ND + *	ND	↓

- : no effect.

ND: not determined

cytotoxic at the highest (100 μM) concentration tested.

* Shown in Rickett et al., 2005 (158)

In the aromatase assay, a general weak effect of the PEs was seen at low concentrations. Mix 1 inhibited the aromatase activity at the lowest concentration (10^{-10} M), but a weak increase in enzyme activity was observed at 10^{-9} M and at 10^{-6} M, an approximately 1.3-fold increase in

activity was seen (Table 2). For mix 2, 3, 4 and for coumestrol aromatase activity was increased at 10^{-6} M and also at 10^{-5} M for mix 2 and mix 4 (Table 2 in papar I and for an overwiew Table 2 in the thesis).

None of the tested PEs or mixtures showed any agonistic or antagonistic effects in the AR assay (Table 2).

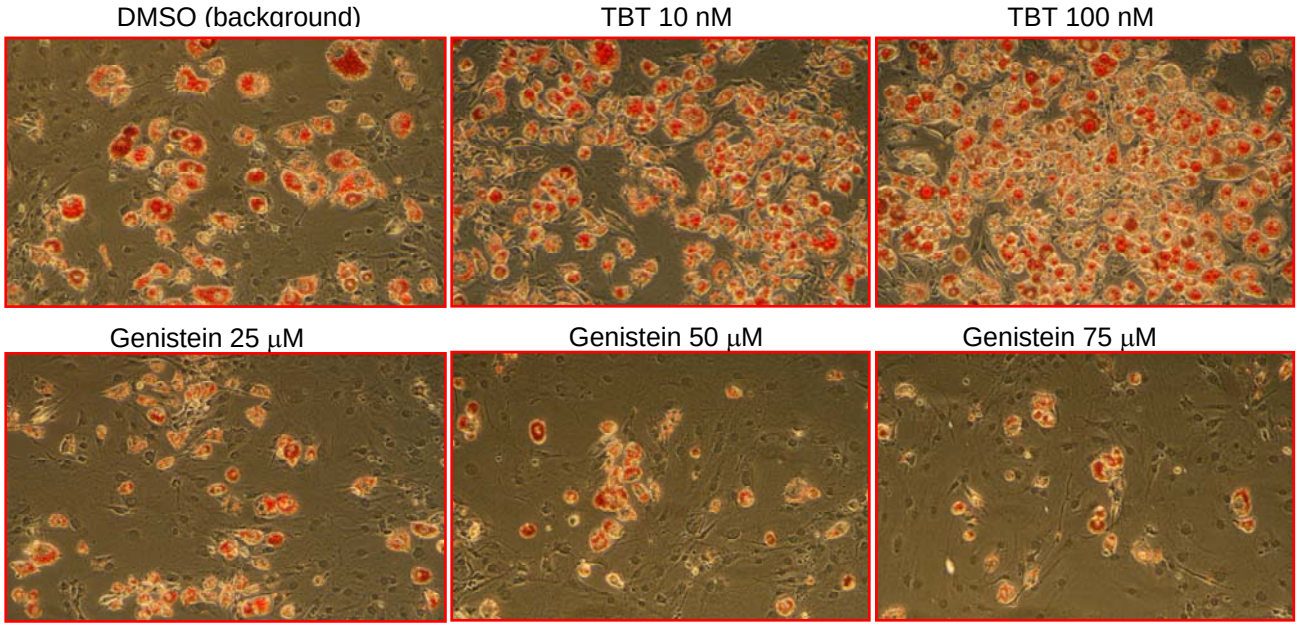

Figure 6- Effect of tributyltin (TBT) and genistein on lipid accumulation in the 3T3-L1 adipocytes.
The 3T3-cells were treated with different concentrations of TBT (nM) or genistein (μM). The background was cells supplemented with 0.1% DMSO. After 6 days exposure during the differentiation period the adipocytes were stained with Oil Red O and the amount of lipid accumulation was analyzed by microscopic inspection and several phototropic images from each well were taken (magnification, x40). The images shown are from one representative experiment out of at least three independent experiments.

Table 3 gives a summery of some of the results presented in paper III regarding the effects found on PPAR activation and lipid accumulation. The effects of the PEs on adipocyte differentiation were measured by scoring the amount of lipid droplets formed in the 3T3-L1 adipocytes. The three tested single PEs (coumestrol, daidzein and genistein) all significantly decreased lipid accumulation, with genistein being the most potent inhibitor showing effect at all tested concentrations (25, 50 and 75 μM) (Figure 6). Coumestrol showed effect at the two highest concentrations 50 and 75 μM and daidzein had significant effect only at 75 μM (Fig. 2A in paper III).

Genistein were also tested in combination with the PPARγ antagonist GW9662, to examine if any possible effect of genistein on lipid accumulation could be influenced by the presence of a PPARγ

antagonist. The co-treatment resulted in an impairment or neutralization of the decreasing effect at 25 μM and 50 μM, giving only a significant decrease at 75 μM, the highest concentration tested (Figure 7).

Of the five mixtures mix 1, mix 2 and mix 3 also showed a significant decrease in lipid accumulation (Table 3). However, for mix 2 the effect was only statistically significant at 50 μM, the second highest concentration, but the effect at 75 μM was comparable to the effect observed at 50 μM (Fig. 2B in paper III). Neither mix 4 nor the total-mix had any significant effect on the lipid accumulation in the 3T3-L1 adipocytes (Table 3).

PPARγ has been shown to play a key regulatory role in adipocyte differentiation (154, 159) and to be necessary for lipid accumulation in adipocytes in mice (160-162). In our study using the PPAR transactivation assay the PEs and mixtures were tested in seven concentrations from 0 to 100 μM. Mix 1, mix 2, and the total-mix showed some PPARγ agonistic effect, with mix 2 being the most potent of the three having effect at a concentration of 6.25 μM up to 50 μM (Fig.5A in paper III). The highest concentration of 100 μM was found to be cytotoxic for mix 2 (Table 3).

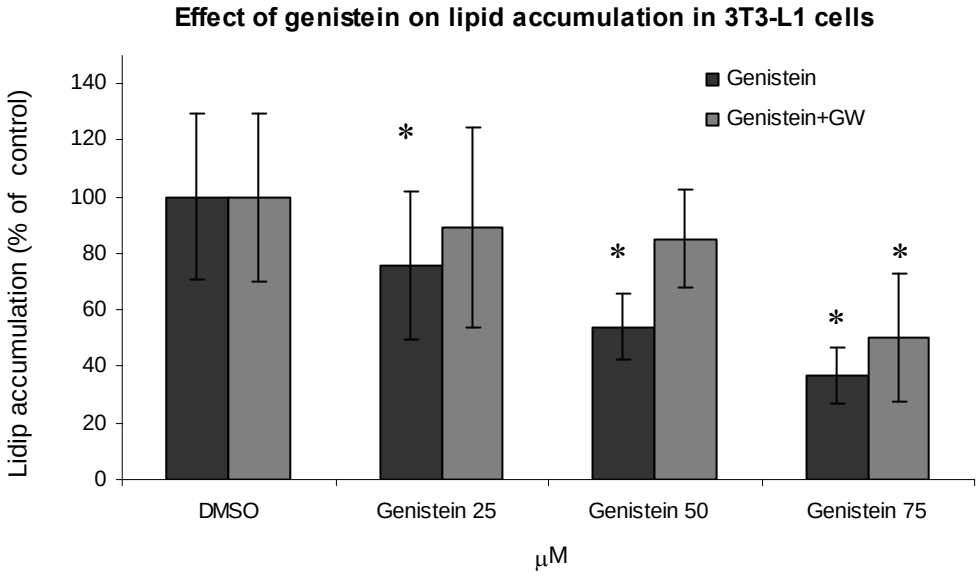

Figure 7- –Effect of the PPARγ antagonist GW9662 on the effect of genistein.
The 3T3-L1 preadipocytes were treated with different concentrations (μM) of genistein either alone or in the presence of 1μM GW9662 (GW). The control was cells supplemented with 0.1% DMSO or 0.1% DMSO plus 1μM GW9662, respectively. After 6 days exposure during the differentiation period, the adipocytes were stained with Oil Red O and the amount of lipid accumulation was analyzed by microscopic inspection and several phototropic images from each well were taken including five to twelve random fields images from each well photographed under phase contrast (magnification, x40). Images were analyzed in ImageJ and data are shown as the mean ± SD from one representative experiment out of three independent experiments.
* Statistically significantly different from control. ($P< 0.05$).

When the mixtures were tested in combination with a PPARγ-selective agonist, a decrease in activity was observed for all mixtures, mix 3 being the most potent showing significant decrease in almost all tested concentrations (Fig.5B in paper III).

For coumestrol alone, no effect was seen in the agonist assay, but in combination with the PPARγ-selective agonist a significant decrease in activity was observed at 50 and 100 μM (Fig.5 in paper III). Overall the tested PEs and mixtures all showed PPARγ antagonistic effects, although in varying degrees (Table 3).

In the PPARα assay, none of the mixtures nor coumestrol alone had any PPARα agonistic effect (Table 3). Contrary, for mix 3 and mix 4, a decrease in activation was observed in the PPARα agonist assay. When testing the PEs in combination with a known PPARα agonist, the only antagonistic effect observed was a weak effect with mix 3 at the two highest concentrations as well as a significant decrease in activity at the highest concentration of mix 1 (Table 3).

For coumestrol alone, a significant decrease in activity was also observed in the antagonist assay at the highest concentration, but this decrease was also observed in the PPARα agonist assay (Table 3). Therefore, this effect could be due to a beginning cytotoxic effect even though cytotoxicity was only found for coumestrol at 50 μM and 100 μM in one out of five independent cytotox-experiments.

Altogether, the results from the studies presented in paper I and III show that the mechanisms underlying the endocrine disrupting effects of the PEs are many and complex. Additionally, many PEs as well as nutrition relevant mixtures of PEs have an inhibiting effect on lipid accumulation *in vitro*, which could suggest a beneficial effect with regard to obesity. However, the concentrations tested in the 3T3-L1 adipocyte assay were much higher than what is normally found in humans. Additionally, based on our current results, as well as the fact that the roles of the different players in adipogenesis and lipolysis is not yet understood, the influence of PEs on adipogeneisis and development of obesity is still unclear.

The current studies cannot be used to conclude, if it is the effects of genestein and/or coumestrol alone that account for most of the endocrine disrupting effects observed with the total PE mix. However, the results from mix 2 in the various *in vitro* assays indicate that it is not only the mammalian metabolites but also the precursor plant isoflavones that have the ability to act as potent endocrine disrupters.

5.2 Use of external metabolizing systems when testing for endocrine disruption in vitro – Paper II

The overall aim of Paper II was to test different *in vitro* metabolising systems for biotransformation of known endocrine disrupting chemicals to find a potential method for the incorporation of metabolic and toxicokinetic aspects into *in vitro* testing for endocrine disruptors.

Table 4 gives a summery of the results obtained with two of the *in vitro* metabolising systems applied.

Table 4 - Metabolism of 10 selected test compounds using different *in vitro* metabolising systems

	Recovery of parent compound (%)	
Compound	**human S9-mix**	**rat microsomes**
Ketoconazol	96%	53% *
Miconazol	97%	85% *
Epoxiconazol	110%	95%
Tebuconazol	96%	86% *
Prochloraz	94%	86% *
Dimethyl phthalate	~0% [a]	< 1% [a]
Diethyl phthalate	~0% [a]	< 1% [a]
Methylparaben	~0% [a]	< 1% [a]
Ethylparaben	~0% [a]	< 1% [a]
Butylparaben	~0% [a]	< 1% [a]

Recovery of the parent compounds (%) after using the indicated *in vitro* metabolizing method.
[a] Indicates that the P-value could not be calculated because the amount of remaining parent compound was below the detection limit.
* Statistically significant difference between the amounts of parent compound in the samples before and after the applied method of metabolism, tested by to-tailed T-test ($p<0.05$).

The LC-MS analysis of the S9 extracts for the azole fungicides showed no indication of any metabolic transformation during the incubation with the human liver S9 mix (Table 4). The parabens and phthalates on the other hand had almost been completely metabolized, with a recovery of the parent compounds from the S9 extracts of less than 1 % for both parabens and phthalates (Table 4).

When using the PCB-induced rat microsomes the results for the parabens and phthalates was the same as seen with the human S9 mix. An extensive metabolic transformation of the parabens and the phthalates was observed, again with a recovery rate of the parent compounds of less that 1% (Table 4). However, in contrast to the human S9 mix, the rat microsomes were able to metabolize the tested amount of azole fungicides to some extent. A statistically significant difference between

the amount of parent compound before and after treatment with the rat microsomes was seen for four out of five azole fungicides (Table 4).

An additional aim of the study presented in paper II was to determine the endogenous metabolic capacity of the cell line applied in the T-screen assay.
The cells used in the T-screen assay are the GH3 cells. The results from the analysis of the metabolic capacity of the GH3 cells showed a statistically significant difference between the amount of parent compound before and after incubation with the GH3 for the two tested phthalates DMP and DEP. No significant transformation of the parabens or azole fungicides was found.

Table 5 - Effects of the 10 parent compounds as well as the S9 extracts of the 3 parabens, and the 2 phthalates in the T-Screen assay

	Effect in the T-screen assay	
Compound	**Parent compound**	**Human S9 metabolic extract**
Ketoconazol	Antagonist [#]	Not tested
Miconazol	Antagonist [#]	Not tested
Epoxiconazol	-	Not tested
Tebuconazol	Antagonist [#]	Not tested
Prochloraz	Antagonist [#]	Not tested
Dimethyl phthalate (DMP)	-	(antagonist)*
Diethyl phthalate (DEP)	-	-
Methylparaben	Agonist	-
Ethylparaben	Agonist	-
Butylparaben	Agonist	Agonist

- : no effect.
[#] : the effect was only seen at the highest concentration tested, so a cytotoxic effect could be the cause
* only at the three highest dilutions

Regarding any possible changes in the thyroid receptor activity of the selected EDCs, after biotransformation, the general picture for the parent compounds was an antagonistic effect of the azole fungicides, while the parabens had an agonistic effect (Table 5). After biotransformation using the human S9 mix, the metabolic extract from butylparaben showed a significant agonistic effect (Table 5). A significant agonistic was also seen for the metabolic extract from methylparaben, however, the effect was only significant at the second lowest dilution and was therefore judged to be a chance finding. For the S9-extracts from the 2 phthalates a statistically significant reduction of the T_3 induced cell proliferation was observed for the DMP extract at the three highest dilutions (Table 5).

The extracts from the 5 azole fungicides were not tested as these compounds had shown very low metabolic transformation after treatment with the human liver S9 mix (Table 4).

Overall, we did not find a marked difference in the effects observed for the parent compounds and the effects of the tested metabolic extracts in the selected *in vitro* assay. However, based on the results from the current study, our conclusion is that the GH3 cells show some metabolic capability in relation to the two tested phthalates DMP and DEP, but almost none towards the tested azole fungicides and parabens.

6 Discussion

The focus of this PhD thesis with the title "Mechanistic evaluation of endocrine disrupting chemicals" has been on different *in vitro* studies of a few groups of already known endocrine disrupting compounds. The overall aim has been to gain more knowledge or information on the many different mechanisms that are either known to or that can have the potential to play a role in the adverse effects of EDCs. An additional focus point has been to improve some of the limitations of *in vitro* assays for endocrine disruption, specifically the lack of metabolism of test substances, and the lack of knowledge regarding the endogenous metabolic capacity of the different cell lines applied in the various *in vitro* assays.

6.1 The studies on phytoestrogens and phytoestrogen mixtures

6.1.1 Effects of PEs on steroidogenesis, aromatase, and estrogen activity

As previously mentioned, many of the studies presented as part of this thesis include testing of different mixtures of PEs. In this regard, it should be emphasised that the *in vitro* studies in this PhD project were not designed with the aim of making predictions of mixture effects.

Two different types of PE-mixtures have been studied, although, the single PEs in each of the five mixtures studied in papers I and III are the same. The mixtures in this project were either designed to be equimolar concentrations as in paper I or based on exposure data as in paper III. Although the the mixtures were designed to represent PEs naturally occurring in Western food, the proportions of the compounds in the mixtures were not representative of the concentrations found in the diet, and the design of the mixtures was not based on effective doses, as different *in vitro* assays were used, and each compound could have very different effect in each assay. Therefore, the results of the studies cannot be used to conclude on additivity.

In paper III the proportions of the single PEs in the mixtures were not equimolar concentrations, but were based on the amount and number of the different classes (e.g. isoflavonoids lignans and coumestans) of PEs naturally found in Scandinavian food (157). These mixtures are therefore very relevant in relation to human food consumption.

However, it would have been very interesting being able to make some kind of predictions and modelling of mixture effects, and this should be kept in mind for future experiments.

Through our diet, we are exposed to many different and complex mixtures of PEs. The overall aim of the study presented in paper I was to evaluate the endocrine disrupting potential of twelve different PEs and mixtures of PEs naturally occurring in Western/Scandinavian food. Until recently many studies have typically been based on single or a small number of individual compounds, or on single dietary compounds like soy or flaxseed. The question we wanted to address was whether the effects of the individual PE compounds are modified when they are combined with other PEs from various classes. One example is the study by Power et al., (2006) where it was found that the isoflavone genistein alone promoted growth of MCF-7 human breast cancer xenografts, but when genistein was given in combination with the mammalian lignans enterolactone and enterodiol, no tumour growth-promoting effect was observed (41).

In the study presented as part of this thesis, the twelve selected PEs were tested alone or in mixtures in a battery of *in vitro* bioassays. In the steroid synthesis assay (H295R), all tested PEs and PE-mixtures caused an increase in the estradiol production. Furthermore, the mixture consisting of the isoflavonoids formononetin and biochanin A, as well as the total-mix containing all twelve PEs caused a dose-dependent reduction in the testosterone production. Genistein alone also caused a dose-dependent reduction in the testosterone production in the H295R cells, and the effect of genistein was in general comparable to the effects observed for mix 1, which contained the isoflavonoid metabolites genistein, daidzein, and equol.

The observed reduction in the testosterone production might be a result of an inhibitory effect of the isoflavones on some of the steroidogenic enzymes involved in testosterone biosynthesis (see Figure 8). This would be in agreement with other studies including a study by Ohno et al. (2002), which showed that genistein, daidzein, formononetin and biochanin A strongly inhibited 3β-hydroxysteroid dehydrogenase (3β-HSD) activity and at high concentrations (12.5 and or 25μM) also inhibited P450c21 activity (163). Also Le Bail et al. (2000) have shown that genistein, daidzein, formononetin and biochanin A are 3β-HSD and/or 17β-HSD inhibitors (Figure 8), and that the inhibitory effect of some of the PEs are related to small structural differences, which can explain the diversity of effects seen for the different PEs (40).

In the MCF-7 cell proliferation assay, the isoflavonoids and coumestrol had a growth stimulatory effect on the MCF-7 cells, whereas the lignans had no effect. The most pronounced effects were seen with mix 1 (genistein, daidzein, and equol) and the total-mix, as well as with the selected single compounds. Overall, the effect of genistein and, at the highest concentration also daidzein

was comparable to the effect observed for mix 1. Coumestrol alone had a significant growth stimulatory effect on the MCF-7 cells, with an effect comparable to that of estradiol at a 1000 fold lower concentration. The growth stimulatory effect of the isoflovonoids and coumestrol are in agreement with what previous studies have shown (40, 164) and our Western blot analysis showed that both the isoflavonoids and coumestrol increased the expression of the estrogen inducible PR A and PR B proteins, supporting an ERα mediated effect.

Le Bail et al. (40) and others (165) have found that many of the PEs show biphasic regulation effects on the cell proliferation of MCF-7 cells, stimulating growth at lower concentrations and inhibiting growth at higher concentrations. This was also seen with coumestrol in the study presented in paper I. Furthermore, studies also showed that the stimulatory effects at lower concentrations correlate with the affinity of the PEs for the ER, supporting our result from the

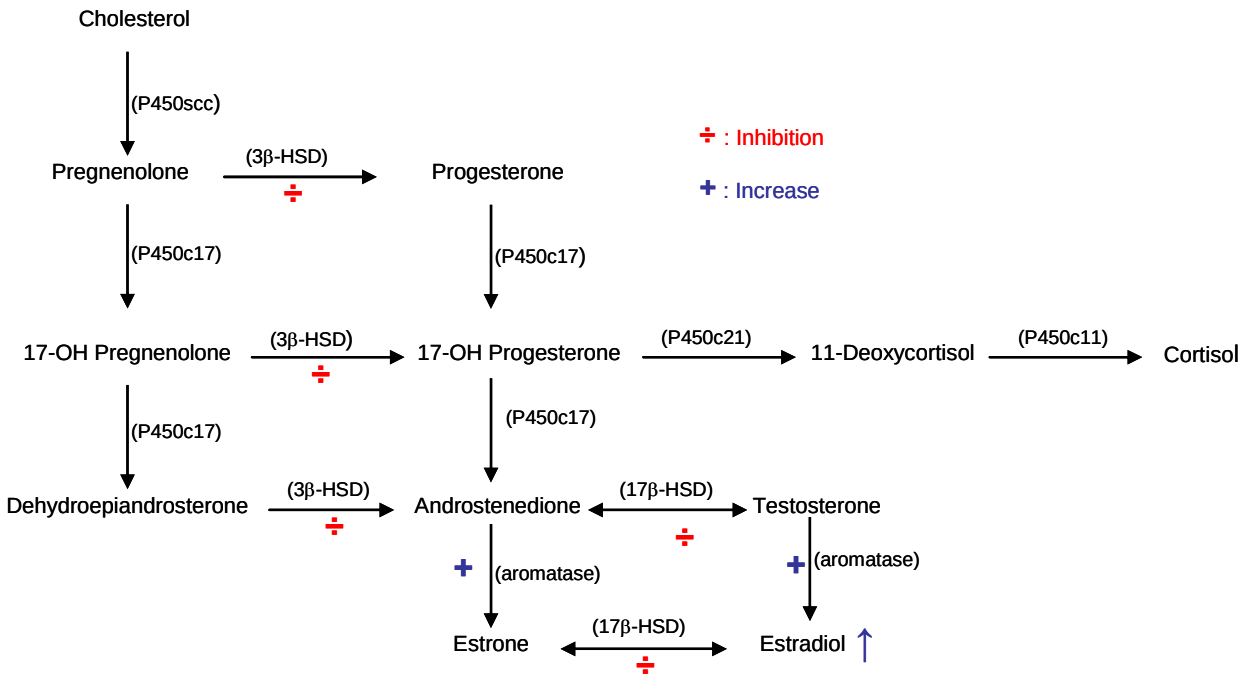

Figure 8 – Potential effects of isoflavonoids on steroiodgenic enzymes found by us and others
The figure shows an overview of the effects found by us and others for PEs, specifically the isoflavonoids, on the different enzymes in the steroid synthesis pathway. Overall, the studies presented in this thesis showed a general increase in aromatase activity for the PE-mixtures. However, the observed effects of the PEs and the mechanisms behind them can be influenced by the specific tissues or cell lines used, as well as the tested concentrations.

Western blot analysis, but that the growth inhibition induced at higher concentrations is probably not mediated through ERα (40, 165). The mechanisms behind the growth inhibitory effect seen at

higher concentrations is currently not understood, but might involve inhibition of one or more of the enzymes involved in the steroid synthesis (e.g., the aromatase and/or 3β-HSD and 17β-HSD) (40, 165).

Overall, the studies presented in this thesis, both the aromatase assay with the JEG-3 cells as well as the steroidogenesis assay with the H295R cells, showed a general increase in aromatase activity for the PE-mixtures, and the use of 4-AOD proved that the observed effects were due to an increase in aromatase activity (Figure 8). This is in contrast to the many studies that indicate that several PEs inhibit the aromatase enzyme (40, 50-52). However, in the study by Le Bail et al. (40), it was found that isoflavonoids and compounds, which presented the phenolic B ring in the 3 position on the pyran ring, are better 3ß-HSD and/or 17ß-HSD inhibitors than aromatase inhibitors.

Nevertheless, there are studies supporting our observations as both genistein and coumestrol have previously been found to induce aromatase activity (53-55). However, it should be taken into consideration, that the effects observed, and the mechanisms underlying them, may be influenced by the specific tissues or cell lines used. Coumestrol and genistein induced aromatase activity in breast cancer cells (SK-BR-3) through a non-genomic action of ERα (53), whereas the genistein increased aromatase activity observed in endometrial stromal cells (ESC) did not seem to be mediated through an ER pathway (55). Again, genistein, flavone, and quercetin have been shown to inhibit phosphodiesterase in several tissues (166, 167), and induction of aromatase activity by genistein, flavone, and quercetin in H295R cells have been suggested to be mediated through the cAMP-dependent protein kinase A (PKA) second messenger pathway (54).

6.1.2 Effects of mixtures of PEs on PPAR activation, and adipocyte differentiation

The hypothesis that there could be a link between exposure to endocrine disrupting chemicals and the development of obesity is being supported by more and more studies. However, the potential contribution of environmental factors to the development of both obesity and type 2 diabetes is far from understood. The aim of the study presented in paper III was to evaluate the different PEs and PE-mixtures, shown previously in paper I to have different endocrine disrupting effects, for effect on parameters involved in obesity using *in vitro* assays. The different mixtures and selected single compounds were evaluated for potential effects on adipocyte differentiation and for their ability to activate the PPARα and PPARγ receptors.

The isoflavones, genistein and daidzein have previously been shown to have both PPARα and PPARγ agonistic effects (158) and some PEs including genistein have also previously been studied for their effect on adipogenesis (168-170). In our study, genistein was found to have a decreasing effect on lipid accumulation in the 3T3-L1 cells. This effect was also observed for coumestrol and daidzein, though not as potent as seen for genistein (Fig 2A in paper III). For mix 1 containing genistein, daidzein and equol, a decrease in lipid accumulation was found as well, the effect being comparable to that of genistein. Genistein alone, but also in combination with the naturally occurring phytoalexin resveratrol and the flavonol quercetin has previously been shown to decrease lipid accumulation in 3T3-L1 cells with the decreasing effect being enhanced when the compounds were combined compared to the effect of the individual compounds (168, 171). Because of the design of the misture study it cannot be concluded, whether it is genistein that is contributing mostly to the effect observed with mix 1. However, the overall decreasing effect of mix 1 is in accordance with what others have reported for genistein (168, 171, 172), and also supported by a study on the effects of equol by Rachon et al.,(2007), who found that dietary equol decreased weight gain, intra-abdominal fat accumulation, and plasma leptin and triglyceride levels in ovariectomized rats (173).

When genistein was tested in combination with the PPARγ antagonist GW9662 the effect of genistein on lipid accumulation was weakened, but not totally blocked. This suggests that the effect genistein exerts on lipid accumulation in the 3T3-L1 is only partly dependent on PPARγ and that lipid accumulation in the 3T3-L1 is mediated by or dependent on other players than PPARγ. One possibility is that the effect of genistein is mediated through the estrogen receptor (ER). This would be in agreement with other studies showing a possible ERα mediated effect of genistein on adipose tissue (170) as well as the reported effect of estrogen on lipid accumulation in 3T3-L1 adipocytes (174).

In addition to mix 1, two other mixtures showed an effect on adipocyte differentiation. Mix 2 (formononetin and biochanin A) and mix 3 (enterolactone and enterodiol) significantly decreased lipid accumulation, though not to the same degree as observed with mix 1. To our knowledge, there are currently only few studies on the effect of formononetin, biochanin A, enterolactone and/or enterodiol on adipogenesis. One such study is by Shen et al., who found that biochanin A and formononetin stimulated lipid accumulation in 3T3-L1 cells at low concentrations (1- 5 μM for biochanin A and 3- 30 μM for formononetin, respectively) (175). However, Shen et al. (175) also found that genestein stimulated lipid accumulation at 15 μM, a result that is different from what we

and others (168, 171, 172) have found. It is known from other types of studies with PEs that many of the PEs show a biphasic concentration-effect curve as also mentioned previously. The difference between the effects found by Shen et al. (175) and others might be due to a difference in the concentrations, as Shen et al. (175) generally have tested lower concentrations.

In regard to the effects of the PEs mixtures on PPAR activation, the overall picture is a PPARγ antagonistic effect, with mix 3 (the lignan metabolites) being the most potent. A PPARγ agonistic effect was also seen for mix 1, mix 2 and the total-mix, with mix 2 showing the strongest effect of the three (Fig.5 in paper III). The PPARγ activation seen for mix 1 are in agreement with previously shown PPARγ activity of genistein and daidzein (158), the main components of mix 1. The PPARγ activation by mix 2 points to PPARγ agonistic activities of either formononectin and/or biochanin A. Interestingly, these two compounds also inhibited lipid accumulation in 3T3-L1 preadipocytes indicating similarities to genistein and daidzein. In contrast, decreases in lipid accumulation were seen for mix 3 (lignans, mainly enterolactone) which showed no PPARγ activiation suggesting that other players besides PPARγ are involved in 3T3-L1 adipocyte differentiation.

No significant activation of PPARα was observed for any of the mixtures and only slight antagonistic effects were seen at high concentrations with mix 1 and 3 as well as with coumestrol alone (Fig. 4 in paper III).

A parallel between the results found on lipid accumulation in the 3T3-L1 cells and the effects found on PPAR activation cannot be drawn for various reasons. One main reason is that, although much progress has been made in regards to understanding adipocyte differentiation and all the different mechanisms underlying the development of adipocytes and adipose tissue, it is complex and still not fully understood. Different studies have shown that, not only PPARγ but also PPARα could play a role in adipocyte differentiation (155, 156). PPARα is not expressed at significant levels in white fat, but it is expressed in brown fat (Figure 9) (176, 177). Data by Brun et al. (155) indicate that, while PPARα possesses some adipogenic potential, PPARγ is a more effective inducer of adipocyte differentiation. However, even though PPARγ is likely to be the predominant receptor in regulation of differentiation of white fat, PPARα and PPARγ may both contribute to the development of brown fat (Figure 9). So, even though PPARγ is thought to play a big role in 3T3-L1 adipocyte differentiation, other pathways are involved as well. Recent work by Choi et al., (2010)

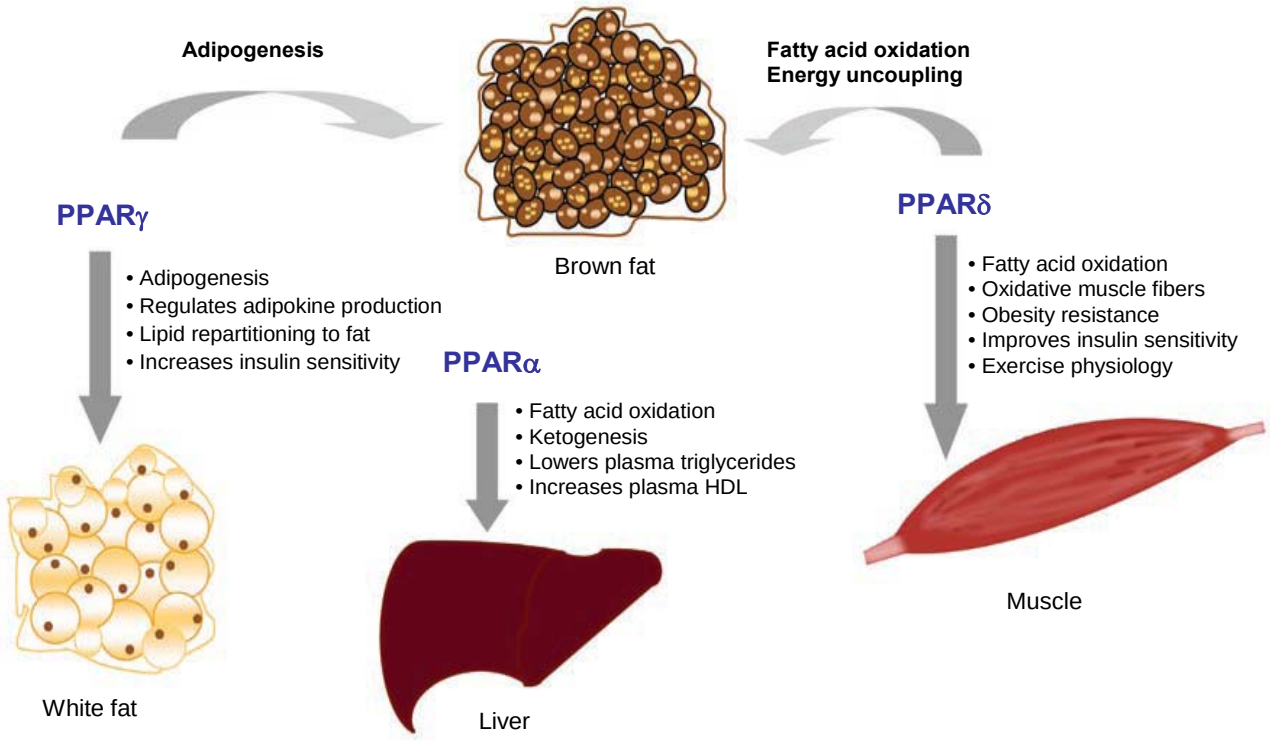

Figure 9- An overwiew of the roles of the different PPARs in energy metabolism. The figure is modified from (178)

suggests that activation of the PPARγ receptor is required for the adipogenic effects of chemicals that bind to the receptor (179). However, the potent effects of organotins on adipogenesis are attributed to dual agonistic activity for PPARγ and RXR, although there is some debate on whether these effects on adipogenesis are primarily due to interactions with PPARγ or RXR (180-183). Tributyltin (TBT) is a known inducer of lipid accumulation in the 3T3-L1 cells, and is also included as a positive control compound in the study presented in paper III. Le Maire et al. have recently shown that the activation of the PPAR-RXR heterodimers by TBT is primarily through its interaction with RXR (182). Overall, based on the result of the study presented in paper III, the different PE mixtures had more effect on PPARγ than PPARα, with some mixtures showing PPARγ agonistic effects while others had more PPARγ antagonistic effects. To my knowledge, nothing is known regarding the interaction between the different PEs and RXR, and such information would be relevant in terms of explaining or interpreting the observed effects of the PEs on adipocyte differentiation and lipid accumulation.

6.2 The use of *in vitro* metabolising systems in connection with *in vitro* testing of EDCs

This study, presented in paper II, on *in vitro* metabolising systems only focused on metabolism through phase I and not phase II metabolism, and it was not the aim of the study to identify the different metabolites.

As mentioned in the result section, the results of the *in vitro* metabolising study using the human cryopreserved hepatocytes are not presented in any of the papers. The reason for this is that our efforts to metabolise the xenobiotics using cryopreserved human hepatocytes failed as the hepatocytes died even though the protocol was followed according to the manufactur's instructions. Several attempts were made with cryopreserved human hepatocytes. The results from the first run were very uneven and generally inconclusive. A new order of cryopreserved human hepatocytes was placed, and this time we measured the number of living cells. It turned out that the number of live cells was so low (less that 3%) that the cell number was too low to run the experiments. A third order of cryopreserved human hepatocytes was placed. This time we also had a very low number of live cells, less than 10%. The low number of living cells could explain the uneven and inconclusive results gathered from the first run, where we had not measured the number of living cells, before starting the experiment. The post-thaw viability promised by the supplier of the hepatocytes was at least 70%. After having revised a total of three new batches of cryopreserved human hepatocytes all with viability less than 10%, it was decided not to pursue with the cryopreserved human hepatocyte metabolism assay.

When working with *in vitro* systems, one is working with isolated systems not whole organisms. Therefore, when looking and evaluating the effects or results obtained from the different test systems, it is important to know how a particular compound is metabolised, both *in vivo* (in the animal) and in the different *in vitro* systems (cell models). In that regard, it is important to have information concerning the endogenous metabolic capacity of the different cell lines used in the various *in vitro* assays now available for the testing of compounds' endocrine disrupting potential. A point of interest in the study presented in paper II was therefore, to investigate the endogenous metabolic capacity of the cells used in the applied *in vitro* assay, which in the current study was the T-screen assay. For the ten selected test compounds representing three different classes of compounds: azole fungicides, parabens and phthalates, we found a significant difference between the amount of parent compound before and after incubation with the GH3 cells for the two phthalates. This could suggest that the GH3 cells, employed in the T-screen assay, have some

metabolic capability in regard to the two tested phthalates, DMP and DEP. In general, such information concerning the metabolic fate of a compound in a given test system, is important when evaluating the results. In the current study, we did not find a marked difference in the effects observed for the parent compounds and the effects of the tested metabolic extracts.

Regarding the aim of looking at different *in vitro* systems for biotransformation of known endocrine disrupting chemicals in connection with testing their effects in *in vitro* assays, two *in vitro* metabolising systems were tested. Human liver S9 mix, and PCB-induced hepatic rat microsomes.

The basis for choosing human liver S9 was initially that we wanted to compare the results from the human liver S9 assay with results from an assay using a rat based *in vitro* metabolising system.

Both test systems gave an almost complete metabolic transformation of the tested parabens and phthalates, with a recovery rate of the parent compounds of less than 1%.

A difference was found between the human S9 and rat microsome assay systems. When using the PCB-induced rat microsomes, a statistically significant difference between the amount of parent compound before and after treatment with the microsomes was seen for four out of the five azole fungicides tested. When using the human liver S9, no significant metabolic transformation of the azole fungicides was detected. This variation could be due to differences in metabolism between humans and rats or it could be due to a general higher metabolic activity of the rat microsomes compared to the human S9 fraction. The use of PCB mixtures is a known and well established method to induce the activity of the different liver enzymes. The induction with PCB can only be done before the rat (or other animal) liver enzymes are recovered, and can be used in connetion with recovery of liver enzymes from humans. The applied human liver S9 mixtures were made from pooled livers from both male and females in the ages from 28 to 77 years of age. So, although the use of human liver enzymes would seem more relevant in regards to a human exposure scenario, at least one question that comes into mind is how representative these human livers and liver enzymes are of a healthy human population? Overall, there is reason to believe that a higher metabolic activity of the rat microsomes could be one reason of the difference in metabolism between the human liver S9 and the PCB induced rat microsomes. However, it is well known that there are many differences in the metabolic capacity between species and also differences within the same species associated with age, gender, nutrition, genetic predisposition, environmental factors and others.

In regards to risk assessment for endocrine disrupting effects and human exposure, it would seem most relevant to use metabolising systems based on materials from humans. However, using

materials from animals compared to humans would most likely be easier and cheaper, particularly because getting enough amount of research material from animals would be less of a challenge. Therefore, it is relevant to get as much data as possible in order to make valid comparisons and extrapolations between results from different species. Another reason for the difference observed between the human S9 and rat microsome metabolising systems could be due to different binding of test compounds to proteins in the enzyme fractions. The azole fungicides are the most apolar of the ten test compounds and they might bind to proteins or lipids making them less accessible for the metabolising enzymes. Several EDs are known to bind strongly to serum proteins and when this occurs *in vivo,* it affects the internal target tissue concentrations of the chemical involved (184). Such binding can occur with components of the metabolising systems like S9 or components in culture media such as proteins in serum added to the media thereby resulting in both variable *in vitro* data, but also in differences in the results obtained in *in vivo* and *in vitro* assays.

The focus of this study was on getting qualitative data, i.e., to see if the test compounds could be metabolised by the different *in vitro* metabolising systems tested and to get information on the metabolic capability of the cells in the selected *in vitro* assay. However, additional studies focusing on determining the metabolic fate of known endocrine disrupters in more detail and identifying metabolites would be relevant.

7 Conclusions and perspectives

The challenge with understanding the effects of EDCs, and the mechanisms behind them is that EDCs involve many different classes of compounds, affecting different systems and pathways, and acting through multiple mechanisms of action, making the picture of the effects of EDCs very complex. The results of the studies presented in this PhD thesis have contributed to the still existing need for more information regarding the effects of EDCs and mechanisms behind the observed effects. Much focus of this PhD project has been on PEs and mixtures of PEs. The contradicting effects and the biphasic nature of the PEs make it difficult to get a clear picture of the effects of the PEs, also in relation to any possible health promoting effects. Overall more research is needed to help understand the mechanisms and the many different effects of PEs.

Based on the studies conducted in this thesis, it cannot be concluded whether it is the potent effects of genestein and/or coumestrol alone that account for most of the effects observed with the total PE mix. However, based on the results from mix 2 in the different *in vitro* assays, it is not only the mammalian metabolites, but also the precursor plant isoflavones that have the ability to act as potent endocrine disrupters. In addition, PEs might act as ER agonists as observed in the MCF-7 proliferation assay reported here and elsewhere, which also correlates with a possible ER mediated effect of PEs like genistein on lipid accumulation in 3T3-L1 adipocytes.

Our conclusion is that many PEs, as well as nutrition relevant mixtures of PEs have an inhibitory effect on lipid accumulation *in vitro*, which could suggest a beneficial effect with regard to obesity. However, the role of the different players involved in adipogenesis and lipolysis, not only the different PPAR isoforms, but RXR and ER as well, are still not understood. Therefore, based on the results of the current studies, the influence of PEs on adipogenisis and their effects on the different pathways involved in the development of obesity remain unclear. Nevertheless, the current results in combination with previous studies and future experiments will hopefully bring us closer to what role exposure to EDCs play in the development of obesity and obesity-related diseases, like type 2 diabetes.

Additionally, the ability of PEs to make human cells (H295R) produce more endogenous estradiol should be emphasised and taken into account, when evaluating the overall effects of PEs on humans.

Further studies are needed in order to investigate mixtures in relation to single compounds. Especially, it could be interesting to test whether the effects of lignans are additive to the effects of an isoflavonoid, such as genistein. Knowledge from such kind of studies would improve risk

assessment of possible adverse effects of the various EDCs that we may come in contact with through our diet.

Finally, in regards to the endogenous metabolic capability of different applied cell lines, and the lack of metabolism in connection with *in vitro* tests for endocrine disruption, our conclusion, based on the results and data from the study presented in paper II, is that the GH3 cells, applied in the T-screen assay, show some metabolic capability in relation to the two tested phthalates DMP and DEP, but none towards the tested azole fungicides and phthalates. However, additional information regarding the metabolic capability of other applied cell lines would be relevant, as well as identification of the metabolites from the different compounds and knowledge concerning the metabolic fate of known EDCs.
Overall, an *in vitro* metabolising system using liver S9 mixtures or hepatic microsomes could be a convenient method for the incorporation of metabolism into *in vitro* tests for endocrine disrupters.

8 Reference list

1. McLachlan JA. Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. Endocr Rev 2001;22:319-41.

2. Devillers J. Endocrine Disruption Modeling: CRC Press; Taylor & Francis Group, 2009.

3. IPCS. Global assessment of the state-of-the-science of endocrine disruptors. In: Damstra T, Barlow, S., Bergman, A., Kavlock, R., Kraak Van der, ed. International Programme on Chemical Safety: World Health Organisation, 2002.

4. Swan SH, Main KM, Liu F, et al. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 2005;113:1056-61.

5. Mouritsen A, Aksglaede L, Sørensen K, et al. Hypothesis: exposure to endocrine-disrupting chemicals may interfere with timing of puberty. International Journal of Andrology 2010;33:346-359.

6. Olefsky JM. Nuclear receptor minireview series. J Biol Chem 2001;276:36863-4.

7. Losel RM, Falkenstein E, Feuring M, et al. Nongenomic steroid action: controversies, questions, and answers. Physiol Rev 2003;83:965-1016.

8. Bjornstrom L, Sjoberg M. Estrogen receptor-dependent activation of AP-1 via non-genomic signalling. Nucl Recept 2004;2:3.

9. Nadal A, Ropero AB, Fuentes E, Soria B. The plasma membrane estrogen receptor: nuclear or unclear? Trends in Pharmacological Sciences 2001;22:597-599.

10. Kenakin T. Inverse, protean, and ligand-selective agonism: matters of receptor conformation. Faseb J 2001;15:598-611.

11. Urban JD, Clarke WP, von Zastrow M, et al. Functional selectivity and classical concepts of quantitative pharmacology. J Pharmacol Exp Ther 2007;320:1-13.

12. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev 2004;25:45-71.

13. Katzenellenbogen BS, Katzenellenbogen JA. Biomedicine. Defining the "S" in SERMs. Science 2002;295:2380-1.

14. Sanderson JT. The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. Toxicol Sci 2006;94:3-21.

15. Miller WL. Molecular biology of steroid hormone synthesis. Endocr Rev 1988;9:295-318.

16. Taxvig C, Hass U, Axelstad M, et al. Endocrine-disrupting activities in vivo of the fungicides tebuconazole and epoxiconazole. Toxicol Sci 2007;100:464-73.

17. Blystone CR, Furr J, Lambright CS, et al. Prochloraz Inhibits Testosterone Production at Dosages below Those that Affect Androgen-Dependent Organ Weights or the Onset of Puberty in the Male Sprague Dawley Rat. Toxicol. Sci. 2007;97:65-74.

18. Westphal U. Steroid-protein interactions II. Monogr Endocrinol 1986;27:1-603.

19. Fortunati N. Sex hormone-binding globulin: not only a transport protein. What news is around the corner? J Endocrinol Invest 1999;22:223-34.

20. Dechaud H, Ravard C, Claustrat F, de la Perriere AB, Pugeat M. Xenoestrogen interaction with human sex hormone-binding globulin (hSHBG). Steroids 1999;64:328-34.

21. Martin ME, Haourigui M, Pelissero C, Benassayag C, Nunez EA. Interactions between phytoestrogens and human sex steroid binding protein. Life Sci 1996;58:429-36.

22. Crain DA, Noriega N, Vonier PM, Arnold SF, McLachlan JA, Guillette LJ, Jr. Cellular bioavailability of natural hormones and environmental contaminants as a function of serum and cytosolic binding factors. Toxicol Ind Health 1998;14:261-73.

23. Nagel SC, vom Saal FS, Welshons WV. The effective free fraction of estradiol and xenoestrogens in human serum measured by whole cell uptake assays: physiology of delivery modifies estrogenic activity. Proc Soc Exp Biol Med 1998;217:300-9.

24. Ho SM, Tang WY, Belmonte de Frausto J, Prins GS. Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. Cancer Res 2006;66:5624-32.

25. Newbold RR, Padilla-Banks E, Jefferson WN. Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. Endocrinology 2006;147:S11-7.

26. Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 2005;308:1466-9.

27. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. Science 2001;293:1068-70.

28. Akhtar A, Cavalli G. The epigenome network of excellence. PLoS Biol 2005;3:e177.

29. Holliday R. DNA methylation and epigenetic mechanisms. Cell Biophys 1989;15:15-20.

30. Herbst AL, Ulfelder H, Poskanzer DC. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. N Engl J Med 1971;284:878-81.

31. McLachlan JA, Newbold RR, Bullock BC. Long-term effects on the female mouse genital tract associated with prenatal exposure to diethylstilbestrol. Cancer Res 1980;40:3988-99.

32. Wu Q, Ohsako S, Ishimura R, Suzuki JS, Tohyama C. Exposure of mouse preimplantation embryos to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters the methylation status of imprinted genes H19 and Igf2. Biol Reprod 2004;70:1790-7.

33. Foster PM. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Int J Androl 2006;29:140-7; discussion 181-5.

34. McLachlan JA, Simpson E, Martin M. Endocrine disrupters and female reproductive health. Best Pract Res Clin Endocrinol Metab 2006;20:63-75.

35. Setchell KD. Soy isoflavones--benefits and risks from nature's selective estrogen receptor modulators (SERMs). J Am Coll Nutr 2001;20:354S-362S; discussion 381S-383S.

36. Ibarreta D, Daxenberger A, Meyer HH. Possible health impact of phytoestrogens and xenoestrogens in food. Apmis 2001;109:161-84.

37. Magee PJ, Rowland IR. Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. Br J Nutr 2004;91:513-31.

38. Adlercreutz H, Mazur W, Bartels P, et al. Phytoestrogens and Prostate Disease. J. Nutr. 2000;130:658-.

39. Jian-Ping Yuan J-HWXL. Metabolism of dietary soy isoflavones to equol by human intestinal microflora - implications for health. Molecular Nutrition & Food Research 2007;51:765-781.

40. Le Bail JC, Champavier Y, Chulia AJ, Habrioux G. Effects of phytoestrogens on aromatase, 3beta and 17beta-hydroxysteroid dehydrogenase activities and human breast cancer cells. Life Sci 2000;66:1281-91.

41. Power KA, Saarinen NM, Chen JM, Thompson LU. Mammalian lignans enterolactone and enterodiol, alone and in combination with the isoflavone genistein, do not promote the growth of MCF-7 xenografts in ovariectomized athymic nude mice. Int J Cancer 2006;118:1316-20.

42. Krista A. Power LUT. Can the combination of flaxseed and its lignans with soy and its isoflavones reduce the growth stimulatory effect of soy and its isoflavones on established breast cancer? Molecular Nutrition & Food Research 2007;51:845-856.

43. Tham DM, Gardner CD, Haskell WL. Clinical review 97: Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. J Clin Endocrinol Metab 1998;83:2223-35.

44. Yang J, Nakagawa H, Tsuta K, Tsubura A. Influence of perinatal genistein exposure on the development of MNU-induced mammary carcinoma in female Sprague-Dawley rats. Cancer Lett 2000;149:171-9.

45. Hilakivi-Clarke L, Cho E, Onojafe I, Raygada M, Clarke R. Maternal exposure to genistein during pregnancy increases carcinogen-induced mammary tumorigenesis in female rat offspring. Oncol Rep 1999;6:1089-95.

46. Allred CD, Ju YH, Allred KF, Chang J, Helferich WG. Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein. Carcinogenesis 2001;22:1667-73.

47. De Lemos ML. Effects of soy phytoestrogens genistein and daidzein on breast cancer growth. Ann Pharmacother 2001;35:1118-1121.

48. Almstrup K, Fernandez MF, Petersen JH, Olea N, Skakkebaek NE, Leffers H. Dual effects of phytoestrogens result in u-shaped dose-response curves. Environ Health Perspect 2002;110:743-8.

49. Simpson ER, Davis SR. Minireview: aromatase and the regulation of estrogen biosynthesis--some new perspectives. Endocrinology 2001;142:4589-94.

50. Rice S, Mason HD, Whitehead SA. Phytoestrogens and their low dose combinations inhibit mRNA expression and activity of aromatase in human granulosa-luteal cells. J Steroid Biochem Mol Biol 2006;101:216-25.

51. Adlercreutz H. Phyto-oestrogens and cancer. Lancet Oncol 2002;3:364-73.

52. Benassayag C, Perrot-Applanat M, Ferre F. Phytoestrogens as modulators of steroid action in target cells. J Chromatogr B Analyt Technol Biomed Life Sci 2002;777:233-48.

53. Kinoshita Y, Chen S. Induction of Aromatase (CYP19) Expression in Breast Cancer Cells through a Nongenomic Action of Estrogen Receptor {alpha}. Cancer Res 2003;63:3546-3555.

54. Sanderson JT, Hordijk J, Denison MS, Springsteel MF, Nantz MH, van den Berg M. Induction and inhibition of aromatase (CYP19) activity by natural and synthetic flavonoid compounds in H295R human adrenocortical carcinoma cells. Toxicol Sci 2004;82:70-9.

55. Edmunds KM, Holloway AC, Crankshaw DJ, Agarwal SK, Foster WG. The effects of dietary phytoestrogens on aromatase activity in human endometrial stromal cells. Reprod Nutr Dev 2005;45:709-20.

56. Jensen AF, Petersen A, Granby K. Cumulative risk assessment of the intake of organophosphorus and carbamate pesticides in the Danish diet. Food Addit Contam 2003;20:776-85.

57. Pitarch E, Serrano R, Lopez FJ, Hernandez F. Rapid multiresidue determination of organochlorine and organophosphorus compounds in human serum by solid-phase extraction and gas chromatography coupled to tandem mass spectrometry. Anal Bioanal Chem 2003;376:189-97.

58. Sanghi R, Pillai MK, Jayalekshmi TR, Nair A. Organochlorine and organophosphorus pesticide residues in breast milk from Bhopal, Madhya Pradesh, India. Hum Exp Toxicol 2003;22:73-6.

59. Swan SH, Kruse RL, Liu F, et al. Semen quality in relation to biomarkers of pesticide exposure. Environ Health Perspect 2003;111:1478-84.

60. Weidner IS, Moller H, Jensen TK, Skakkebaek NE. Cryptorchidism and hypospadias in sons of gardeners and farmers. Environ Health Perspect 1998;106:793-6.

61. Ghannoum MA, Rice LB. Antifungal Agents: Mode of Action, Mechanisms of Resistance, and Correlation of These Mechanisms with Bacterial Resistance. Clin. Microbiol. Rev. 1999;12:501-517.

62. Zarn JA, Bruschweiler BJ, Schlatter JR. Azole fungicides affect mammalian steroidogenesis by inhibiting sterol 14 alpha-demethylase and aromatase. Environ Health Perspect 2003;111:255-61.

63. Barton HA, Tang J, Sey YM, et al. Metabolism of myclobutanil and triadimefon by human and rat cytochrome P450 enzymes and liver microsomes. Xenobiotica 2006;36:793-806.

64. Tully DB, Bao W, Goetz AK, et al. Gene expression profiling in liver and testis of rats to characterize the toxicity of triazole fungicides. Toxicol Appl Pharmacol 2006;215:260-73.

65. Sun G, Thai SF, Tully DB, et al. Propiconazole-induced cytochrome P450 gene expression and enzymatic activities in rat and mouse liver. Toxicol Lett 2005;155:277-87.

66. Mason JI, Carr BR, Murry BA. Imidazole antimycotics: selective inhibitors of steroid aromatization and progesterone hydroxylation. Steroids 1987;50:179-89.

67. Sanderson JT, Boerma J, Lansbergen GW, van den Berg M. Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. Toxicol Appl Pharmacol 2002;182:44-54.

68. Vinggaard AM, Hnida C, Breinholt V, Larsen JC. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol In Vitro 2000;14:227-34.

69. Vinggaard AM, Christiansen S, Laier P, et al. Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring. Toxicol Sci 2005;85:886-97.

70. Vinggaard AM, Jacobsen H, Metzdorff SB, Andersen HR, Nellemann C. Antiandrogenic effects in short-term in vivo studies of the fungicide fenarimol. Toxicology 2005;207:21-34.

71. Byford JR, Shaw LE, Drew MG, Pope GS, Sauer MJ, Darbre PD. Oestrogenic activity of parabens in MCF7 human breast cancer cells. J Steroid Biochem Mol Biol 2002;80:49-60.

72. Oishi S. Effects of propyl paraben on the male reproductive system. Food Chem Toxicol 2002;40:1807-13.

73. Okubo T, Yokoyama Y, Kano K, Kano I. ER-dependent estrogenic activity of parabens assessed by proliferation of human breast cancer MCF-7 cells and expression of ERalpha and PR. Food Chem Toxicol 2001;39:1225-32.

74. Pedersen KL, Pedersen SN, Christiansen LB, Korsgaard B, Bjerregaard P. The preservatives ethyl-, propyl- and butylparaben are oestrogenic in an in vivo fish assay. Pharmacol Toxicol 2000;86:110-3.

75. Routledge EJ, Parker J, Odum J, Ashby J, Sumpter JP. Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. Toxicol Appl Pharmacol 1998;153:12-9.

76. Nakagawa Y, Moldeus P. Mechanism of p-hydroxybenzoate ester-induced mitochondrial dysfunction and cytotoxicity in isolated rat hepatocytes. Biochem Pharmacol 1998;55:1907-14.

77. Darbre PD, Harvey PW. Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks. J Appl Toxicol 2008;28:561-78.

78. Casals-Casas C, Desvergne B. Endocrine Disruptors: From Endocrine to Metabolic Disruption. Annu Rev Physiol 2011.

79. Halden RU. Plastics and health risks. Annu Rev Public Health 2010;31:179-94.

80. Arcadi FA, Costa C, Imperatore C, et al. Oral toxicity of bis(2-ethylhexyl) phthalate during pregnancy and suckling in the Long-Evans rat. Food Chem Toxicol 1998;36:963-70.

81. Li LH, Jester WF, Jr., Orth JM. Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured Sertoli cells and gonocytes from neonatal rats. Toxicol Appl Pharmacol 1998;153:258-65.

82. Sharpe RM. Phthalate exposure during pregnancy and lower anogenital index in boys: wider implications for the general population? Environ Health Perspect 2005;113:A504-5.

83. Christiansen S, Boberg J, Axelstad M, et al. Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats. Reprod Toxicol 2010;30:313-21.

84. Gayathri NS, Dhanya CR, Indu AR, Kurup PA. Changes in some hormones by low doses of di (2-ethyl hexyl) phthalate (DEHP), a commonly used plasticizer in PVC blood storage bags & medical tubing. Indian J Med Res 2004;119:139-44.

85. Heudorf U, Mersch-Sundermann V, Angerer J. Phthalates: toxicology and exposure. Int J Hyg Environ Health 2007;210:623-34.

86. Stahlhut RW, van Wijngaarden E, Dye TD, Cook S, Swan SH. Concentrations of urinary phthalate metabolites are associated with increased waist circumference and insulin resistance in adult U.S. males. Environ Health Perspect 2007;115:876-82.

87. Desvergne B, Feige JN, Casals-Casas C. PPAR-mediated activity of phthalates: A link to the obesity epidemic? Molecular and Cellular Endocrinology 2009;304:43-48.

88. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, et al. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev 2009;30:293-342.

89. Grun F, Blumberg B. Minireview: the case for obesogens. Mol Endocrinol 2009;23:1127-34.

90. Grun F, Blumberg B. Endocrine disrupters as obesogens. Mol Cell Endocrinol 2009;304:19-29.

91. Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenbergh JG, vom Saal FS. Exposure to bisphenol A advances puberty. Nature 1999;401:763-4.

92. Newbold RR, Padilla-Banks E, Snyder RJ, Jefferson WN. Developmental exposure to estrogenic compounds and obesity. Birth Defects Res A Clin Mol Teratol 2005;73:478-80.

93. Rubin BS, Soto AM. Bisphenol A: Perinatal exposure and body weight. Mol Cell Endocrinol 2009;304:55-62.

94. Longnecker MP, Michalek JE. Serum dioxin level in relation to diabetes mellitus among Air Force veterans with background levels of exposure. Epidemiology 2000;11:44-8.

95. Newbold RR. Impact of environmental endocrine disrupting chemicals on the development of obesity. Hormones (Athens) 2010;9:206-17.

96. Baillie-Hamilton PF. Chemical toxins: a hypothesis to explain the global obesity epidemic. J Altern Complement Med 2002;8:185-92.

97. Heindel JJ. Endocrine disruptors and the obesity epidemic. Toxicol Sci 2003;76:247-9.

98. Boberg J, Metzdorff S, Wortziger R, et al. Impact of diisobutyl phthalate and other PPAR agonists on steroidogenesis and plasma insulin and leptin levels in fetal rats. Toxicology 2008;250:75-81.

99. Grun F. Obesogens. Curr Opin Endocrinol Diabetes Obes 2010;17:453-9.

100. Swedenborg E, Ruegg J, Makela S, Pongratz I. Endocrine disruptive chemicals: mechanisms of action and involvement in metabolic disorders. J Mol Endocrinol 2009;43:1-10.

101. Rosen ED, Spiegelman BM. Molecular regulation of adipogenesis. Annu Rev Cell Dev Biol 2000;16:145-71.

102. Larsen TM, Toubro S, Astrup A. PPARgamma agonists in the treatment of type II diabetes: is increased fatness commensurate with long-term efficacy? Int J Obes Relat Metab Disord 2003;27:147-61.

103. Jones ME, Thorburn AW, Britt KL, et al. Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity. Proc Natl Acad Sci U S A 2000;97:12735-40.

104. Kim HK, Nelson-Dooley C, Della-Fera MA, et al. Genistein decreases food intake, body weight, and fat pad weight and causes adipose tissue apoptosis in ovariectomized female mice. J Nutr 2006;136:409-14.

105. Goodman-Gruen D, Kritz-Silverstein D. Usual dietary isoflavone intake and body composition in postmenopausal women. Menopause 2003;10:427-32.

106. Naaz A, Yellayi S, Zakroczymski MA, et al. The soy isoflavone genistein decreases adipose deposition in mice. Endocrinology 2003;144:3315-20.

107. Wu J, Oka J, Tabata I, et al. Effects of isoflavone and exercise on BMD and fat mass in postmenopausal Japanese women: a 1-year randomized placebo-controlled trial. J Bone Miner Res 2006;21:780-9.

108. Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, et al. Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. Endocr Rev 2009;30:293-342.

109. Achard V, Boullu-Ciocca S, Desbriere R, Grino M. Perinatal programming of central obesity and the metabolic syndrome: role of glucocorticoids. Metab Syndr Relat Disord 2006;4:129-37.

110. Nyirenda MJ, Lindsay RS, Kenyon CJ, Burchell A, Seckl JR. Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. J Clin Invest 1998;101:2174-81.

111. Combes RD. The in vivo relevance of in vitro genotoxicity assays incorporating enzyme activation systems. . In: Gibson GG, ed. Progress in Drug Metabolism London: Taylor and Francis Limited, 1992:295 - 321.

112. Coecke S, Ahr H, Blaauboer BJ, et al. Metabolism: a bottleneck in in vitro toxicological test development. The report and recommendations of ECVAM workshop 54. Altern Lab Anim 2006;34:49-84.

113. Werck-Reichhart D, Feyereisen R. Cytochromes P450: a success story. Genome Biol 2000;1:REVIEWS3003.

114. Wong C, Kelce WR, Sar M, Wilson EM. Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. J Biol Chem 1995;270:19998-20003.

115. Setchell KDRaHA. Mammalian lignans and phytoestrogens. Recent studies on their formation, metabolism, and biological role in health and disease. In: Rowland IR, ed. Role of the Gut Flora in Toxicity and Cancer. London: Academic Press, 1988:315-345.

116. Coldham NG, Horton R, Byford MF, Sauer MJ. A binary screening assay for pro-oestrogens in food: metabolic activation using hepatic microsomes and detection with oestrogen sensitive recombinant yeast cells. Food Addit Contam 2002;19:1138-47.

117. Guillouzo A. Liver cell models in in vitro toxicology. Environ Health Perspect 1998;106 Suppl 2:511-32.

118. Daston GP, Cook JC, Kavlock RJ. Uncertainties for endocrine disrupters: our view on progress. Toxicol Sci 2003;74:245-52.

119. Kortenkamp A. Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology. Int J Androl 2008;31:233-40.

120. Kortenkamp A, Backhaus T, Faust M. State of the Art Report on Mixture Toxicity - Final Report, Executive Summary. 2009:1-391.

121. Cedergreen N, Christensen AM, Kamper A, et al. A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. Environ Toxicol Chem 2008;27:1621-32.

122. Rider CV, Furr J, Wilson VS, Gray LE, Jr. A mixture of seven antiandrogens induces reproductive malformations in rats. Int J Androl 2008;31:249-62.

123. Hass U, Scholze M, Christiansen S, et al. Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. Environ Health Perspect 2007;115 Suppl 1:122-8.

124. Rajapakse N, Silva E, Kortenkamp A. Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. Environ Health Perspect 2002;110:917-21.

125. Silva E, Rajapakse N, Kortenkamp A. Something from "nothing"--eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ Sci Technol 2002;36:1751-6.

126. Payne J, Rajapakse N, Wilkins M, Kortenkamp A. Prediction and assessment of the effects of mixtures of four xenoestrogens. Environ Health Perspect 2000;108:983-7.

127. Birkhoj M, Nellemann C, Jarfelt K, et al. The combined antiandrogenic effects of five commonly used pesticides. Toxicol Appl Pharmacol 2004;201:10-20.

128. Nellemann C, Dalgaard M, Lam HR, Vinggaard AM. The combined effects of vinclozolin and procymidone do not deviate from expected additivity in vitro and in vivo. Toxicol Sci 2003;71:251-62.

129. Metzdorff SB, Dalgaard M, Christiansen S, et al. Dysgenesis and histological changes of genitals and perturbations of gene expression in male rats after in utero exposure to antiandrogen mixtures. Toxicol Sci 2007;98:87-98.

130. Muller AK, Bosgra S, Boon PE, van der Voet H, Nielsen E, Ladefoged O. Probabilistic cumulative risk assessment of anti-androgenic pesticides in food. Food Chem Toxicol 2009;47:2951-62.

131. Charles GD. In vitro models in endocrine disruptor screening. Ilar J 2004;45:494-501.

132. Connolly L, Ropstad E, Verhaegen S. In vitro bioassays for the study of endocrine-disrupting food additives and contaminants. TrAC Trends in Analytical Chemistry 2011;In Press, Corrected Proof.

133. Mueller SO. Overview of in vitro tools to assess the estrogenic and antiestrogenic activity of phytoestrogens. J Chromatogr B Analyt Technol Biomed Life Sci 2002;777:155-65.

134. Rainey WE, Bird IM, Sawetawan C, et al. Regulation of human adrenal carcinoma cell (NCI-H295) production of C19 steroids. J Clin Endocrinol Metab 1993;77:731-7.

135. Hecker M, Newsted JL, Murphy MB, et al. Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis: hormone production. Toxicol Appl Pharmacol 2006;217:114-24.

136. Lephart ED, Simpson ER. Assay of aromatase activity. Methods Enzymol 1991;206:477-83.

137. Brueggemeier RW. Update on the use of aromatase inhibitors in breast cancer. Expert Opinion on Pharmacotherapy 2006;7:1919-1930.

138. OECD. Detailed revue paper on thyroid hormone disruption assays, . OECD Environment, Health and Safety Publications Series on Testing and Assessment 2006;No. 57:1-434.

139. Capen CC. Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. Toxicol Pathol 1997;25:39-48.

140. Cheek AO, Kow K, Chen J, McLachlan JA. Potential mechanisms of thyroid disruption in humans: interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. Environ Health Perspect 1999;107:273-8.

141. Craft ES, DeVito MJ, Crofton KM. Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. Toxicol Sci 2002;68:372-80.

142. DeVito M, Biegel L, Brouwer A, et al. Screening methods for thyroid hormone disruptors. Environ Health Perspect 1999;107:407-15.

143. Kato Y, Haraguchi K, Shibahara T, Yumoto S, Masuda Y, Kimura R. Reduction of serum thyroxine concentrations by methylsulfonyl metabolites of tetra-, penta- and hexachlorinated biphenyls in male Sprague-Dawley rats. Chemosphere 2000;40:1233-40.

144. Zhou T, Ross DG, DeVito MJ, Crofton KM. Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. Toxicol Sci 2001;61:76-82.

145. Zoeller RT, Bansal R, Parris C. Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist in vitro, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. Endocrinology 2005;146:607-12.

146. Hallgren S, Sinjari T, Hakansson H, Darnerud PO. Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. Arch Toxicol 2001;75:200-8.

147. Gauger KJ, Kato Y, Haraguchi K, et al. Polychlorinated biphenyls (PCBs) exert thyroid hormone-like effects in the fetal rat brain but do not bind to thyroid hormone receptors. Environ Health Perspect 2004;112:516-23.

148. Fritsche E, Cline JE, Nguyen NH, Scanlan TS, Abel J. Polychlorinated biphenyls disturb differentiation of normal human neural progenitor cells: clue for involvement of thyroid hormone receptors. Environ Health Perspect 2005;113:871-6.

149. Hinkle PM, Kinsella PA. Thyroid hormone induction of an autocrine growth factor secreted by pituitary tumor cells. Science 1986;234:1549-52.

150. Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. Physiol Rev 1998;78:783-809.

151. Klein J, Fasshauer M, Klein HH, Benito M, Kahn CR. Novel adipocyte lines from brown fat: a model system for the study of differentiation, energy metabolism, and insulin action. Bioessays 2002;24:382-8.

152. Auwerx J. PPARgamma, the ultimate thrifty gene. Diabetologia 1999;42:1033-49.

153. Ferre P. The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. Diabetes 2004;53 Suppl 1:S43-50.

154. Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 1994;79:1147-56.

155. Brun RP, Tontonoz P, Forman BM, et al. Differential activation of adipogenesis by multiple PPAR isoforms. Genes Dev 1996;10:974-84.

156. Yu K, Bayona W, Kallen CB, et al. Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J Biol Chem 1995;270:23975-83.

157. Hedelin M, Klint A, Chang ET, et al. Dietary phytoestrogen, serum enterolactone and risk of prostate cancer: the cancer prostate Sweden study (Sweden). Cancer Causes Control 2006;17:169-80.

158. Ricketts ML, Moore DD, Banz WJ, Mezei O, Shay NF. Molecular mechanisms of action of the soy isoflavones includes activation of promiscuous nuclear receptors. A review. J Nutr Biochem 2005;16:321-30.

159. Hu E, Tontonoz P, Spiegelman BM. Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. Proc Natl Acad Sci U S A 1995;92:9856-60.

160. Barak Y, Nelson MC, Ong ES, et al. PPAR gamma is required for placental, cardiac, and adipose tissue development. Mol Cell 1999;4:585-95.

161. Kubota N, Terauchi Y, Miki H, et al. PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. Mol Cell 1999;4:597-609.

162. Rosen ED, Sarraf P, Troy AE, et al. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Mol Cell 1999;4:611-7.

163. Ohno S, Shinoda S, Toyoshima S, Nakazawa H, Makino T, Nakajin S. Effects of flavonoid phytochemicals on cortisol production and on activities of steroidogenic enzymes in human adrenocortical H295R cells. J Steroid Biochem Mol Biol 2002;80:355-63.

164. van Meeuwen JA, Ter Burg W, Piersma AH, van den Berg M, Sanderson JT. Mixture effects of estrogenic compounds on proliferation and pS2 expression of MCF-7 human breast cancer cells. Food Chem Toxicol 2007;45:2319-30.

165. Ying C, Hsu J-T, Yang C-D, et al. The Combination Effects of Phytoestrogens on Cell Proliferation, Cell Cycle Progression and Estrogen Receptor Alpha. Journal of Food and Drug Analysis 2005;13:30-37.

166. Kuppusamy UR, Das NP. Effects of flavonoids on cyclic AMP phosphodiesterase and lipid mobilization in rat adipocytes. Biochem Pharmacol 1992;44:1307-15.

167. Nichols MR, Morimoto BH. Differential inhibition of multiple cAMP phosphodiesterase isozymes by isoflavones and tyrphostins. Mol Pharmacol 2000;57:738-45.

168. Park HJ, Yang JY, Ambati S, et al. Combined effects of genistein, quercetin, and resveratrol in human and 3T3-L1 adipocytes. J Med Food 2008;11:773-83.

169. Liao QC, Li YL, Qin YF, et al. Inhibition of adipocyte differentiation by phytoestrogen genistein through a potential downregulation of extracellular signal-regulated kinases 1/2 activity. J Cell Biochem 2008;104:1853-64.

170. Cooke PS, Naaz A. Effects of estrogens and the phytoestrogen genistein on adipogenesis and lipogenesis in males and females. Birth Defects Res A Clin Mol Teratol 2005;73:472-3.

171. Rayalam S, Della-Fera MA, Yang J-Y, Park HJ, Ambati S, Baile CA. Resveratrol Potentiates Genistein's Antiadipogenic and Proapoptotic Effects in 3T3-L1 Adipocytes. J. Nutr. 2007;137:2668-2673.

172. Harmon AW, Harp JB. Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. Am J Physiol Cell Physiol 2001;280:C807-13.

173. Rachon D, Vortherms T, Seidlova-Wuttke D, Wuttke W. Effects of dietary equol on body weight gain, intra-abdominal fat accumulation, plasma lipids, and glucose tolerance in ovariectomized Sprague-Dawley rats. Menopause 2007;14:925-32.

174. Homma H, Kurachi H, Nishio Y, et al. Estrogen suppresses transcription of lipoprotein lipase gene. Existence of a unique estrogen response element on the lipoprotein lipase promoter. J Biol Chem 2000;275:11404-11.

175. Shen P, Liu MH, Ng TY, Chan YH, Yong EL. Differential effects of isoflavones, from Astragalus membranaceus and Pueraria thomsonii, on the activation of PPARalpha, PPARgamma, and adipocyte differentiation in vitro. J Nutr 2006;136:899-905.

176. Issemann I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 1990;347:645-50.

177. Beck F, Plummer S, Senior PV, Byrne S, Green S, Brammar WJ. The ontogeny of peroxisome-proliferator-activated receptor gene expression in the mouse and rat. Proc Biol Sci 1992;247:83-7.

178. Wang YX. PPARs: diverse regulators in energy metabolism and metabolic diseases. Cell Res 2010;20:124-37.

179. Choi K, Kim Y-B. Molecular Mechanism of Insulin Resistance in Obesity and Type 2 Diabetes. Korean J Intern Med 2010;25:119-129.

180. Hiromori Y, Nishikawa J-i, Yoshida I, Nagase H, Nakanishi T. Structure-dependent activation of peroxisome proliferator-activated receptor (PPAR) [gamma] by organotin compounds. Chemico-Biological Interactions 2009;180:238-244.

181. Inadera H, Shimomura A. Environmental chemical tributyltin augments adipocyte differentiation. Toxicol Lett 2005;159:226-34.

182. le Maire A, Grimaldi M, Roecklin D, et al. Activation of RXR-PPAR heterodimers by organotin environmental endocrine disruptors. EMBO Rep 2009;10:367-373.

183. Kirchner S, Kieu T, Chow C, Casey S, Blumberg B. Prenatal exposure to the environmental obesogen tributyltin predisposes multipotent stem cells to become adipocytes. Mol Endocrinol 2010;24:526-39.

184. Safe S, Connor K, Ramamoorthy K, Gaido K, Maness S. Human exposure to endocrine-active chemicals: hazard assessment problems. Regul Toxicol Pharmacol 1997;26:52-8.

Appendix 1 – Papers I to III

Paper I

Nutrition and Cancer, 62(1), 122–131
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Effects of Nutrition Relevant Mixtures of Phytoestrogens on Steroidogenesis, Aromatase, Estrogen, and Androgen Activity

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Phytoestrogens (PEs) are naturally occurring plant components produced in a large range of plants. They can induce biologic responses in vertebrates by mimicking or modulating the action or production of endogenous hormones. This study examined mixtures of 12 food relevant PEs for effects on steroid hormone production, aromatase activity, estrogenic activity, and for interaction with the androgen receptor. The results show that a mixture of all tested PEs increased estradiol production and decreased testosterone production in H295R human adrenal corticocarcinoma cells, indicating an induced aromatase activity. Furthermore, exposure of the H295R cells to isoflavonoids caused a decrease in testosterone production, and various mixtures of PEs significantly stimulated MCF-7 human breast adenocarcinoma cell growth and induced aromatase activity in JEG-3 choriocarcinoma cells. The estrogenic effect in the MCF7 cells of the isoflavonoid mixture and coumestrol was supported by an observed increase in progesterone receptor protein expression as well as a decreased ERα expression.Overall, the results support that nutrition-relevant concentrations of PEs both alone and in mixtures possess various endocrine disrupting effects, all of which need to be considered when assessing the effects on human health.

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INTRODUCTION

Phytoestrogens (PEs) are a diverse group of naturally occurring plant compounds that because of their structural similarity with estradiol, have the ability to cause estrogenic or/and antiestrogenic effects (1).

The broad classes of PEs include the isoflavonoids (genistein, daidzein, formonetin, biochanin A, and equol) found predominantly in soya beans and red clover, the lignans (enterolactone, enterodiol, pinoresinol, lariciresinol, secoisolariciresinol, matairesinol) found particularly in flax seed and fruits, and the coumestans (coumestrol) found in red clover and a variety of other plants. Several of the PEs are ingested as precursors and then converted by the microflora in the mammalian gut. Lignans, for instance, are metabolized by intestinal bacteria to form the mammalian lignans known as enterodiol and enterolactone (2). The major isoflavonoids daidzin and genistin are metabolized in mammals to form the active compounds daidzein and genistein. Daidzein is further metabolized to the compound equol (3).

Many of the metabolites have been shown to have more pronounced effects compared to the mother compound (3,4). Several in vivo studies have indicated that PEs may elicit cancer-preventing effects in laboratory animals exposed to a cancer-inducing agent (3,5,6). Furthermore, epidemiological data suggests that a diet rich in phytoestrogens decreases the risk of breast cancer, among others, because the breast cancer incidence is much lower in Asian populations in comparison

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with Western populations (7). However, other studies, both animal and in vitro, have shown that the effects of phytoestrogens may increase cancer risk (8–11).

The vast majority of experimental studies in vitro and in vivo have typically been based on a single diet (containing primarily soy or flaxseed) or on single or a small number of individual components. However, PEs occur in complex matrices in our diet and it is relevant to conduct experimental designs in order to evaluate the effect of mixtures of these compounds. The selection of PEs for the current study was based on a Scandinavian epidemiological study, estimating the intake of a number of PEs in the Swedish population (12). Western diet contains several more lignans than the traditionally studied isoflavones.

The extent of the metabolism of PEs by the intestinal bacteria appears to be highly variable among individuals; for example, only one-third to one-half of the population can metabolize the soy isoflavone daidzein to equol (13,14). PEs are weak estrogens, but may also function as estrogen antagonists by blocking the binding of the much more potent endogenous estrogen to its receptor. In addition, it has been shown that PEs can affect the synthesis or metabolism of steroid hormones, for example, by affecting the aromatase activity. Aromatase activity is important for the balance between the endogenous testosterone and 17ß-estradiol, and changes in this balance may be beneficial or adverse in relation to proliferation of, for example, breast cancer tumor cells, as 17ß-estradiol is considered the main determinant of proliferation of estrogen-dependent breast cancer cells (15). The major site of aromatase dependent estradiol synthesis is in the ovary in premenopausal women and in peripheral tissues in postmenopausal women (such as the adrenals) (16). Many papers have referred to the inhibitory effects of PEs on the aromatase activity (4,17–19), but it cannot be generalized that these compounds elicit inhibitory effects, and it has been found that some PEs in fact induce aromatase activity (20–22). The available literature provides conflicting findings as to the effects of PEs, which probably results from the multiple mechanisms of action of these compounds. The complexity of the influence of PEs at the cellular and molecular level is further increased by the fact that their effects are dependent on the dose, the class to which they belong, the presence or absence of endogenous estrogens, their different affinity for the estrogen receptor (ER)α and ERß receptor, and the type of tissue or cell considered (19).

The aim of this study was to evaluate the endocrine disrupting potential of different PEs and mixtures of PEs naturally occurring in Western/Scandinavian food. The proportions of the different compounds in the mixtures were in equimolar concentrations; so although representing PEs naturally occurring in Western food, the proportions of the compounds in the mixtures are not a representative of the concentrations found in the diet. The PEs were analyzed in a panel of in vitro bioassays, designed to look for effects on 1) steroid hormone production in human adrenal corticocarcinoma cells (H295R), 2) aromatase activity in human JEG-3 choriocarcinoma cells, 3) estrogenic activity using the human MCF-7 cell proliferation assay, and 4) interaction with the androgen receptor (AR) in a reporter gene assay. The design of mixtures was not based on effective dose, as 4 different in vitro assays were used, and each chemical could have very different effect in each assay. In addition only genistein, daidzein, equol, and coumestrol were tested as single compounds. The results of this study, therefore, cannot be used to conclude on additivity. An overview of the different PEs and mixtures are presented in Fig. 1.

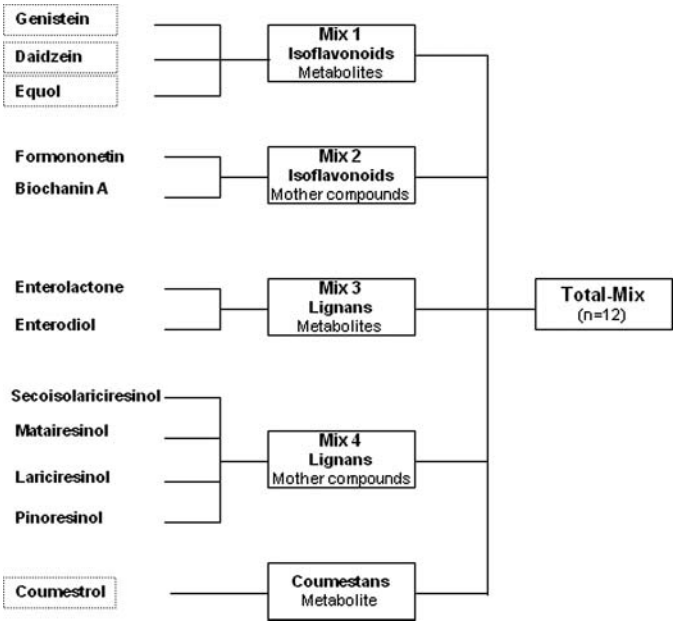

FIG. 1. Overview of the different phytoestrogens (PEs) and mixtures tested in the in vitro assays. The selected PEs consists of both mother compounds (biochanin A, formononetin, secoisolariciresinol, matairesinol, lariciresinol, pinoresinol, and coumestrol) and metabolites (genistein, daidzein, equol, enterodiol, and enterolactone). The different mixtures and the total mixture are presented in boxes, and the compounds, which were (also) tested alone, are represented in dotted line boxes.

MATERIALS AND METHODS

Test Compounds

The 12 PEs used and their data are listed in Table 1. The mixtures were made by mixing the PEs in equimolar concentrations. Thus a 10 μM concentration of mixture 1 is composed of 3.33 μM of each of the 3 PEs, genistein, daidzein, and equol. Mixture 2 is composed of the 2 isoflavonoids, formononetin and biochanin A; and mixture 3 is composed of enterolactone and enterodiol. Mixture 4 is a mixture of the 4 lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol. Coumestrol was part of the total mixture but was also tested alone, as it was the only coumestan included in the study. An overview of the different PEs and mixtures included in the study is given in Fig. 1.

[1β-^{3}H]Androst-4-ene-3,17-dione was from PerkinElmer (Boston, MA), and both 4-androstene-3,17-dione (4-AD) and 4-androsten-4-ol-3,17-dione (4-AOD) were from Sigma Aldrich

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(Milwaukee, WI). The test compounds were dissolved in dimethyl sulfoxide (DMSO).

Steroid Synthesis Assay (H295R)

The H295R cells are human adrenocortical carcinoma cells. The cell line has been characterized in detail and shown to be a useful tool for the study of adrenocortical function and steroidogenesis, as it express all the key enzymes necessary for steroidogenesis and is able to produce many steroid hormones, glucocorticoids, and mineralocorticoids.

H295R cells were obtained from the American Type Culture Collection (ATCC, CRL-2128; ATCC, Manassas, VA) and grown in 75 cm^2 flasks at 37°C with 5% CO_2. The cells were maintained in a 1:1 mixture of DMEM/F12 (Sigma) supplemented with 1.2g/l Na_2CO_2, 5.0 ml/l of ITS + premix (BD Bioscience), and 12.5 ml/l NU-serum (BD Bioscience). Cells were grown until almost confluent and seeded in 24-well cell culture plates at a density of 300,000 cells/ml. After a 24 h attachment period, the medium was removed, and new media containing the test compounds was added. Each test compound or mix was tested in triplicate. After incubation for 48 h, the media was removed and stored at –20°C until hormones were measured. Prochloraz (known inhibitor of testosterone/estradiol synthesis) was used as positive control, and DMSO was used as vehicle control. As positive control for aromatase inhibition, the H295R cells were exposed to 100 nM/1 μM of the steroidal aromatase inhibitor 4-AOD. Cytotoxicity was evaluated in each well by the use of a Live/Dead kit (following instructions supplied by the manufacturer, Molecular Probes, Invitrogen A/S, Taastrup, Denmark) and AlamarBlue (Serotec, Kidlington, UK). Fluorescence was measured after 1, 2, and 3 h (excitation 10 nm/emission 10 nm) using a PerkinElmer luminescence spectrophotometer, equipped with a microtiter plate reader.

The hormones were extracted from the medium by solid phase extraction using IST Isolute C18 SPE columns of 200 mg (Mikrolab Aarhus). The medium samples (800 μl) were diluted twofold with purified water and applied to columns preconditioned and rinsed with methanol and water, respectively. Interfering substances were eluted with 2 ml methanol:water (20:80 vol/vol), and the steroids were eluted with 2*2.4 ml methanol. Subsequently, the solvent was evaporated and samples were resuspended in 200 μl Diluent (PerkinElmer Life Sciences, Wallac). Testosterone and estradiol were measured in the extracts using Delfia kits from PerkinElmer Life Sciences, Wallac, for each tested compound/mixture. The H295R assay was repeated 3 times in the concentration range 10^{-10} to 10^{-5} M, and furthermore twice with 5 concentrations within the range 10^{-6} to 10^{-5} M, to study this concentration range in detail.

MCF-7 Cell Proliferation Assay

The MCF-7 (MCF-7-BUS) cell line was kindly provided by Ana Soto (Boston, MA). The cells were maintained in DMEM (Gibco, supplied by Invitrogen A/S, Taastrup, Denmark) without phenol red, supplemented with 10% heat-inactivated fetal calf serum (FCS) (Life Technologies, Bethesda, MD), 6 ng/ml bovine insulin (Novo Nordic, Bagsværd, Denmark), 4 mM glutamine, 20 mM HEPES and 1 mM sodium pyruvate (all from Invitrogen). Cells were subcultivated by trypsination once a week and were incubated in a 5% CO_2 humidified incubator at 37°C. For experiments, MCF-7 cells (2.5×10^4) were seeded into 24-well plates (1.9 cm^2 wells) and left to attach for 24 h before initiating 5 days exposure to PEs. At the onset of exposure, FCS in the medium was replaced with serum depleted of steroids by dextrane-coated charcoal treatment (DCC-FCS) (23), and the medium was supplemented with 2.5×10^5 U penicillin and 31.25 μg/l streptomycin (DCC medium). Cells were exposed to PEs in the combinations and doses shown in Fig. 4. Effects on proliferation of the PEs were tested in the absence and presence of 17β-estradiol (Sigma) in order to detect both estrogenic and antiestrogenic effects. A suboptimal stimulatory concentration of estradiol (1 pM) was used to enable detection of either antiestrogenic effects or additive estrogenic effects in

TABLE 1
Information on test compounds

Compund	MW	Supplier	CAS no.	Purity(%)
Coumestrol	267.0	Fluka, Sigma-Aldrich	479-13-0	>95
Genistein	270.2	Sigma-Aldrich	446-72-0	98
Daidzein	254.2	Sigma-Aldrich	486-66-8	98
Equol	242.3	Sigma-Aldrich	94105-90-5	99
Formononetin	268.3	Fluka BioChemika, Sigma-Aldrich	485-72-3	>99
Biochanin A	284.3	Sigma, Sigma-Aldrich	491-80-5	>99
Enterolactone	298.3	Fluka BioChemika, Sigma-Aldrich	78473-71-9	>95
Enterodiol	301.2	Fluka, Sigma-Aldrich	80226-00-2	>95
Lariciresinol	360.0	Arbo-Nova, Finland	27003-73-2	>95
Pinoresinol	358.4	Arbo-Nova, Finland	487-36-5	>95
Matairesinol	358.4	Arbo-Nova, Finland	580-72-3	>95
Secoisolarici resinol	362.4	Fluka BioChemika, Sigma-Aldrich	29388-59-8	>95

the same assay. Exposure to 100 pM estradiol (resulting in maximal stimulation of cell growth) was used as a positive control for estrogenic effects, and exposure to 1 pM estradiol + 1 nM ICI 182,780 was used as a control for antiestrogenic effects. Vehicle (DMSO) was added to the control cultures. Exposure was sustained for 5 days (Day 0 to 5), with replacement of medium and compounds on Day 0, 1, and 4. On Day 5, the cells were rinsed in phosphate buffered saline (PBS), and a crystal violet colorimetric assay staining DNA was used to obtain an indirect measure of the cell number (24). The obtained optical density for each sample was expressed as a relative value in percent of the DCC control culture.

Western Analysis

Expression of ERα, 67 kDa, progesterone receptor (PR A, 81 kDa and PR B, 116 kDa) and β-actin (42 kDa) was determined by Western analysis. The MCF-7 cells were grown in control medium for 2 days before exposure to vehicle (DMSO), 100 pM estradiol, 1 μM Mix 1 or 1 μM Mix 1 plus 100 nM ICI 182,780, 100 nM coumestrol, and 100 nM coumestrol plus 100 nM ICI 182,780. After 3 days, whole cell extracts were prepared by harvest in RIPA buffer (100 nM NaCl; 20 mM Trizma-base; 0.1% Triton X-100; 0.5% sodium desoxycholate; 0.1% sodium dodecyl sulphate; 1 mM ethylenediaminetetraacetic acid, pH 8.0). 10 μg cell extracts were separated by SDS-PAGE and immuno detected as described previously (25). Three independent experiments were performed.

Aromatase Assay

The aromatase assay was performed in human JEG-3 choriocarcinoma cells (no. HTB-36; ATCC, Manassas, VA) as described in (26), with minor modifications. The test compounds were dissolved in DMSO. Cells were seeded in 24-well culture plates (Nunc) at 4×10^4 cells/well in 1 ml culture medium for 2 days. Day 3, at approximately 80% confluence, serum-free medium ± test compound and/or control were added (max 0.1% DMSO).

After 18-h incubation (Day 4), medium was carefully removed, cells were washed with PBS and 0.5 ml substrate medium without serum containing 0.2 μCi [1β-^{3}H]androst-4-ene-3,17-dione, and 10 nM unlabelled 4-AD was added. After 2 h of incubation, the aromatization was terminated by placing the 24-well plates on ice. A total of 300 μl of the culture medium was withdrawn and extracted; and to remove remaining substrate, 150 μl of the aqueous phase was treated with 100 μl dextran-charcoal in PBS (5%; Sigma Aldrich). The positive solvent control (PSC) was cells incubated for 18 h (Day 4) without test compounds, and after cell wash added 0.5 ml substrate medium without serum containing 0.2 μCi [1β-^{3}H]androst-4-ene-3,17-dione and 10 nM unlabeled 4-AD and 0.1% DMSO.

The determined aromatase activity was subtracted from background level (culture media alone minus cells), corrected to cell protein concentration, and related to the PSC.

For protein determination, cells were lyzed with 0.5 ml 1X lysis buffer (5x lysis buffer, Reporter Gene Assay, Roche 1897 675, Denmark) for 20 min under mixing at RT. After quick-spin centrifugation, 40 μl was applied for protein determination in parallel with bovine serum albumin standards (10^{-6} to 10^{-4} g) in lysis buffer using 40 μl lysis buffer as blank. A total of 50 μl of Fluorescamin solution (100 mg Fluorescamin dissolved in 200 ml Acetonitril) was added, and the plate was incubated for 20 min. while mixing at room temperature in a fume hood. The protein concentration was then determined using a fluorometer at 355/460 nm wavelength.

In parallel, the 4-AOD was used as an aromatase inhibitor control at 10 μM [effective concentration (EC) of 100%] and 10 nM [50% EC (EC_{50})]. All compound and control incubations were performed in triplicate in at least 3 independent assays. In each assay, all data were related to the positive control (media + substrate: labeled and unlabeled 4-AD), which were set to 100%. Cell cytotoxicity was determined on the cell culture media Day 4 using the cytotoxicity detection kit (LDH) from Roche (Mannheim, Germany) as described previously (27).

AR Reporter Gene Assay

Effects of PEs on AR activity were tested in a gene assay based on transiently transfected Chinese hamster ovary (CHO) cells. The assay was performed as previously described by (28) with minor modifications. The cells were seeded in microtiter plates at a density of 5×10^3 cells per well and incubated at 37°C in a humidified atmosphere of 5% CO_2/air. After 24 h, the PEs to be tested were added and dissolved in culture media. The test solutions were prepared from stock solutions in DMSO (final concentration in the media was 0.1%) with or without 0.1 nM of the positive control R1881 (NEN, Boston, MA). Hydroxyflutamide (Schering-Plough, Berlin, Germany) was included as a positive control in every experiment. Chemicals were tested at 8 concentrations within the range of 1–50 μM. Shortly after addition of test compounds, each well was transfected with a total of 75 ng cDNA consisting of the human AR expression vector pSVAR0 and the MMTV-LUC reporter plasmid (both provided by Albert Brinkmann, Erasmus University, Rotterdam, The Netherlands) in a ratio of 1:100 using 25 μl of the nonliposomal transfection reagent FuGene (Boehringer, Mannheim, Germany).

After an incubation period of 24 h, the media was aspirated, and the cells were lysed. Five microliters of a substrate containing 1 mM luciferin (Sigma) and 1 mM ATP in lysis buffer was injected automatically and the chemiluminescence was measured over a 1-s interval after an incubation time of 2 s. After 24 h incubation, cytotoxicity was measured by replacing the pSVAR0 expression vector with the constitutively active AR expression vector, pSVAR13, as described in (28). Cytotoxicity was defined as the ability of the compounds to affect the transcription process in the cell. Furthermore, the cytotoxicity was also measured using AlamarBlue (Serotec, Kidlington, UK). The AR assay was repeated 3 times for each test compound/mixture.

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Statistical Analysis

Steroid synthesis and AR reporter gene assay. Data were examined for normal distribution and homogeneity of variances. Effects of PEs on steroid synthesis and AR activation/inhibition were analyzed with a 1-way parametric analysis of variance (ANOVA); and if ANOVA results were significant ($P < 0.05$), Dunnett's tests were used to compare the means between the controls and the means of the different concentrations.

MCF-7 cell proliferation. Levene's test was used to analyze for homogeneity of variance in all experiments, and log transformation of data was performed in case of unequal variance. A linear model of analysis of variance, followed by 2-sided, pairwise *t*-tests with Bonferroni's correction was used to detect differences between treatments. Results were considered significant when $P < 0.05$. Calculations were performed using SAS, version 8.2 (SAS institute, Cary, NC). For mixture 2, cytotoxicity at approximately 10 μM resulted in large SDs, as the cells in some wells had died, whereas others had only initiated the death process. The large SDs for this data point confounded statistical analysis of the rest of the data set, which is why the data point was removed from the data material before statistical analysis.

Aromatase assay. The statistical analysis was performed in SPSS 14.0 (SPSS, Inc., Chicago, IL). Due to unequal of variances and relatively few data points per concentration, nonparametric statistics was performed. The Kruskal–Wallis test was used to compare differences between different concentrations, and the Jonckheere–Tepstra test was used to analyze for a linear trend between concentrations and response. If one or both tests showed a significant difference ($P \leq 0.05$), the Mann–Whitney test was used to compare the difference between each test concentration and the respective control.

The EC_{50} was calculated by fitting dose-response data to a 3-parameter sigmodal Hill curve using Sigma Plot (SPSS, Chicago, IL) as described previously (26).

RESULTS

The PEs alone or in the different mixtures was tested in the *in vitro* panel for endocrine activity. The different mixtures and single PEs tested can be seen in Fig. 1.

Effects on Steroid Production in H295R Cells

Experiments in the H295R cells showed that none of the PEs had any effect on the synthesis of testosterone or estradiol in the low concentration range from 10^{-10} M to 10^{-7} M (data not shown). At the highest concentration (10 μM), all compounds/mixtures tested caused an increase in the estradiol production (Fig. 2). Furthermore, the highest concentration of the isoflavonoids in Mix 2 (formononetin and biochanin A) and the total mixture caused a significant decrease in the testosterone production (Fig. 3). Additional examination of the concentrations ranging from 1–10 μM revealed that increasing concentrations of Mix 1 (genistein, daidzein, and equol), Mix 2 (for-

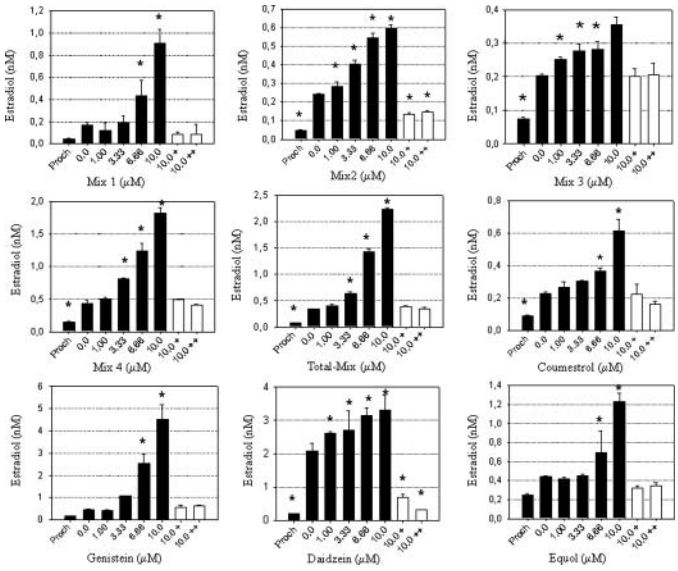

FIG. 2. Effect of phytoestrogens (PEs) on estradiol production in the H295R cells. In vitro effects of PEs on estradiol production in human adrenocortical carcinoma cells (H295R) are shown. Data represent the mean ± SD for 3 independent experiments. + and ++ represent 100 nM and 1 μM of the aromatase inhibitor 4-androstene-4-ol-3,17-dione, respectively. Proch, 3 μM prochloraz. *, Statistically significantly different from control ($P < 0.05$).

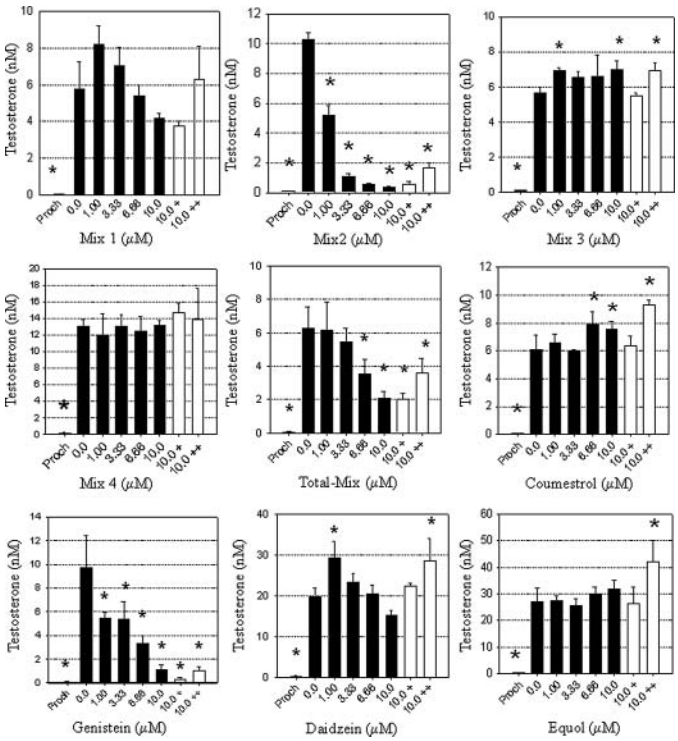

FIG. 3. Effect of phytoestrogens (PEs) on testosterone production in the H295R cells. In vitro effects of PEs on testosterone production in human adrenocortical carcinoma cells (H295R) are shown. Data represent the mean ± SD for 3 independent experiments. + and ++ represent 100 nM and 1 μM of the aromatase inhibitor 4-androstene-4-ol-3,17-dione, respectively. Proch, 3 μM Prochloraz. *, Statistically significantly different from control ($P < 0.05$).

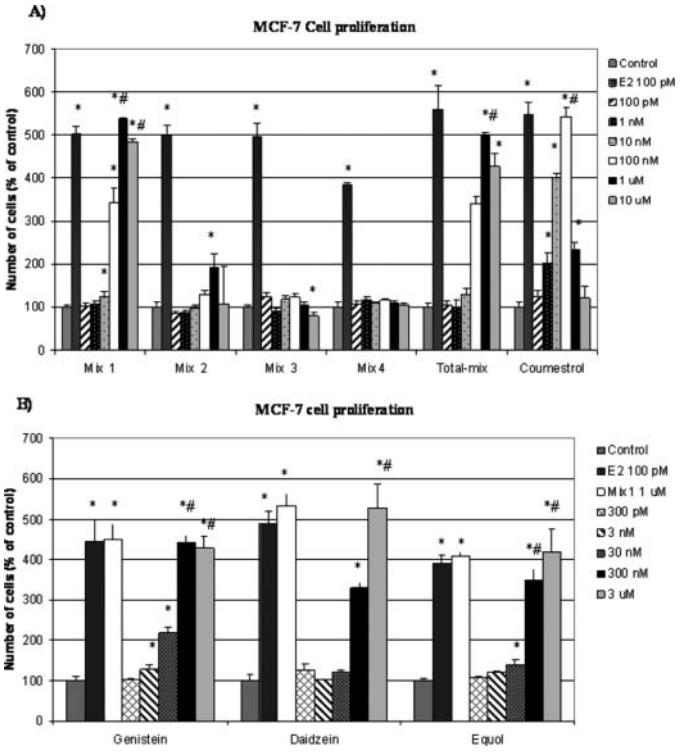

FIG. 4. Effects of phytoestrogens (PEs) exposure on MCF-7 cell proliferation. Results are expressed as cell number in percent of the dextrane-coated charcoal (DCC) control culture ± SD. Cells were exposed to PEs for 5 days. Estradiol (E_2) treatment served as a positive control in all experiments. The effect of genistein, daidzein, and equol was further compared to the effect of 1 μM of Mix 1. An indirect measure of the cell number was obtained using a crystal violet colorimetric assay. Figure shows results from representative experiments with triplicate measures. For each PE mix, 3 independent experiments in triplicate were performed and gave comparable results. *, Denotes significantly different from the DCC control; #, Indicates a cell number equal to the E_2-treated culture.

mononetin and biochanin A), the total mixture, and genistein alone caused a dose-dependent reduction in the testosterone production, although not statistically significant for Mix 1 (Fig. 3). Exposure to secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol (Mix 4) to enterolactone and enterodiol (Mix 3) as well as to coumestrol did not reduce the testosterone production in H295R cells. On the contrary, coumestrol and Mix 3 caused a slight but significant increase in testosterone production (Fig. 3).

To elucidate the mechanism behind the increased levels of estradiol, the tested compounds, at the highest concentration (10 μM), were studied in combination with a specific inhibitor of aromatase, 4-AOD. The results of these experiments revealed that the increased levels of estradiol following PE exposure was reduced to background levels following treatment with 100 nM or 1 μM of 4-AOD (Fig. 2). Of the 3 single compounds tested, genistein was the compound that had most effect on the steroid hormone production in the H295R cells. No significant cytotoxic effects were observed for the PEs in the tested concentrations.

MCF-7 Cell Proliferation

Figure 4 shows the effect of PE exposure on MCF-7 cell proliferation using the setup for detection of estrogenic effects. The results are expressed as cell number in percent of the DCC control culture. Significant stimulatory effects were observed with the isoflavone metabolites from 10 nM (Mix 1), whereas the precursor plant isoflavones were less potent (Mix 2) and only resulted in growth stimulation at 1 μM. For Mix 2, cytotoxicity occurred at 10 μM. Exposure to 1–10 μM Mix 1 resulted in MCF-7 cell growth stimulation comparable to the maximal growth stimulation obtained with estradiol. No effects were observed with any concentrations of the lignans (Mix 3 and 4). The mix of all tested PEs (total mix) resulted in stimulation of MCF-7 cell growth at concentrations from 100 nM to 10 μM. In 2 out of 3 independent experiments, significant growth stimulation was observed already at 10 nM. At 1 μM, the effect was comparable to the maximal effect obtained with estradiol. Experiments with selected single compounds were conducted in order to investigate whether single compounds could account for the observed mixture effects or whether combination effects were present. The isoflavone metabolites in Mix 1 all resulted in growth stimulation of MCF-7 cell growth (potency: genistein > equol > daidzein). The potent stimulatory effect of genistein alone was comparable to the effects observed with Mix 1 and may have marked detection of any potential mixture effect of the PEs in Mix 1. Coumestrol stimulated cell growth at concentrations from 1 nM to1 μM and the stimulatory effect at 100 nM was comparable to that of 100 pM estradiol. At 1–10 μM, coumestrol appeared to be cytotoxic after prolonged exposure (5 days), following an initial growth stimulation at these concentrations. None of the tested PEs showed antiestrogenic effects on MCF-7 cell growth (data not shown).

Effect of Mix 1 and Coumestrol on PR and ERα Expression

To establish the mechanism behind the growth stimulatory effects observed for the MCF-7 cells, expression of the PR and ERα protein was assessed using Western blot analysis. PR protein is expressed in 2 forms, PR A and PR B, and the PR gene is an estrogen-regulated gene, the expression of which is often used as a marker for the presence of functional ERα (29). It should also be mentioned that MCF-7 cells express extremely low level of ERβ (30), and therefore expression of ERβ was not measured.

Addition of estradiol in this experimental setup showed an expected increase in PR expression and decrease in ERα expression (Fig. 5). Addition of Mix 1 as well as coumestrol resulted in increased expression of PR up to the level observed for 100 pM estradiol (Fig. 5), indicating that the PEs can act as potent estrogens via ERα. Furthermore, the antiestrogen ICI 182,780 could completely inhibit the stimulation of the PR expression (Fig. 5), again supporting an ERα-mediated effect of the PEs. As expected, estradiol downregulated the ERα expression (31), and Mix 1 and coumestrol also reduced ERα

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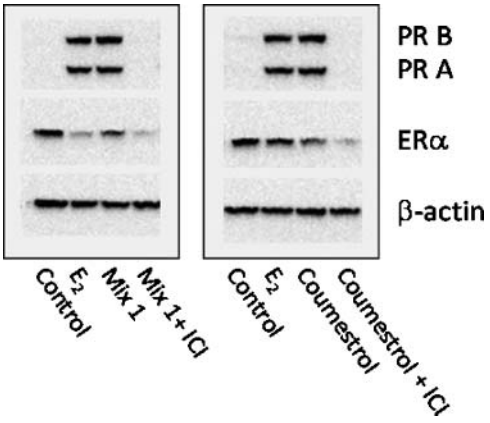

FIG. 5. Effect of Mix 1 and coumestrol on progesterone receptor (PR) and estrogen receptor alpha (ERα) expression. Expression of ERα (67 kDa), the PR A (81 kDa) and PR B (116 kDa), and β-actin (42 kDa) determined by Western analysis. The MCF-7 cells were grown in medium with 10% steroid depleted FCS and exposed for 3 days to vehicle (DMSO, control), 100 pM estradiol (E_2), 1 μM Mix 1 or 1 μM Mix 1 plus 100 nM ICI 182,780 (ICI), or 100 nM coumestrol and 100 nM coumestrol plus 100 nM ICI. 10 μg cell extracts were separated by SDS-PAGE as described in **Materials and Methods.**

expression. Downregulation of ERα protein with the PEs was not reversed by ICI treatment, as this antiestrogen in itself downregulates ER expression (31).

Aromatase activity. In general, weak effects of the PEs were observed at low concentrations. Mix 1 inhibited the aromatase activity at the lowest concentration (10^{-10} M), but a weak increase in enzyme activity was observed at 10^{-9} M, and at 10^{-6} M an approximately 1.3-fold increase in activity was seen (Table 2). For Mix 2, 3, 4, and coumestrol, the aromatase activity was significantly increased at 1 μM and also at 10 μM for Mix 2 and Mix 4. No effect on the aromatase activity was observed for the single compounds of Mix 1, genistein, daidzein, and equol and neither for the mixture of all PEs (Table 2).

Androgenicity (AR–assay). None of the tested PEs exerted any agonistic or antagonistic effects in the androgen reporter assay in the tested concentration range (data not shown).

DISCUSSION

Through our diet, we are exposed to many different and complex mixtures of PEs. The overall aim of this study was to evaluate the endocrine disrupting potential of 12 different PEs and mixtures of PEs naturally occurring in Western/Scandinavian food. As mentioned previously, many of the conducted experimental studies have typically been based on single or a small number of individual compounds, or on single diet compounds such as soy or flaxseed. However, focus has now been brought on the question of what happens to the effect of the individual PE compounds when they are combined with other PEs from different classes. One such example is the study by Power et al. (5) in which it was found that the isoflavone genistein alone promoted growth of MCF-7 human breast cancer xenografts; but when genistein was given in combination with the mammalian lignans enterolactone and enterodiol, no tumor growth-promoting effects were observed.

In this study, the 12 selected PEs were tested alone or in mixtures in a panel of 4 in vitro bioassays. In the steroid synthesis assay (H295R), all tested PEs and mixtures caused an increase in the estradiol production. Furthermore, Mix 2, consisting of formononetin and biochanin A, and the total mix containing all 12 PEs caused a dose-dependent reduction in the testosterone production. Genistein alone also caused a dose-dependent reduction in the testosterone production in the H295R cells, and the effect of genistein was in general comparable to the effects observed for Mix 1. The observed reduction in the testosterone production might be a result of an inhibitory effect of the isoflavones on some of the steroidogenic enzymes involved in testosterone biosynthesis. This is in agreement with a study (32) that showed that genistein, daidzein, formononetin, and biochanin A strongly inhibited 3β-hydroxysteroid dehydrogenase (3β-HSD) activity and at high concentrations (12.5 and or 25 μM) also inhibited P450c21 activity. Also Le Bail et al. (4) showed that genistein, daidzein, formononetin, and biochanin A are 3β-HSD and/or 17β-HSD inhibitors and that the inhibitory effect of some of the PEs are related to small structural differences, which can explain the diversity of effects seen for the different PEs.

In the MCF-7 cell proliferation assay, the isoflavonoids and coumestrol had growth stimulatory effect on the MCF-7 cells, whereas the lignans had no effect. The most pronounced effects were seen with Mix 1 and the total mix as well as with the selected single compounds. Overall, the effect of genistein, and at the highest concentration, also daidzein, was comparable to the effect observed for Mix 1. Coumestrol alone also had a significant growth stimulatory effect on the MCF-7 cells, with an effect comparable to that of estradiol at 1,000-fold lower concentration. The growth stimulatory effect of the isoflovonoids and coumestrol are in agreement with what previous studies have shown (4,33). Concomitant with the growth stimulation, both the isoflavonoids and coumestrol increased the expression of the estrogen inducible PR A and PR B proteins, supporting an ERα-mediated effect. Le Bail et al. (4) and others (34) have found that many of the PEs show biphasic regulation effects on the cell proliferation of MCF-7 cells, stimulating growth at lower concentrations and inhibiting growth at higher concentrations, as seen with coumestrol in this study. Furthermore, studies have also shown that the stimulatory effects at lower concentrations correlate with the affinity of the PEs for the ER, supporting our result from the Western blot analysis, but that the growth inhibition induced at higher concentrations is probably not mediated through ERα (4,34). Altogether, this further adds to the difficulty of getting a clear picture of the effects of the PEs, also in relation to any possible health promoting effects.

Finally, this study (both with the JEG-3 and the H295R cells) showed a general increase in aromatase activity for the PE-mixtures, and the use of 4-AOD proved that the observed effects were due to an increase in aromatase activity. This is in contrast

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TABLE 2
Effect of phytoestrogens on aromatase activity[a]

PEs	Mix No.	PSC control	IC50 10 nM	100 pM	1 nM	10 nM	100 nM	1 μM	10 μM
Isoflavonoids (metabolites)	1 (n = 3)	100 (4.6)	0.558* (0.22)	0.836* (0.14)	1.138* (0.15)	0.960 (0.17)	0.918 (0.13)	1.274* (0.23)	0.959 (0.28)
Isoflavonoids (mother compounds)	2 (n = 2)	100 (0.08)	0.603* (0.21)	0.942 (0.18)	1.129 (0.29)	1.123* (0.0.9)	1.060 (0.17)	1.466* (0.21)	1.344* (0.37)
Lignanns (metabolites)	3 (n = 2)	100 (0.06)	0.586* (0.23)	0.995 (0.19)	0.996 (0.18)	1.040 (0.14)	1.050 (0.27)	1.504* (0.18)	1.296 (0.46)
Lignans (mother comp.)	4 (n = 4)	0.937 (0.16)	0.490* (0.04)	0.980 (0.05)	1.041 (0.13)	0.984 (0.10)	1.069 (0.18)	1.129* (0.08)	1.172* (0.10)
All PEs	n = 12	100 (0.03)	0.429* (0.09)	1.000 (0.11)	0.989 (0.13)	1.026 (0.15)	1.019 (0.11)	1.035* (0.12)	1.067* (0.12)
Coumestrol	Single PE	100 (0.04)	0.586* (0.23)	1.007 (0.38)	1.135 (0.32)	1.008 (0.24)	1.205 (0.27)	1.503* (0.37)	1.332 (0.52)
Genistein	Single PE	100 (0.08)	0.454* (0.07)	1.050 (0.09)	0.964 (0.26)	0.981 (0.14)	0.946 (0.08)	1.048 (0.10)	1.206 (0.33)
Daidzein	Single PE	100 (0.08)	0.454* (0.07)	1.007 (0.27)	1.006 (0.27)	1.032 (0.33)	1.080 (0.29)	1.078 (0.19)	1.015 (0.25)
Equol	Single PE	100 (0.03)	0.429* (0.09)	1.000 (0.11)	0.989 (0.13)	1.026 (0.15)	1.019 (0.11)	1.035 (0.12)	1.067 (0.12)

[a]Abbreviations are as follows: PSC, solvent positive control; IC_{50}, inhibitory concentration of 50%; PEs, phytoestrogens. The aromatase activity was measured in human JEG-3 choriocarcinoma cells as described in **Materials and Methods.** The PSC was cells + substrate: labeled and unlabeled 4-AD (see **Methods**). In parallel, the 4-AOD was added and used as an aromatase inhibitor control at 10 μM (100% effective concentration) and 10 nM (IC_{50}). Means and SD are shown for n= 3–6. *, Significantly different ($P \leq 0.05$) from the respective PSC control in the Mann–Whitney test.

to the many studies that have indicated that several PEs inhibit the aromatase enzyme (4,17–19). However, in another study (4), it was found that isoflavonoids and compounds, which presented the phenolic B ring in the 3 position on the pyran ring, are better 3ß-HSD and/or 17ß-HSD inhibitors than aromatase inhibitors.

Nevertheless, there are studies supporting our observations, as both genistein and coumestrol have previously been found to induce aromatase activity (20–22). However, it should be taken into consideration that the effects observed, and the mechanisms underlying them, may be influenced by the specific tissues or cell lines used. Coumestrol and genistein induced aromatase activity in breast cancer cells (SK-BR-3) through a nongenomic action of ERα (20), whereas the genistein increased aromatase activity, observed in the endometrial stromal cells (ESC), did not seem to be mediated through an ER-mediated pathway (22). Induction of aromatase activity by genistein, flavone, and quercetin in H295R cells (21) again indicates that the effects seen in the H295R cells could be mediated through the cAMP-dependent protein kinase (PKA) second messenger pathway.

Altogether, the mechanisms underlying the different endocrine disrupting effects of the PEs are many and complex. Based on this study, it cannot be concluded if it is the potent effects of PEs such as genestein and/or coumestrol alone that account for most of the effects observed with the total PE mix. However, based on the results from Mix 2 in the various in vitro assays, it is not only the mammalian metabolites but also the precursor plant isoflavones that have the ability to act as potent endocrine disrupters. In addition, PEs might act as ER agonists as observed in the MCF-7 assay reported here and elsewhere, but the ability of PEs to make human cells (H295R) produce more endogenous estradiol should be emphasized and taken into account.

Further studies are needed in order to investigate mixtures in relation to single compounds. Especially, it could be interesting to test whether the effects of lignans are additive to the effects of an isoflavonoid such as genistein. Knowledge from these kinds of studies will help us in risk assessment of possible adverse effects of the different mixtures of PEs that we may come in contact with through our diet.

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REFERENCES

1. Setchell KD: Soy isoflavones—benefits and risks from nature's selective estrogen receptor modulators (SERMs). *J Am Coll Nutr* **20,** 354S–362S, 381S–383S, 2001.
2. Ibarreta D, Daxenberger A, and Meyer HH: Possible health impact of phytoestrogens and xenoestrogens in food. *Apmis* **109,** 161–184, 2001.
3. Magee PJ and Rowland IR: Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. *Br J Nutr* **91,** 513–531, 2004.
4. Le Bail JC, Champavier Y, Chulia AJ, and Habrioux G: Effects of phytoestrogens on aromatase, 3beta and 17beta-hydroxysteroid dehydrogenase activities and human breast cancer cells. *Life Sci* **66,** 1281–1291, 2000.
5. Power KA, Saarinen NM, Chen JM, and Thompson LU: Mammalian lignans enterolactone and enterodiol, alone and in combination with the isoflavone genistein, do not promote the growth of MCF-7 xenografts in ovariectomized athymic nude mice. *Int J Cancer* **118,** 1316–1320, 2006.
6. Krista A and Power LUT: Can the combination of flaxseed and its lignans with soy and its isoflavones reduce the growth stimulatory effect of soy and its isoflavones on established breast cancer? *Mol Nutr Food Res* **51,** 845–856, 2007.
7. Tham DM, Gardner CD, and Haskell WL: Clinical review 97: Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J Clin Endocrinol Metab* **83,** 2223–2235, 1998.
8. Yang J, Nakagawa H, Tsuta K, and Tsubura A: Influence of perinatal genistein exposure on the development of MNU-induced mammary carcinoma in female Sprague-Dawley rats. *Cancer Lett* **149,** 171–179, 2000.
9. Hilakivi-Clarke L, Cho E, Onojafe I, Raygada M, and Clarke R: Maternal exposure to genistein during pregnancy increases carcinogen-induced mammary tumorigenesis in female rat offspring. *Oncol Rep* **6,** 1089–1095, 1999.
10. Allred CD, Ju YH, Allred KF, Chang J, and Helferich WG: Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein. *Carcinogenesis* **22,** 1667–1673, 2001.
11. De Lemos ML: Effects of soy phytoestrogens genistein and daidzein on breast cancer growth. *Ann Pharmacother* **35,** 1118–1121, 2001.
12. Hedelin M, Klint A, Chang ET, Bellocco R, Johansson JE, et al.: Dietary phytoestrogen, serum enterolactone and risk of prostate cancer: the cancer prostate Sweden study (Sweden). *Cancer Causes Control* **17,** 169–180, 2006.
13. Adlercreutz H, Mazur W, Bartels P, Elomaa VV, Watanabe S, et al.: Phytoestrogens and Prostate Disease. *J. Nutr* **130,** 658S–658S, 2000.
14. Jian-Ping Yuan J-HWXL: Metabolism of dietary soy isoflavones to equol by human intestinal microflora—implications for health. *Mol Nutr Food Res* **51,** 765–781, 2007.
15. Almstrup K, Fernandez MF, Petersen JH, Olea N, Skakkebaek NE, et al.: Dual effects of phytoestrogens result in u-shaped dose-response curves. *Environ Health Perspect* **110,** 743–748, 2002.
16. Simpson ER and Davis SR: Minireview: aromatase and the regulation of estrogen biosynthesis—some new perspectives. *Endocrinology* **142,** 4589–4594, 2001.
17. Rice S, Mason HD, and Whitehead SA: Phytoestrogens and their low dose combinations inhibit mRNA expression and activity of aromatase in human granulosa-luteal cells. *J Steroid Biochem Mol Biol* **101,** 216–225, 2006.
18. Adlercreutz H: Phyto-oestrogens and cancer. *Lancet Oncol* **3,** 364–373, 2002.
19. Benassayag C, Perrot-Applanat M, and Ferre F: Phytoestrogens as modulators of steroid action in target cells. *J Chromatogr B Analyt Technol Biomed Life Sci* **777,** 233–248, 2002.
20. Kinoshita Y and Chen S: Induction of aromatase (CYP19) expression in breast cancer cells through a nongenomic action of estrogen receptor {alpha}. *Cancer Res* **63,** 3546–3555, 2003.
21. Sanderson JT, Hordijk J, Denison MS, Springsteel MF, Nantz MH, et al.: Induction and inhibition of aromatase (CYP19) activity by natural and

synthetic flavonoid compounds in H295R human adrenocortical carcinoma cells. *Toxicol Sci* **82,** 70–79, 2004.

22. Edmunds KM, Holloway AC, Crankshaw DJ, Agarwal SK, and Foster WG: The effects of dietary phytoestrogens on aromatase activity in human endometrial stromal cells. *Reprod Nutr Dev* **45,** 709–720, 2005.
23. Horwitz KB and McGuire WL: Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. *J Biol Chem* **253,** 2223–2228, 1978.
24. Lundholt BK, Briand P, and Lykkesfeldt AE: Growth inhibition and growth stimulation by estradiol of estrogen receptor transfected human breast epithelial cell lines involve different pathways. *Breast Cancer Res Treat* **67,** 199–214, 2001.
25. Rasmussen LM, Zaveri NT, Stenvang J, Peters RH, and Lykkesfeldt AE: A novel dual-target steroid sulfatase inhibitor and antiestrogen: SR 16157, a promising agent for the therapy of breast cancer. *Breast Cancer Res Treat* **106,** 191–203, 2007.
26. Bonefeld-Jorgensen EC, Long M, Hofmeister MV, and Vinggaard AM: Endocrine-disrupting potential of bisphenol A, bisphenol A dimethacrylate, 4-n-nonylphenol, and 4-n-octylphenol in vitro: new data and a brief review. *Environ Health Perspect* **115**(**1**Suppl), 69–76, 2007.
27. Bonefeld-Jorgensen EC, Grunfeld HT, and Gjermandsen IM: Effect of pesticides on estrogen receptor transactivation in vitro: a comparison of stable transfected MVLN and transient transfected MCF-7 cells. *Mol Cell Endocrinol* **244,** 20–30,2005.
28. Vinggaard AM, Joergensen EC, and Larsen JC: Rapid and sensitive reporter gene assays for detection of antiandrogenic and estrogenic effects of environmental chemicals. *Toxicol Appl Pharmacol* **155,** 150–160, 1999.
29. Kastner P, Krust A, Turcotte B, Stropp U, Tora L, et al.: Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *Embo J* **9,** 1603–1614, 1990.
30. Grünfeld HT and Bonefeld-Jorgensen EC: Effect of in vitro estrogenic pesticides on human estrogen receptor alpha and beta mRNA levels. *Toxicol Lett* **151,** 467–480, 2004.
31. Jensen BL, Skouv J, Lundholt BK, and Lykkesfeldt AE: Differential regulation of specific genes in MCF-7 and the ICI 182780-resistant cell line MCF-7/182R-6. *Br J Cancer* **79,** 386–392, 1999.
32. Ohno S, Shinoda S, Toyoshima S, Nakazawa H, Makino T, et al.: Effects of flavonoid phytochemicals on cortisol production and on activities of steroidogenic enzymes in human adrenocortical H295R cells. *J Steroid Biochem Mol Biol* **80,** 355–363, 2002.
33. van Meeuwen JA, Ter Burg W, Piersma AH, van den Berg M, and Sanderson JT: Mixture effects of estrogenic compounds on proliferation and pS2 expression of MCF-7 human breast cancer cells. *Food Chem Toxicol* **45,** 2319–2330, 2007.
34. Ying C, Hsu J-T, Yang C-D, Hsung S-J, Wang L-K, et al.: The combination effects of phytoestrogens on cell proliferation, cell cycle progression and estrogen receptor alpha. *J Food Drug Anal* **13,** 30–37, 2005.

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Paper II

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Use of external metabolizing systems when testing for endocrine disruption in the T-screen assay

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ABSTRACT

Although, it is well-established that information on the metabolism of a substance is important in the evaluation of its toxic potential, there is limited experience with incorporating metabolic aspects into *in vitro* tests for endocrine disrupters. The aim of the current study was a) to study different *in vitro* systems for biotransformation of ten known endocrine disrupting chemicals (EDs): five azole fungicides, three parabens and 2 phthalates, b) to determine possible changes in the ability of the EDs to bind and activate the thyroid receptor (TR) in the *in vitro* T-screen assay after biotransformation and c) to investigate the endogenous metabolic capacity of the GH3 cells, the cell line used in the T-screen assay, which is a proliferation assay used for the *in vitro* detection of agonistic and antagonistic properties of compounds at the level of the TR.
The two *in vitro* metabolizing systems tested the human liver S9 mix and the PCB-induced rat microsomes gave an almost complete metabolic transformation of the tested parabens and phthalates. No marked difference the effects in the T-screen assay was observed between the parent compounds and the effects of the tested metabolic extracts. The GH3 cells themselves significantly metabolized the two tested phthalates dimethyl phthalate (DMP) and diethyl phthalate (DEP). Overall the results and qualitative data from the current study show that an *in vitro* metabolizing system using liver S9 or microsomes could be a convenient method for the incorporation of metabolic and toxicokinetic aspects into *in vitro* testing for endocrine disrupting effects.

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Introduction

Both *in vivo* and *in vitro* tests are presently being proposed for the testing of endocrine disrupting activity of different compounds. The *in vivo* or animal tests are currently necessary because it is not possible to model the complex responses of a whole body to endocrine disrupters (EDs), including biotransformation. Biokinetic processes, like absorption, distribution, biotransformation and excretion, determine the relation of the administered and the internal dose. For *in vivo* animal studies, regulatory toxicokinetic studies can determine the relation between the administered dose via the oral, dermal or inhalation route and the actual dose at the point of interest for example a specific organ or cell When carrying out *in vitro* studies the same biokinetic information is crucial when relating the administered *in vitro* dose to the internal dose (i.e. free concentration) reaching the cellular targets.

It is well-established that information on the metabolism of a substance is important in the evaluation of its toxic potential. For example, the determination of metabolic stability can provide information on the potential for bioaccumulation and whether a chemical is likely to be converted to a more or less active form. With regard to endocrine disruptors, one also needs to consider that metabolism of the xenobiotic can alter not only its own potency but also the toxicokinetics of other substances. Hormones and in particular steroid hormones are formed or metabolized by enzymes that participate in the metabolism of xenobiotics. A chemical that is metabolized by these enzymes may, by mere interaction with the enzyme, act as a competitor for the hormone or hormone precursor and therefore disturb hormone levels and hormonal equilibrium. However, so far there is little or no experience with incorporating metabolic and toxicokinetic aspects into *in vitro* tests for endocrine disrupters, which is in contrast to other areas of toxicity testing such as the genotoxicity area.

Metabolism occurs especially in the liver, but also in extra-hepatic tissues (Combes, 1992; Coecke et al., 2006), and can be divided into phases I and II. Phase I metabolism involves the oxidation, reduction or hydrolysis of the parent compound into a more polar substance. The key enzymes for phase I oxidation are the isoforms of the CYP family of enzymes (Werck-Reichhart and Feyereisen, 2000). However, CYP enzymes can often be induced or inhibited by exogenous chemicals that may exert endocrine effects through this mechanism. Phase I metabolism has been shown to metabolize xenobiotics to both more and less active compounds (Setchell, 1988; Wong et al., 1995; Coldham et al., 2002). Phase II metabolism often involves the further

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conjugation of the metabolite with polar molecules, such as sulphate, amino acids, glutathione or glucuronic acid, generating metabolites that are more soluble and thus easily eliminated (Combes, 1992; Coecke et al., 2006). In the current study focus will only be on metabolism through phase I.

A number of *in vitro* systems are available for studying metabolism. They include both cellular (tissue slices, isolated and cultured hepatocytes or liver cell lines) and subcellular (microsomes, recombinant enzymes) systems (Guillouzo, 1998).

The aim of the current study was a) to study different *in vitro* systems for biotransformation of ten known endocrine disrupting chemicals (EDs), b) to determine possible changes in the ability of the EDs to bind and activate the thyroid receptor (TR) in the *in vitro* T-screen assay after biotransformation and c) to investigate the endogenous metabolic capacity of the GH3 cells, the cell line used in the T-screen assay.

The GH3 cells employed in the T-screen assay is a rat pituitary tumor cell line. The cell line growth is dependent on the thyroid hormone (TH) 3,3′,5-triiodothyronine (T3) and it has a high expression of TR. The growth stimulatory effect of T3 is mediated by specific, high-affinity TRs that upon binding of THs bind to thyroid hormone responsive elements (TREs) in the cell nucleus ultimately leading to gene expression (Hinkle and Kinsella, 1986). Thus the T-screen assay can be used for *in vitro* detection of agonistic and antagonistic properties of compounds at the level of the TR.

The interest in the TR and the thyroid hormones (T3 and T4) has arisen because they play a role in the control of development (e.g. metamorphosis in amphibians and brain development in mammals), and chemicals may alter their synthesis, transport and catabolism (Capen, 1997; Cheek et al., 1999; DeVito et al., 1999; Kato et al., 2000; Zhou et al., 2001; Craft et al., 2002). Increasingly, more evidence is accruing showing that xenobiotics may act at the thyroid hormone receptors, and more chemicals are suspected of being able to interfere with the thyroid receptor function (Zoeller et al., 2005). Compounds such as polybrominated diphenyl ethers (Hallgren et al., 2001) and PCB's have been shown to have the potential to interfere with the thyroid receptor function (Gauger et al., 2004) and some of these chemicals seem to suppress transcription that is linked to thyroid receptor activation (Fritsche et al., 2005).

The ten compounds selected for the current study were 5 azole fungicides: prochloraz, ketoconazole, miconazole, tebuconazole and epoxiconazole; 3 parabens: butyl-, ethyl- and methyl paraben, and 2 phthalates: dimethyl phthalate (DMP) and diethyl phthalate (DEP).

The compounds were selected from a preliminary study of 20 compounds. The 10 compounds were selected based both on analytical parameters involving their ease of detection in the HPLC analysis but also as examples of compounds with known endocrine disrupting effects on different hormone systems.

Materials and methods

Test compounds

The ten tested compounds and their data are listed in Table 1. For the *in vitro* assay all compounds were dissolved in dimethylesulfoxide (DMSO). MS-grade acetonitrile (99.9%), MS-grade methanol (99.9%) and MS-grade formic acid (98%) was purchased from Sigma-Aldrich (Milwaukee, WI, USA). DMSO, 99.8% was purchased from Merck KGaA (Darmstadt, Germany). Milli-Q quality water was supplied from water purification system from Millipore (Billerica, MA, USA).

In vitro metabolizing systems

Human liver S9. The human liver S9 (Product number X008023) was purchased from Celsis (Brussels, Belgium) The purchased liver S9 was a pooled human mixed gender fraction. The pooled fraction was from ten individuals, five females and five males, in the age between 28 and 81 years old. The original stock had a protein concentration of 23 mg/mL. The procedure for human liver S9 assay was based on preliminary experiments for optimization of protein concentration as well as the instructions provided by the supplier (Celsis).

Table 1
Information on test compounds.

Compound	MW	Supplier	CAS no.
Prochloraz	376.67	Dr.Ehrendorfer GmBH, Augsburg Germany	67747-09-5
Miconazole	479.14	Sigma-Aldrich (St. Louis, MO, USA)	22832-87-7
Ketoconazole	531.4	Sigma-Aldrich (St. Louis, MO, USA)	65277-42-1
Tebuconazole	307.8	Sigma-Aldrich/(Riedel-de Haën)	107534-96-3
Epoxiconazole	329.76	Dr.Ehrendorfer GmBH, Augsburg Germany	106325-08-8
Ethyl paraben	166.18	Sigma-Aldrich (St. Louis, MO, USA)	120-47-8
Butyl paraben	194.23	Sigma-Aldrich (St. Louis, MO, USA)	94-26-8
Methyl paraben	152.15	Sigma-Aldrich (St. Louis, MO, USA)	99-76-3
Dimethyl phthalate (DMP)	194.19	Sigma-Aldrich (St. Louis, MO, USA)	131-11-3
Diethyl phthalate (DEP)	222.24	Sigma-Aldrich (St. Louis, MO, USA)	84-66-2

The liver S9 fraction was diluted in Tris buffer to a concentration of 20 mg/mL. Glass test tubes were placed into an ice bath and 50 μL of diluted S9 was added followed by 320 μL of Tris buffer. 5 μL of 100× (20 mM for the phthalates and 10 mM for all the other test compounds) test article stock was added and the test tubes were placed into a 37 °C shaking water bath, along with a 3.4 mg/mL NADPH solution, shaking at 150 rpm for 5 min. 125 μL of the 3.4 mg/mL NADPH solution was then added to each test tube, and the reaction timer was started at the addition of NADPH to the first sample. Total reaction mixture was exactly 0.5 mL in each tube giving a final protein concentration of 2.0 mg/mL in sample. The samples were incubated for 60 min. After incubation the reaction was stopped by adding 1.5 mL ice-cold methanol and all samples were placed in a −20 °C freezer for 1 h, before the samples were centrifuged at 16,000×*g*, 4 °C and the supernatants were transferred to new tubes and stored at −20 °C until they were analysed on the HPLC.

Samples containing NADPH solution and DMSO (0.1%) were used as background. Furthermore samples containing only NADPH solution, DMSO (0.1%) and S9 were used as negative controls. Reference samples were identical to the test samples, but with the addition of test compound after the methanol induced inactivation. The background and the negative controls samples contain no test compounds but all other components such as cell media, NADHP and DMSO in the same concentration as the maximum amount of DMSO found in the test solutions and the for the negative control samples they also contain S9 or microsomes. The reference samples contained all components including test compound, but because the test compounds were added after inactivation of the enzymes with cold methanol, we would not expect any metabolism of the test compound to occur. All samples were run in triplicates.

PCB-induced hepatic rat microsomes. The polychlorinated biphenyl (PCB)-induced hepatic rat microsomes were prepared from adult male Wistar rats (age 7–8 weeks, weight ~200 g) as described in (Frandsen et al., 1994; Frederiksen and Frandsen, 2002). Four rats were used to prepare a pool of PCB-induced hepatic rat microsomes. PCB (Aroclor 1254, 500 mg/kg, dissolved in corn oil) was injected intraperitoneally 5 days before termination as described in (Frandsen et al., 1994; Frederiksen and Frandsen, 2002). The metabolism assay using the PCB-induced rat microsomes was run as described above with the human lever S9 fraction with a final protein concentration of 2 mg/mL.

In the current study the primary interest was only Phase I metabolism and therefore NADPH (phase I oxidation) was the only exogenous cofactor added, both in the S9 and microsome study.

The endogenous metabolic capacity of the GH3 cells

For the evaluation of the endogenous metabolic capacity of the GH3 cells all 10 compounds were tested in triplicates at a single concentration of 30 μM using the standard assay protocol as described in (Taxvig et al., 2008). At the end of the assay time period (4 days) the exposure of the GH3 cells and potential further metabolism of the test compounds was stopped by adding 3× volume of ice-cold methanol in relation to the cell suspension volume (e.g. 300 μL methanol to 100 μL cell suspension) to all the samples.

The samples were transferred to test tubes and placed in a −20 °C freezer for 1 h, before the samples were centrifuged at 16,000×*g*, 4 °C and the supernatants were transferred to new tubes and stored at −20 °C until they were analysed on the HPLC.

LC–MS/MS analysis

The LC–MS/MS analyses were conducted on an 1100 series LC coupled to a MSD ion trap SL mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) using positive and negative electrospray ionisation depending on the analysed compounds. A sample volume of 0.5 μL was injected onto a Gemini RP C18 column (3 μm, 150×2.0 mm, Phenomenex, Torrance, CA, USA). Analytes were determined using a gradient of water containing 0.01% formic acid (solvent A) and acetonitrile (solvent B): 0 min: 5% B, 1.0 min: 5% B, 2.0 min: 60% B, 8.0 min: 100% B, 15.5 min: 100% B and 16.0 min: 5% B. The flow rate was constant at 0.2 ml/min and column was 50 °C. The mass spectrum was scanned from 100 *m/z* to *1000 m/z* (a mass spectrum is intensity vs. *m/z* (mass-to-charge ratio)). Analytes were semi quantified using external standard calibration. Semi quantification was based on the total sum of the dominant ions for each analyte. Ketoconazole (positive ionisation), extracted ions: *m/z* 531; 532; 533; 266; 267; Miconazole (positive ionisation), extracted ions: *m/z* 415; 417; 419; Epoxyconazole (positive ionisation), extracted ions: *m/z* 330; 331; 332; Tebuconazole (positive ionisation), extracted ions: *m/z* 308; 309; 310; Prochloraz (positive ionisation), extracted ions: *m/z* 376; 378; 380; 308; 310; Dimethyl phthalate (positive ionisation), extracted ions: *m/z* 163; 195; 217; 411; Diethyl phthalate (positive ionisation), extracted ions: *m/z* 149; 177; 223; 245; 467; Methyl paraben (negative ionisation), extracted ions: *m/z* 151; Ethyl paraben (negative ionisation), extracted ions: *m/z* 165; Butyl paraben (negative ionisation), extracted ions: *m/z* 193.

The T-screen assay

The rat pituitary cell line GH3 obtained from American Type Culture Collection, ATCC, were cultured and the assay carried out as described in (Taxvig et al., 2008).

All 10 mother compounds were tested in triplicate (0, 10, 375, 1000, 3000, 10,000 and 30,000 nM) and were tested both in the absence or presence of 0.1 nM T3 to test for agonistic and antagonistic potency. A T3 concentrations response curve from 0 to 10 nM was included in each assay (Fig. 1). The human S9 metabolic extracts from the 3 parabens and the 2 phthalates were also tested for effect in the T-screen assay. The extracts were tested in five dilutions (dilution 1/16 to 1, with dilution 1/16 being the extract sample that was the most diluted). As for the mother compounds the extracts were tested both alone and in combination with 0.1 nM T3.

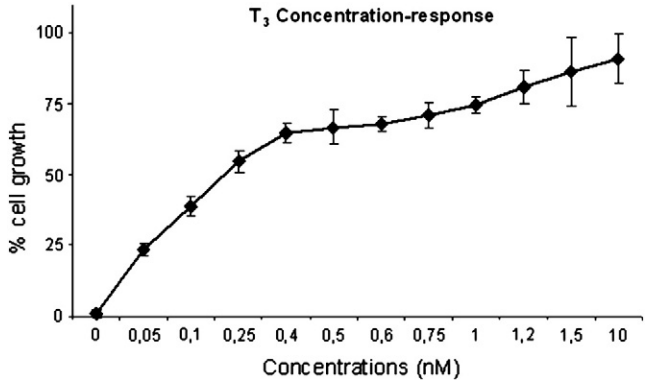

Fig. 1. Effect of T3 the on proliferation of GH3 cells. Solvent control (0.1% DMSO) was set to 0%. Data represent mean ± SD for three independent experiments.

Table 2
Metabolism of the 10 selected test compounds by use of human S9-fraction.

	Recovery of parent compound (%)	Significance (*p*-value)
Ketoconazol	96%	0.68
Miconazol	97%	0.38
Epoxiconazol	110%	0.47
Tebuconazol	96%	0.66
Prochloraz	94%	0.26
Dimethyl phthalate	~0%	– [a]
Diethyl phthalate	~0%	– [a]
Methylparaben	~0%	– [a]
Ethylparaben	~0%	– [a]
Butylparaben	~0%	– [a]

Recovery of the parent compounds (%) after incubation with human S9 according to the protocol described in the Material and methods section.

[a] Indicates that the *P*-value could not be calculated because the amount of remaining parent compound was below the detection limit. Statistically significant difference between the amounts of parent compound in the samples before and after incubation with S9 was tested by two-tailed *T*-test ($p<0.05$).

Statistical analyses

All data showing normal distribution and homogeneity of variance were analysed by one-way analyses of variance (ANOVA) and, if significant, followed by the *post hoc* test, Dunnett's test. Significance was judged at $p<0.05$.

The HPLC data was evaluated for statistical significance using a two-tailed *T*-test ($p<0.05$).

Results

In vitro metabolizing systems

Human liver S9 and rat microsomes. The LC–MS analysis of the S9 extracts for the azole fungicides showed no indication of any metabolic transformation during the incubation with the human liver S9 mix (Table 2). The parabens and phthalates on the other hand had almost been completely metabolized. The recovery of the parent compounds from the S9 extracts were less than 1% for both parabens and phthalates (Table 2).

Table 3
Metabolism of the 10 selected test compounds treatment with rat microsomes.

	Recovery of parent compound (%)	Significance (*p*-value)
Ketoconazol	53%	<0.01*
Miconazol	85%	0.02*
Epoxiconazol	95%	0.06
Tebuconazol	86%	<0.01*
Prochloraz	86%	0.02*
Dimethyl phthalate	<1%	– [a]
Diethyl phthalate	<1%	– [a]
Methylparaben	<1%	– [a]
Ethylparaben	<1%	– [a]
Butylparaben	<1%	– [a]

Recovery of the parent compounds (%) after incubation with PCB-induced rat microsomes as described in the Material and methods section.

* Statistically significant difference between the amounts of parent compound in the samples before and after incubation with rat microsomes using a two-tailed *T*-test ($p<0.05$).

[a] The compound was below the detection limit.

When using the PCB-induced rat microsomes the results for the parabens and phthalates was the same as seen with the human S9 mix. An extensive metabolic transformation of the parabens and the phthalates was observed, with a recovery rate of the parent compounds of less that 1% (Table 3). However, in contrast to the human S9 mix, the rat microsomes were able to metabolize the tested amount of azole fungicides to some extent. A statistically significant difference between the amount of parent compound before and after treatment with the rat microsomes was seen for four out of the five azole fungicides (Table 3).

Effects in the T-screen assay

Parent compounds. The effect on the proliferation of GH3 cells of the ten selected test compounds is illustrated in Figs. 2 and 3. For all five azole fungicides, except epoxiconazole, a significant decrease in the GH3 cell proliferation was observed at the highest concentration (30,000 nM), both when tested alone and in combination with T3 (Figs. 2A and B). This effect could be due to a cytotoxic effect, as others have demonstrated cytotoxicity at these concentrations (at and above 25,000 nM), for some of the same chemicals (Ghisari and Bonefeld-Jorgensen, 2005).

For miconazole a significant decrease in the T3-induced cell growth was observed with the second highest concentration (10,000 nM) (Fig. 2A). Furthermore, ketoconazole alone showed a decreasing effect on the cell proliferation at 6000 nM and above (Fig. 2B).

The three parabens, butyl paraben, ethyl paraben and methyl paraben, all showed TR agonistic effect, with butyl paraben being the most potent of the three (butyl paraben>ethyl paraben>methyl

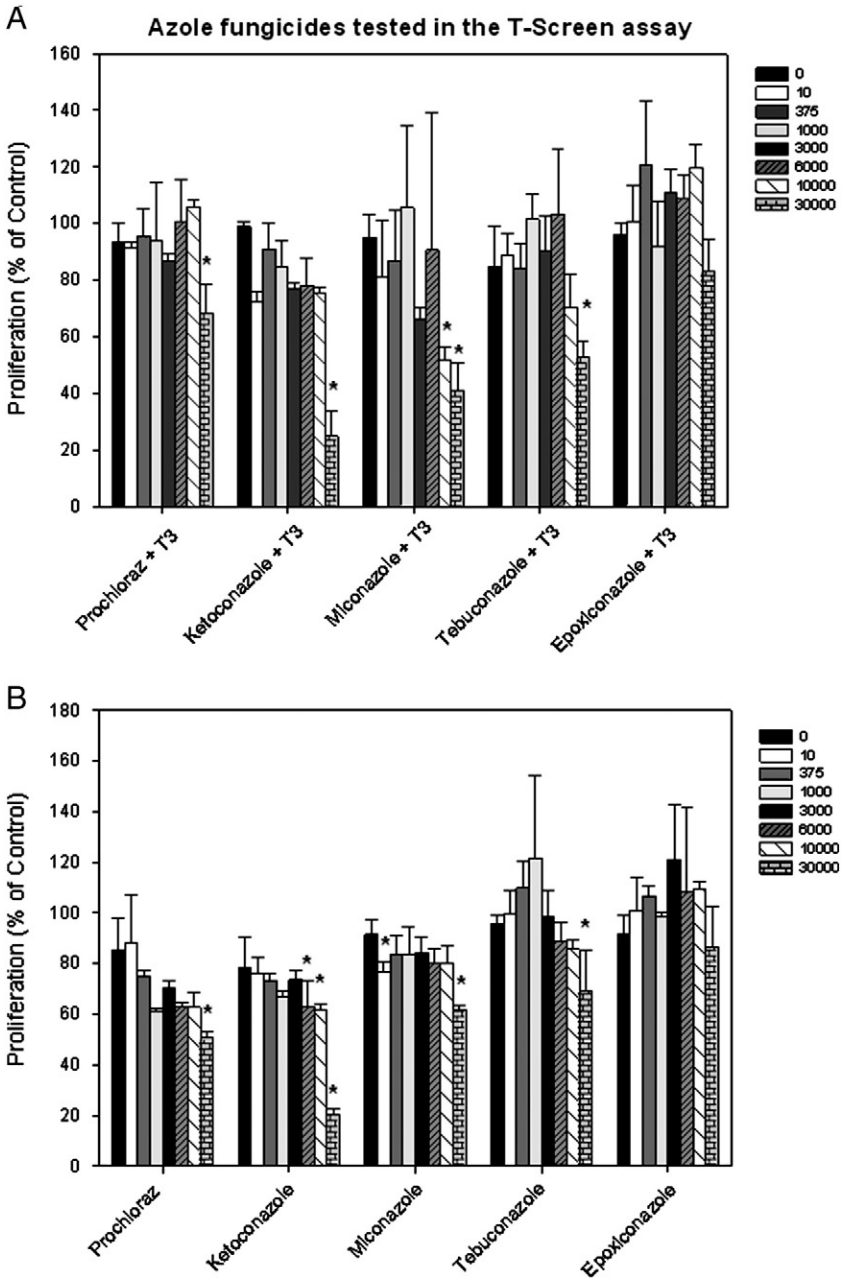

Fig. 2. Effects of the 5 azole fungicides: prochloraz, ketoconazole, miconazole, tebuconazole and epoxiconazole in the T-screen assay. The GH3 cells were treated with different concentrations of the 5 compounds A) in the presence of 0.1 nM T3 or B) alone. Data represent mean ± SD. The concentrations listed on the right are in nM. *Statistically significantly different from control ($P<0.05$).

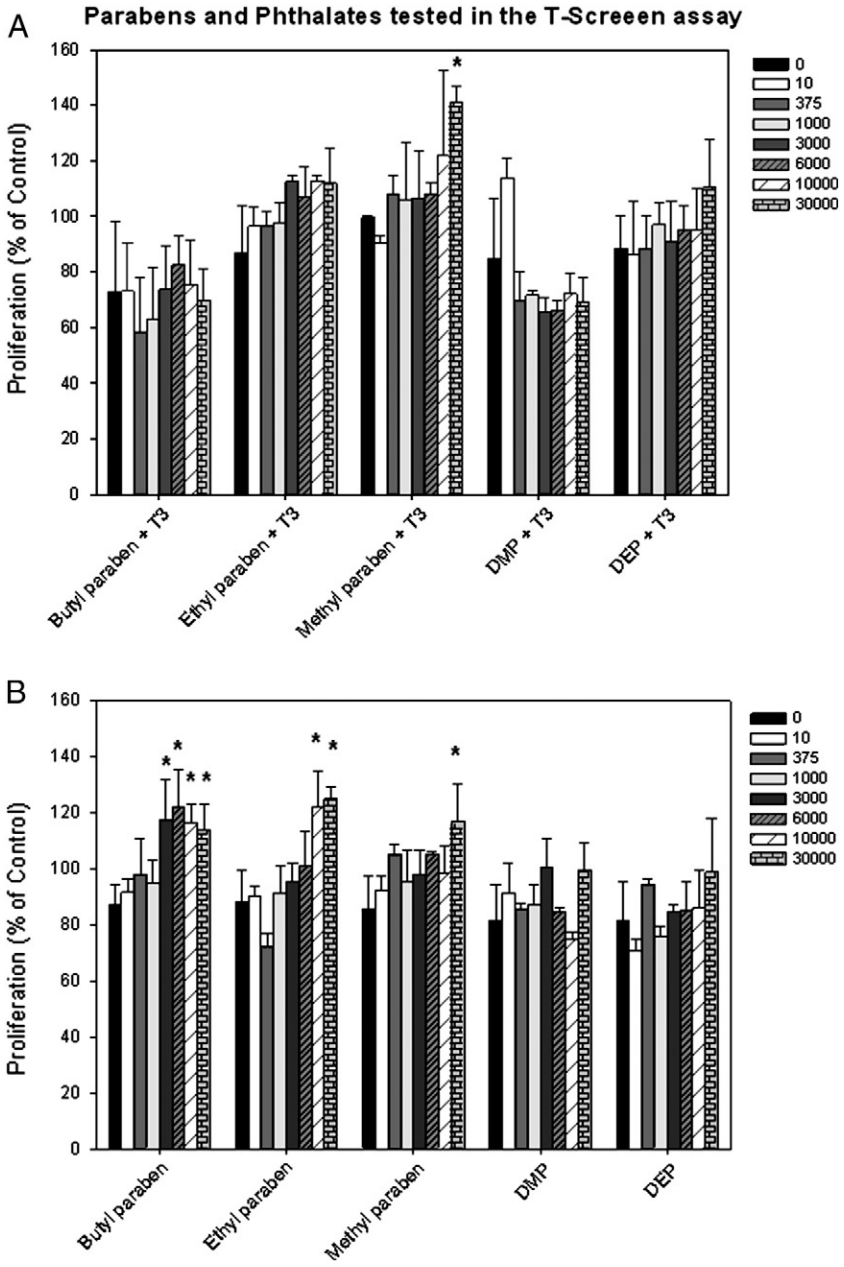

Fig. 3. Effects of the 3 parabens and 2 phthalates: methyl-, ethyl- and butyl paraben, DMP and DEP in the T-screen assay: The GH3 cells were treated with different concentrations of the compounds A) in the presence of 0.1 nM T3 or B) alone. Data represent mean ± SD. The concentrations listed on the right are in nM. *Statistically significantly different from control ($P<0.05$).

paraben) showing significant effect from 3 μM and above (Fig. 4B). None of the tested parabens had any TR antagonistic effect *in vitro* (Fig. 4A).

For the two tested phthalates, DMP and DEP, no effect was seen neither alone nor in combination with T3 (Figs. 3A and B).

Metabolic extracts from the human liver S9 assay. The extracts from the 3 parabens and the 2 phthalates after incubation with the human S9 were tested for effect in the T-screen assay. The extracts from the 5 azole fungicides were not tested as these compounds had shown very low metabolic transformation after treatment with the human liver S9 mix (Table 2).

The metabolic extract from butyl paraben showed a significant agonistic effect at the three lowest dilutions of the extract: 1/16, 1/8 and 1/14 (Fig. 4). The metabolic extract from methyl paraben also showed a significant agonistic but only at the second lowest dilution (1/8 diluted). As seen for the parent compounds, none of the paraben extracts had any antagonistic effect on the TR (Fig. 4).

For the S9-extracts from the 2 phthalates DMP and DEP a statistically significant reduction of the T_3 induced cell proliferation was observed for the DMP extract at the three highest dilutions (Fig. 4).

The endogenous metabolic capacity of the GH3 cells

Table 4 lists the results from the analysis of the samples after the parent compounds have been incubated the GH3 cells according to the protocol for testing of compounds in the T-screen assay.

A statistically significant difference between the amount of parent compound before and after incubation with the GH3 cells had taken place was found for the two phthalates, suggesting some metabolic capability of the GH3 cells in regard to DMP and DEP.

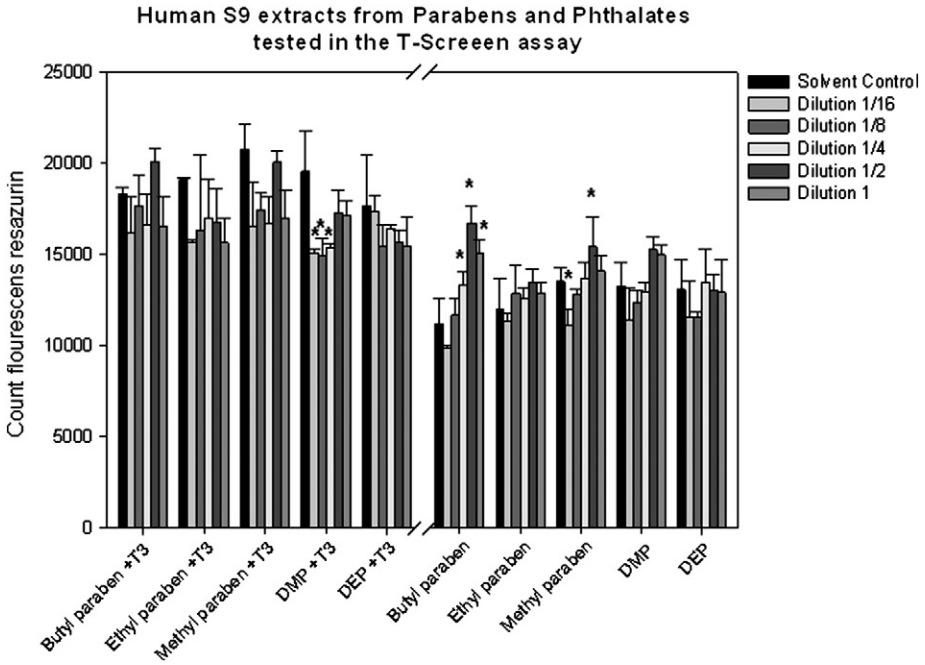

Fig. 4. Effects of the S9 extracts from methyl-, ethyl- and butyl paraben, and from DMP and DEP in the T-screen assay. GH3 cells treated with different dilutions of the extracts A) in the presence of 0.1 nM T3 or B) alone. Data represent mean ± SD. *Statistically significantly different from the solvent control ($P<0.05$).

Discussion

In vitro methods are a valuable and important tool for screening of many compounds for potential endocrine disrupting effects. However, when working with *in vitro* systems one is working with isolated systems not whole organisms. Therefore, when looking and evaluating the effects or results obtained from the different test systems, it is important to know how a particular compound is metabolized both *in vivo* (in the animal) and in the different in *in vitro* systems (cell models). In that regard it is important to have information concerning the endogenous metabolic capacity of the different cells used in the many different *in vitro* assays now available for the testing of a compounds endocrine disrupting potential. A focus point of the current study was therefore to investigate the endogenous metabolic capacity of the cells used in the applied *in vitro* assay, which in the current study was the T-screen assay. For the 10 selected test compounds representing 3 different classes of compounds: azole fungicides, parabens and phthalates, we found a significant difference between the amount of parent compound before and after incubation with the GH3 cells for the two phthalates. This could suggest that the GH3 cells have some metabolic capability in regard to the two tested phthalates, DMP and DEP. In general, such information concerning the metabolic fate of a compound in a given test system, is important when evaluating the results. In the current study we did not find a marked difference in the effects observed for the parent compounds and the effects of the tested metabolic extracts.

Table 4
The endogenous metabolic capacity of the GH3 cells.

	Recovery of parent compound (%)	Significance (p-value)
Ketoconazol	85%	0.13
Miconazol	83%	0.11
Epoxiconazol	96%	0.63
Tebuconazol	105%	0.63
Prochloraz	90%	0.12
Dimethyl phthalate	64%	<0.01*
Diethyl phthalate	45%	<0.01*
Methylparaben	100%	0.97
Ethylparaben	95%	0.32
Butylparaben	96%	0.71

Recovery of the parent compounds (%) after the compounds had been incubation with the GH3 cells according to the protocol for the T-screen assay as described in the Material and Methods section.

* Statistically significant difference between the amounts of parent compound in the samples before and after T-screen assay termination was tested by two-tailed T-test ($p<0.05$).

One other objective of the current study was to look at different *in vitro* systems for biotransformation of known endocrine disrupting chemicals before testing for their effects in *in vitro* assays. Two *in vitro* metabolizing systems were tested in the current study. Human liver S9 mix and PCB-induced hepatic rat microsomes. Both test systems gave an almost complete metabolic transformation of the tested parabens and phthalates, with a recovery rate of the parent compounds of less than 1%.

A difference was found between the human S9 and rat microsome assay systems. When using the PCB-induced rat microsomes a statistically significant difference between the amount of parent compound before and after treatment with the microsomes was seen for four out of the five azole fungicides tested. When using the human liver S9 we were not able to detect any significant metabolic transformation of the azole fungicides. This variation could be due to differences in metabolism between humans and rats or it could be due to a general higher metabolic activity of the rat microsomes compared to the human S9 fraction. It is well known that there are many differences in the metabolic capacity between species and also differences within the same species associated with age, gender, nutrition, genetic predisposition, environmental factors and others. The basis for choosing human liver S9 was initially that we wanted to compare the results from the human liver S9 assay with results from an assay using a rat based *in vitro* metabolizing systems.

In regards to risk assessment for endocrine disrupting effects and human exposure it would seem most relevant to use metabolizing systems based on materials from humans. However, using materials coming from animals compared to humans would most likely be easier and cheaper, particularly because getting enough amounts of research material from animals would be less of a challenge.

Therefore, it is relevant to get as much data as possible in order to make valid comparisons and extrapolations between results from different species.

Another reason for the difference observed between the human S9 and rat microsome metabolizing systems could be due to different binding of test compounds to proteins in the enzyme fractions. The azole fungicides are the most apolar of the 10 test compounds and they might bind to proteins or lipids making them less assessable for the metabolizing enzymes. Several EDs are known to bind strongly to serum proteins, and when this occurs *in vivo*, it affects the internal target tissue concentrations of the chemical involved (Safe et al., 1997). Such binding can occur with components of the metabolizing systems like S9 or components in culture media such as proteins in serum added to the media thereby resulting in both variable *in vitro* data, but also in differences in the results obtained in *in vivo* and *in vitro* assays.

It was not an aim of the current study to get any quantitative information on the metabolic fate of the selected test compounds as we were not going to identify the metabolites from the different compounds. We were interested in getting qualitative data, to see if the test compounds could be metabolized by the different *in vitro* metabolizing systems tested and to get information on the metabolic capability of the cells in the selected *in vitro* assay. However, additional studies focusing on determining the metabolic fate of known endocrine disrupters in more detail and identifying metabolites would certainly be relevant.

Based on the results and data from the current study our conclusion is that the GH3 cells show some metabolic capability in relation to the two tested phthalates DMP and DEP but none towards the tested azole fungicides and phthalates. Furthermore, an *in vitro* metabolizing system using liver human S9 or hepatic rat microsomes could be a convenient method for the incorporation of metabolic and toxicokinetic aspects into *in vitro* tests for endocrine disrupters.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Capen, C.C., 1997. Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. Toxicol. Pathol. 25, 39–48.

Cheek, A.O., Kow, K., Chen, J., McLachlan, J.A., 1999. Potential mechanisms of thyroid disruption in humans: interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. Environ. Health Perspect. 107, 273–278.

Coecke, S., Ahr, H., Blaauboer, B.J., Bremer, S., Casati, S., Castell, J., Combes, R., Corvi, R., Crespi, C.L., Cunningham, M.L., Elaut, G., Eletti, B., Freidig, A., Gennari, A., Ghersi-Egea, J.F., Guillouzo, A., Hartung, T., Hoet, P., Ingelman-Sundberg, M., Munn, S., Janssens, W., Ladstetter, B., Leahy, D., Long, A., Meneguz, A., Monshouwer, M., Morath, S., Nagelkerke, F., Pelkonen, O., Ponti, J., Prieto, P., Richert, L., Sabbioni, E., Schaack, B., Steiling, W., Testai, E., Vericat, J.A., Worth, A., 2006. Metabolism: a bottleneck in in vitro toxicological test development. The report and recommendations of ECVAM workshop 54. Altern. Lab. Anim. 34, 49–84.

Coldham, N.G., Horton, R., Byford, M.F., Sauer, M.J., 2002. A binary screening assay for pro-oestrogens in food: metabolic activation using hepatic microsomes and detection with oestrogen sensitive recombinant yeast cells. Food Addit. Contam. 19, 1138–1147.

Combes, R.D., 1992. The in vivo relevance of in vitro genotoxicity assays incorporating enzyme activation systems. In: Gibson, G.G. (Ed.), Progress in Drug Metabolism. Taylor and Francis Limited, London, pp. 295–321.

Craft, E.S., DeVito, M.J., Crofton, K.M., 2002. Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long–Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. Toxicol. Sci. 68, 372–380.

DeVito, M., Biegel, L., Brouwer, A., Brown, S., Brucker-Davis, F., Cheek, A.O., Christensen, R., Colborn, T., Cooke, P., Crissman, J., Crofton, K., Doerge, D., Gray, E., Hauser, P., Hurley, P., Kohn, M., Lazar, J., McMaster, S., McClain, M., McConnell, E., Meier, C., Miller, R., Tietge, J., Tyl, R., 1999. Screening methods for thyroid hormone disruptors. Environ. Health Perspect. 107, 407–415.

Frandsen, H., Nielsen, P.A., Grivas, S., Larsen, J.C., 1994. Microsomal metabolism of the food mutagen 2-amino-3, 4, 8-trimethyl-3H-imidazo[4, 5-f]-quinoxaline to mutagenic metabolites. Mutagenesis 9, 59–65.

Frederiksen, H., Frandsen, H., 2002. In vitro metabolism of two heterocyclic amines, 2-amino-9H-pyrido[2, 3-b]indole (A(alpha)C) and 2-amino-3-methyl-9H-pyridol2, 3-b]indole (MeA(alpha)C) in human and rat hepatic microsomes. Pharmacol. Toxicol. 90, 127–134.

Fritsche, E., Cline, J.E., Nguyen, N.H., Scanlan, T.S., Abel, J., 2005. Polychlorinated biphenyls disturb differentiation of normal human neural progenitor cells: clue for involvement of thyroid hormone receptors. Environ. Health Perspect. 113, 871–876.

Gauger, K.J., Kato, Y., Haraguchi, K., Lehmler, H.J., Robertson, L.W., Bansal, R., Zoeller, R.T., 2004. Polychlorinated biphenyls (PCBs) exert thyroid hormone-like effects in the fetal rat brain but do not bind to thyroid hormone receptors. Environ. Health Perspect. 112, 516–523.

Ghisari, M., Bonefeld-Jorgensen, E.C., 2005. Impact of environmental chemicals on the thyroid hormone function in pituitary rat GH3 cells. Mol. Cell. Endocrinol. 244, 31–41.

Guillouzo, A., 1998. Liver cell models in in vitro toxicology. Environ. Health Perspect. 106 (Suppl 2), 511–532.

Hallgren, S., Sinjari, T., Hakansson, H., Darnerud, P.O., 2001. Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. Arch. Toxicol. 75, 200–208.

Hinkle, P.M., Kinsella, P.A., 1986. Thyroid hormone induction of an autocrine growth factor secreted by pituitary tumor cells. Science 234, 1549–1552.

Kato, Y., Haraguchi, K., Shibahara, T., Yumoto, S., Masuda, Y., Kimura, R., 2000. Reduction of serum thyroxine concentrations by methylsulfonyl metabolites of tetra-, penta- and hexachlorinated biphenyls in male Sprague–Dawley rats. Chemosphere 40, 1233–1240.

Safe, S., Connor, K., Ramamoorthy, K., Gaido, K., Maness, S., 1997. Human exposure to endocrine-active chemicals: hazard assessment problems. Regul. Toxicol. Pharmacol. 26, 52–58.

Setchell, K.D.R.a.H.A., 1988. Mammalian lignans and phytoestrogens. Recent studies on their formation, metabolism, and biological role in health and disease. In: Rowland, I.R. (Ed.), Role of the Gut Flora in Toxicity and Cancer. Academic Press, London, pp. 315–345.

Taxvig, C., Vinggaard, A.M., Hass, U., Axelstad, M., Boberg, J., Hansen, P.R., Frederiksen, H., Nellemann, C., 2008. Do parabens have the ability to interfere with steroidogenesis? Toxicol. Sci. 106, 206–213.

Werck-Reichhart, D., Feyereisen, R., 2000. Cytochromes P450: a success story. Genome Biol. 1 (REVIEWS3003).

Wong, C., Kelce, W.R., Sar, M., Wilson, E.M., 1995. Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. J. Biol. Chem. 270, 19998–20003.

Zhou, T., Ross, D.G., DeVito, M.J., Crofton, K.M., 2001. Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. Toxicol. Sci. 61, 76–82.

Zoeller, R.T., Bansal, R., Parris, C., 2005. Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist in vitro, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. Endocrinology 146, 607–612.

Paper III

Effects of mixtures of phytoestrogens on adipocyte differentiation, and PPARα and PPARγ activation *in vitro*

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Running title: Phytoestrogens and obesity parameters *in vitro*

Keywords: Phytoestrogens; 3T3-L1; adipocyte differentiation; lipid accumulation; PPARγ; PPARα

Abstract

Many studied phytoestrogens (PEs) have been reported to exhibit anti-adipogenic effects in adipocytes and anti-proliferative and pro-apoptotic activities in several cell types. The current study examines different mixtures of twelve food relevant PEs, hereunder both isoflavones, lignans and coumestrol, for effects on adipogenesis in the 3T3-L1 preadipocyte cell line as well as their effect on PPAR α and γ in a transactivation assay. Overall the results shoved that the different PE mixtures had more effect on PPARγ activation than on PPARα. Furthermore, the tested single PEs as well as the nutrition relevant mixtures of PEs had a decreasing effect on lipid accumulation *in vitro*, which suggests that PEs can affect pathways known to play a role in the development of obesity. However, based on the results of the current study as well as the fact, that the role of different players in adipogenesis and lipolysis is not fully understood, the influence of PEs on adipogenesis, and pathways involved in development of obesity, remains unclear. Nevertheless, the current study adds to an increasing understanding of what effects exposure to different endocrine disrupting chemicals can exert on the different pathways playing a role in the development obesity.

1. Introduction

It is now well documented that numerous environmental chemicals can interfere with complex endocrine signalling pathways and cause adverse effects (Diamanti-Kandarakis et al., 2009). The centre of attention for endocrine disrupting chemicals (EDCs) has been broadened by the knowledge that multiple organ systems can be affected by EDCs. One of the new emerging modes of action for EDCs is that exposure to EDCs may play a role in obesity. Different reports have played a part in starting the hypothesis that there could be a link between environmental chemical exposure and the development of obesity. Some of the work supporting this hypothesis is studies describing excess weight gain in animals treated developmentally with certain EDCs, including diethylstilbestrol (DES) and bisphenol A (BPA) (Howdeshell et al., 1999; Newbold et al., 2005; Rubin and Soto, 2009) as well as data from epidemiologic studies showing a possible association between serum dioxin levels and the onset and incidence rate of diabetes (Longnecker and Michalek, 2000).

Additionally, in one of our own studies, we found that diisobutyl phthalate (DiBP), butylparaben, and rosiglitazone reduced plasma leptin and/or insulin levels in 21-day old male and female fetuses after exposure of pregnant rats from gestation day (GD) 7–21 (Boberg et al., 2008).

The purpose of the current study was to evaluate 12 different phytoestrogens (PEs), naturally occurring in Western/Scandinavian food. We previously investigated the endocrine disrupting abilities of these PEs on different endpoints such as steroidogenesis as well as estrogen and androgen activity, and found different effects including estrogen activity in MFC-7 cells, as well as increased estradiol production in the H295R steroidogenesis assay (Taxvig et al., 2010). Here we examined whether any of these naturally occurring PEs could affect the ability of preadipocyte 3T3-L1 cells to differentiate into adipocytes and the subsequent accumulation of lipid droplets, and

studied how the PEs affected transactivation of peroxisome proliferator-activated receptors (PPARs).

The need to develop new effective strategies in controlling obesity has become more acute because obesity leads to a wide range of negative impacts on health. This has lead to progress in understanding the process of adipocyte differentiation. Adipose tissue growth involves formation of new adipocytes from precursor cells, further leading to an increase in adipocyte size. The transition from undifferentiated fibroblast-like preadipocytes into mature adipocytes constitutes the adipocyte life cycle, and a better understanding of how size and number of adipocytes is regulated may be required to improve understanding and treatment of obesity (Rayalam et al., 2008). Different *in vitro* systems exist for studying adipocyte differentiation. One of the most frequently employed cell lines is the 3T3-L1 cell line. The 3T3-L1 is a mouse embryonic fibroblast pre-adipocyte cell line, and the 3T3-L1 differentiation assay is a well established *in vitro* model of adipocytes to study signaling pathways involved in adipocyte differentiation, as well as pathways playing a role obesity or obesity related diseases. The preadipocyte 3T3-L1 cells have a fibroblast-like morphology during the growth phase. However, under appropriate conditions, the cells differentiate into an adipocyte-like phenotype, where the preadipocyte converts to a spherical shape, accumulates lipid droplets, and progressively acquires the morphological and biochemical characteristics of a mature white adipocyte (Gregoire et al., 1998).

Adipogenesis and the many different mechanisms involved in the regulation of adipocyte differentiation is complex. However, one of the transcription factors shown to play a key role in adipocyte differentiation is the PPAR family. Generally the PPARs are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes that play essential roles in cellular differentiation, development, and metabolism. PPARγ is primarily expressed in adipose tissue, and promotes adipocyte differentiation and activates transcription of genes involved

in lipid storage and control of insulin sensitivity (Auwerx, 1999; Ferre, 2004; Shen et al., 2006; Tontonoz et al., 1994). PPARα is predominantly expressed in tissues with high rates of mitochondrial and peroxisomal fatty acid catabolism, such as liver, brown adipose tissue, heart, skeletal muscle, kidney, and intestinal mucosa. PPARα ligands have been shown to be hypolipidemic, to improve insulin sensitivity and to reduce adiposity, through a direct regulatory action on lipid metabolism (Guerre-Millo et al., 2000; Yoon, 2009), and different studies have also shown that PPARα could have a role in adipocyte differentiation (Brun et al., 1996; Yu et al., 1995). Therefore, a second interest point for the current study was to test the different mixtures of the 12 PEs for their effect on PPAR α and γ, to see if they had any ability activate PPAR α and/or γ in a transactivation assay.

The selection of PEs for the current study was based on a Scandinavian epidemiological study, estimating the intake of a number of PEs in the Swedish population (Hedelin et al., 2006). The proportions of the single PEs in the mixtures were based on the amount and number of the different classes (e.g. isoflavonoids lignans and coumestans) of PEs naturally found in Scandinavian food (Hedelin et al., 2006). Therefore, the design of the mixtures was based on representing PEs naturally occurring in Western food, but the proportions of the compounds in the mixtures were not representative of the concentrations found in the diet, and the design of the mixtures was not based on effective doses.

Five different mixtures were tested: Mix 1 composed of the isoflavonoid metabolites genistein, daidzein and equol, Mix 2 composed of the two isoflavonoids formononetin and biochanin A; Mix 3 composed of the lignan metabolites enterolactone and enterodiol; Mix 4 containing the four lignans secoisolariciresinol, matairesinol, lariciresinol and pinoresinol, and finally a total mixture including all 12 selected PEs. An overview of the different PEs and mixtures is presented in Figure 1. Additionally the single compound coumestrol was also tested in the PPAR transactivation assay,

and in the 3T3-L1 assay, coumestrol, genistein, and daidzein were also tested as single compounds as well tributyltin (TBT), which was included because of its reported positive effects in the 3T3-L1 differentiation assay (Collins, 2005; Grun et al., 2006; Inadera and Shimomura, 2005).

The experiments involving the PPARγ antagonist GW9662, was included to examine if any possible effects on lipid accumulation in the 3T3-L1 cells could be influenced by the presence of a PPARγ antagonist. Rosigiltazone is a known PPARγ agonist and was also run in the 3T3-L1 assays as a reference compound, because it is known to induce adipocyte differentiation.

2. Methods and Materials

2.1 Test compounds

Genistein (CAS 446-72-0), daidzein (CAS 486-66-8), equol (CAS 66036-38-2), formononetin (CAS 485-72-3), biochanin A (CAS 491-80-5), enterolactone (CAS 78473-71-9), enterodiol (CAS 80226-00-2), secoisolarici resinol (CAS 29288-59-8), and coumestrol (CAS 479-13-0) were all purchased from Sigma-Aldrich (Milwaukee, WI, USA). Matairesinol (CAS 580-72-3), lariciresinol (CAS 27003-73-2), and pinoresinol (CAS 487-36-5) were purchased from Arbo-Nova, Finland.

Rosiglitazone (BRL 49653) cas no. 122320-73-4 was from Cayman Chemicals (cat no. 71740). WY 14,643, cas no. 50892-23-4, was from Calbiochem (cat no. 681725). Phosphate Buffered Saline (PBS Tablets; Oxoid, Cat no BR1400G)Biotin (CAS 58-85-5), Calcium pantothenate (CAS 137-08-06), Dexamethasone (CAS 50-02-2), Isobutylmethylxanthine (IBMX) (CAS 28822-58-4), Insulin (CAS 11070-73-8), Tributyltin chloride (TBT) (CAS 1461-22-9), Oil Red O (CAS 1320-06-5), Sodium dihydrogen phosphate, NaH_2PO_4 (CAS 7558-80-7), Sodium hydrogenphosphate, Na_2HPO_4 (CAS 7558-79-4), Calcium chloride (CAS 10043-52-4), Magnesium chloride hexahydrate, $MgCl_26H_2O$ (CAS 7791-18-6), 2-Propanol (CAS 67-63-0), Formaldehyde (CAS 50-00-0), and GW9662 (CAS 22978-25-2) all from Sigma Aldrich (Milwaukee, WI, USA). The test compounds

were dissolved in dimethyl sulfoxid (DMSO, CAS 67-68-5) also from Sigma Aldrich. The twelve phytoestrogens used, their data, estimated daily intake, and their proportions in the different mixtures are listed in Table 1.

2.2 Adipocyte differentiation assays

3T3-L1 cells (ATCC number: CL-173) were cultured in a humid atmosphere at 37˚C and 95% air/5% CO_2 in DMEM (Gibco-Invitrogen, Paisley, UK) supplemented with 1% Antibiotic/Antimycotic (PSF), 10% (vol/vol) calf serum (FBS) and 1 mM sodium pyruvate (Invitrogen). When starting the assay cells were switched from growing in 10 cm culture dished (Falkon) to 6 well culture plates (Falcon) and the media were also switched to DMEM with 10% FBS. The cells were cultured in the 6-well plates to confluence and exposed to the induction cocktail: 1 μg/ml insulin, 600 nM dexamethasone, 500 μM isobutylmethylxanthine (IBMX) (MDI cocktail) as well as 33 μM biotin and 17 μM calcium pantothenate. In the experiments where rosiglitazone was giving during the during the induction period the cells were exposed to 0.5 μM rosiglitazone in addition to the MDI induction cocktail. After 48 hr in the medium with the added induction substances the medium was replaced with differentiation medium containing 33 μM biotin, 17 μM calcium pantothenate and 1 ug/ml insulin as well as the respective test compounds or test mixtures. The phytoestrogens and mixtures were tested duplicates in three concentrations: 25, 50 and 75 μM. Included as a background control on each test plate was a well with 0.1% DMSO instead of test compound. The differentiation medium with test compounds were changed every 2 days until day 6 where the plates were stained with Oil red O. For the assays including the PPARγ antagonist GW9662 a final concentration of 1 μM GW9662 was added to each well every 6 hour for the 6 days the cells were exposed to test compound.

For the Oil Red O staining each well was washed with 2 mL PBS and then fixed with 10% formaldehyde (0.4 g sodium phosphate; 0.65 g disodium hydrogen phosphate dissolved in 10 mL formaldehyde and 90 mL distilled water) for 15-20 min. After the formaldehyde treatment, the wells were washed with PBS and then stained for 15 min with 2 mL/well filtered Oil Red O solution (0.36 g Oil Red O added to 100 mL 60% isopropanol) After 15 min. the staining solution was removed and the wells were rinsed once with 2 mL/well 60% isopropanol for 30 sec. followed by washing 3 times with 2 mL/well PBS.

After the last wash 2 ml PBS was left in each well to prevent it from drying out before photographing was completed. The Oil Red O stained wells were inspected under a microscope and five to twelve random field images from each well were photographed under phase contrast. These images were analyzed in ImageJ, where they were converted into high-contrast black and white images to visualize the lipid droplets and score them as percentage area per field using the ImageJ software.

All compounds and mixtures were run in three independent experiments and data are shown as the mean ± SD from one representative experiment.

2.3 PPARα and PPARγ transactivation assay

The NIH-3T3 cells (ATCC number: CL-1658) were maintained in phenol red-free DMEM/F12 (Gibco, Paisley, UK) with 10 % charcoal-filtered calf serum (Biological Industries Ltd, Israel) and 1 % Antibiotic/Antimycotic (PSF) (PAA Laboratories GmbH, Austria) at 37˚C in 5 % CO_2/air. To examine the effect of the test compounds on ligand-induced PPAR transactivation, cells were transiently transfected with a plasmid expressing the ligand binding domain of mouse PPARα or PPARγ coupled to Gal4 and a plasmid containing an UAS linked luciferase reporter construct, 4xUAS-TK-luc. Used as positive controls was the specific PPARα agonist WY14.643 or the

PPARγ-selective agonist rosiglitazone. On day 1 of the experiment, cells were counted and transferred to 96-well cell culture plates (PerkinElmer, Massachusetts, USA) at a density of 7000 cells per well. After 20 hours, cells were transfected with the luciferase reporter plasmid 4xUAS-TK and either gal4-DBD_mPPARαLBD or gal4-DBD_mPPARγLBD expression vectors using FuGene® (Roche. Mannheim, Germany) as a transfection reagent. One hour before transfection medium was changed: FuGene and serum-free medium was mixed using 4.5 μL FuGene in 10 μL medium per well and added to a mixture of the plasmids with a total cDNA content of 75 ng per well. Plasmids were mixed in a 1:2 ratio for gal4-DBD_mPPARαLBD and 4xUAS-TK-luc and a ratio of 1:1 for gal4-DBD_mPPARγLBD and 4xUAS-TK-luc. These ratios and cDNA concentrations had been selected as the optimal for each receptor giving the highest activation level compared to other plasmid ratios and DNA contents. The FuGene-cDNA mixture was incubated for 30 minutes before being added to the cells. 5 hours after transfection, media was removed and new media containing test compounds was added and left for incubation at 37˚C in 5 % CO_2/air over night (22 h). All test compounds and mixtures were tested in triplicate in concentrations from 0 to 100 μM (0; 3.13; 6.25; 12.5; 25; 50, and 100 μM), with a maximal concentration of DMSO below 0.3 % which had previously been found not to be cytotoxic.

Cytotoxicity was evaluated in each well by incubating the cells with resazurin solution (CAS 62758-13-8, Sigma Aldrich; Milwaukee, WI, USA) for 2 h followed by measuring fluorescence (excitation wavelength 560 nm/emission. 590 nm) on a Wallac Victor 1420 multilabel counter (PerkinElmer Life Sciences).

Each test compound and mixture was run in three to five independent experiments and data are presented as the mean ± SD from three comparable independent experiments.

2.4 Statistical analyses

All data were tested for homogeneity of variance and normal distribution. Data were analyzed by one-way analyses of variance (ANOVA) and, if significant, followed by Dunnett's *post hoc* test. Significance was judged at $p < 0.05$.

3. Results

3.1 Effects of naturally occurring PEs on adipogenesis in 3T3-L1 cells

The 3T3-L1 preadipocyte cell line was used as an *in vitro* model for studying adipogenesis and specifically to look at the effect of nutritionally relevant concentrations and mixtures of PEs on the differentiation of 3T3-L1 preadipocytes, a process in which PPARγ is thought to play a major role. The 3T3-L1 cells were grown to confluence and induced to differentiate into adipocytes by treatment with a mixture containing isobutylmethylxanthine (IBMX), dexamethasone, and insulin (MDI cocktail). After 2 days in the induction medium the medium was changed to adipogenesis differentiation medium containing biotin, calcium pantothenate and insulin as well as the respective test compounds or test mixtures. The effects of the PEs on adipocyte differentiation were measured by scoring the amount of lipid droplets formed in the 3T3-L1 adipocytes using Oil Red O staining (Figure 5). Along with the different PEs and mixtures, TBT was also included in the assays, as TBT previously has been shown to be a potent inducer of lipid accumulation in these 3T3-L1 adipocytes (Grun et al., 2006; Inadera and Shimomura, 2005). As presented in Figure 2A, all three selected single PEs (genistein, daidzein, and coumestrol) significantly decreased lipid accumulation, with genistein being the post potent inhibitor showing effect at all tested concentrations (25, 50 and 75 μM). Coumestrol showed effect at the two highest concentrations 50 and 75 μM and daidzein had significant effect only at 75 μM (Fig 2A).

For the five different mixtures tested mix 1 (genistein, daidzein and equol), mix 2 (formononetin and biochanin A) and mix 3 (enterolactone and enterodiol) also showed significant decrease in lipid accumulation (Fig 2B). However, for mix 2 the effect was only statistically significant at 50 μM, the second highest concentration but the effect at 75 μM were comparable to the effect observed at 50 μM (Fig 2B). Neither mix 4 (secoisolariciresinol, matairesinol, lariciresinol and pinoresinol) nor the total-mix had any significant effect on the lipid accumulation in the 3T3-L1 adipocytes (Fig 2B).

Rosiglitazone is a known PPARγ agonist and the single compounds genistein, daidzein and coumestrol as well as mixture 1, 2 and 3 were also tested for the effect on rosiglitazone induced differentiation. Rosiglitazone alone showed a concentration dependent increase in lipid assumulation (Figure 3A). When the 3T3-L1 celle were induced to differentiate by standard MDI adipogenic treatment in the presence of 0,5 μM rosiglitazone (Rosi), all three single PEs as well as the tested PE-mixtures showed a significant ability to decrease the rosiglitazone induce lipid accumulation in the 3T3-L1 cells as seen in figure 3B (the results for mix 2 and mix 3 are not presented, but were comparable with the effects seen in figure 2B).

In figure 5 are shown representative pictures of the Oil Red O staining from both the experiments using standard MDI induction and from the experiments with induction in the presence of 0.5 μM rosiglitazone.

3.1.1 Effect of PEs in combination with a PPARγ antagonist on 3T3-L1 differentiation

PPARγ has been shown to play a key regulatory role in adipocyte differentiation (Hu et al., 1995; Tontonoz et al., 1994) and to be necessary for lipid accumulation in adipocytes in mice (Barak et al., 1999; Kubota et al., 1999; Rosen et al., 1999).

In the current study we found that treatment of the 3T3-L1 with TBT in combination with the PPARγ antagonist GW9662 did not significantly influence the effect of TBT on lipid accumulation in the 3T3-L1 adipocytes (Fig 4A). These results are in accordance with results from a previous study by Inadera and Shimomura (Inadera and Shimomura, 2005) where it was found that induction of aP2 (adipocyte protein 2) - a well known marker of adipocyte differentiation - by TBT was unaffected by co-treatment of TBT with the PPARγ antagonist GW9662, suggesting that the effect of TBT on 3T3-L1 differentiation is not PPARγ-dependent.

In the present study genistein and the mixture containing all 12 PEs were also tested in combination with GW9662, to examine if any possible effect of genistein or the mixture on lipid accumulation could be influenced by the presence of a PPARγ antagonist. For genistein which alone significantly decreased lipid accumulation at all tested concentrations the co-treatment with GW resulted in an impairment or neutralization of the decreasing effect at 25 μM and 50 μM, giving only a significant decrease at 75 μM, the highest concentration tested (Fig. 4B), indicating that other players besides PPARγ are involved in 3T3-L1 differentiation. For the total-mix, which alone had shown no effect, the co-treatment with GW9662 had no effect (data not shown).

3.2 Effect of PE-mixtures on the activation of PPARα and PPARγ

Transactivation of PPARα and PPARγ was examined in a reporter gene assay by adding different concentrations of test compound or mixture to cells transiently transfected with expression plasmid for the ligand binding domain of either mouse PPARα or PPARγ coupled to Gal4 and a plasmid containing an UAS linked luciferase reporter construct (UAS-TK-luc).

PPARα

None of the mixtures or coumestrol alone had any PPARα activating effect (Fig. 6A and Table2). Contrary, for Mix 3 (enterolactone and enterodiol) and mix 4 (secoisolariciresinol, matairesinol, lariciresinol and pinoresinol) a decrease in activation was observed in the PPARα (Fig. 6A).
For coumestrol alone a significant decrease in activity was observed at 100 μM (Fig 6A), which could be due to a beginning cytotoxic effect even though cytotoxicity was only found for coumestrol at 50 μM and 100 μM in one out of five independent cytotox-experiments (data not shown). The response observed for the positive control the PPARα agonist WY14.643 was 382 % of the background control (data not shown).

PPARγ

In the PPARγ assay, mix 1 (genistein, daidzein and equol), mix 2 (formononetin and biochanin A) and the total-mix showed some activating effect, with mix 2 being the most potent of the three shoving an activating effect at a concentration of 6.25 μM up to 50 μM, whit a maximum around 200% of the background control (Fig. 6B and Table 2). For comparison the response observed with the positive control rosiglitazone was 8583% of the background control (data not shown)
The highest concentration of 100 μM was found to be cytotoxic for mix 2.
For coumestrol alone no effect was, seen (Fig 6B).

4. Discussion

There are only limited data on the possible role of EDCs in the development of obesity in humans. Several pregnancy cohort studies have examined chemicals in relation to childhood or adult BMI or height. In general, findings from these studies are inconsistent. Different studies on associations

between levels of dichlorodiphenyldichloroethylene (DDE) and PSB and endpoints such as BMI and growth outcomes have reported both positive associations as well as either negative or null associations (Blanck et al., 2002; Gladen et al., 2004; Gladen et al., 2000; Karmaus et al., 2009; Ribas-Fito et al., 2006; Verhulst et al., 2009). However, despite conflicting results from epidemiological studies, the hypothesis that there could be a link between exposure to endocrine disrupting chemicals and the development of obesity is being supported by more and more studies. The modes of action and the contribution of environmental factors to the development of both obesity and obesity related diseases is though, far from understood and the aim of the current study was to evaluate 12 different PEs, naturally occurring in Western/Scandinavian food, and previously shown to have different endocrine disrupting effects (Taxvig et al., 2010), for their effect on obesity parameters *in vitro*. Different mixtures but also a few single compounds were evaluated for potential effects on adipocyte differentiation and their ability to activate peroxisome proliferator activated receptor PPARα and PPARγ. The isoflavones, genistein and daidzein, have already been shown to have both PPARα and PPARγ agonistic effects (Ricketts et al., 2005; Shen et al., 2006) and some PEs including genistein have also previously been studied for their effect on adipogenesis (Cooke and Naaz, 2005; Liao et al., 2008; Park et al., 2008). In the present study genistein had a decreasing effect on lipid accumulation in the 3T3-L1 cells for all concentrations tested (25-75 μM). This effect was seen both when differentiation induced using standard MDI treatment, but genistein also decreased rosiglitazone-induced adipocyte differentiation. The same effects were observed for coumestrol and daidzein, and in the experiments with the rosiglitazone-induced differentiation the effects on lipid accumulation observed for the three single PEs were comparable (Fig 3A). For mix 1 containing a combination of genistein, daidzein and equol a decrease in lipid accumulation was found as well, the effect being comparable to that of genistein, and in the rosiglitazone-induction experiments, the effect of Mix1 was comparable that seen for each of the

single compounds (Fig 3A). Genistein both alone but also in combination with the naturally occurring phytoalexin resveratrol and the flavonol quercetin has previously been shown to decrease lipid accumulation in 3T3-L1 cells with the decreasing effect being enhanced when the compounds were combined compared to the effect of the single compounds (Park et al., 2008; Rayalam et al., 2007).

The overall decreasing effect of mix 1 is in accordance with what others have reported for genistein (Harmon and Harp, 2001; Park et al., 2008; Rayalam et al., 2007), and also supported by a study on the effects of equol by Rachon et al.,(2007) (Rachon et al., 2007), which found that dietary equol decreased weight gain, intra-abdominal fat accumulation, and plasma leptin and triglyceride levels in ovariectomized rats. However, our results contradicts studies by (Cho et al., 2010) and (Relic et al., 2009) who found that daidzein and genistein enhanced adipogenesis, although for the Relic et al. study this was not shown in 3T3-L1 cells but in cultured synovial fibroblasts.

In order to examine whether the effect of genistein was PPARγ dependent, genistein was tested in combination with the PPARγ antagonist GW9662. This showed that the effect of genistein on lipid accumulation was weakened but not totally blocked, suggesting that the effect genistein exerts on lipid accumulation in the 3T3-L1 is only partly dependent on PPARγ and/or that lipid accumulation in the 3T3-L1 is mediated or dependent on other players than PPARγ. One possibility is that the effect of genistein is mediated through the estrogen receptor (ER). This would be in agreement with other studies showing a possible ERα mediated effect of genistein on adipose tissue (Cooke and Naaz, 2005) as well as the reported decreasing effect of estrogen on lipid accumulation in 3T3-L1 adipocytes (Homma et al., 2000). Additionally, when TBT was tested in combination with the PPARγ antagonist GW9662, it did not affect the increase in lipid accumulation brought on by TBT. This is in agreement with the results of Inadera and Shimomura (Inadera and Shimomura, 2005), who found that combined treatment with TBT and GW9662 did not prevent the induction of the

well known marker of adipocyte differentiation aP2, suggesting that the effect of TBT on the 3T3-L1 cells is PPARγ-independent, or at least that other players beside PPARγ are involved. The potent effects of organotins on adipogenesis have been found to be attributed to dual agonistic activity for PPARγ and RXR, although there is some debate on whether these effects on adipogenesis are primarily do to interactions with PPARγ or RXR (Hiromori et al., 2009; Inadera and Shimomura, 2005; Kirchner et al., 2010; le Maire et al., 2009). Le Maire et al. have recently shown that the activation of the PPAR-RXR heterodimers by TBT is primarily through its interaction with RXR (le Maire et al., 2009). Our results with TBT however, are in contrast to the effects found by Kirchner et al. (2010), who were able to block the lipid accumulating effect of TBT in mouse adipose-derived stromal stem cells (mADSCs) by the addition of the potent PPARγ antagonist T0070907 (Kirchner et al., 2010). Whether this difference is due to a different involvement of PPARγ in lipid accumulation in the 3T3-L1 cells compared to the mADSCs is currently not known.

Also mix 2 (formononetin and biochanin A) and mix 3 (enterolactone and enterodiol) significantly decreased lipid accumulation, though not in the same order of magnitude as observed with mix 1. Few studies have examined the effect of formononetin, biochanin A, enterolactone and/or enterodiol on adipogenesis. One such study showed that biochanin A and formononetin stimulated lipid accumulation in 3T3-L1 cells at low concentrations (1- 5 μM for biochanin A and 3- 30 μM for formononetin, respectively)(Shen et al., 2006). However, the study by Shen et al, (2006) also found that genistein stimulated lipid accumulation at 15 μM, a result that is different from what we and others have found (Harmon and Harp, 2001; Park et al., 2008; Rayalam et al., 2007; Shen et al., 2006). It is known from other types of studies with PEs that many of the PEs show a biphasic concentration-effect curve. One such example is the effect of PEs on the cell proliferation of MCF-7 cells, where Le Bail et al.,(2000) and others have found that some of the PEs stimulated growth at lower concentrations but inhibited growth at higher concentrations (Le Bail et al., 2000; Taxvig et

al., 2010; Ying et al., 2005). As Shen et al., (2006) have generally tested lower concentrations than applied in our and other studies, this may cause the observed differences in the effects found in the different studies (Shen et al., 2006).

In regards to the effects of the PEs mixturets on PPAR activation, no significant PPARα activating effect was observed for any of the mixtures (Fig. 6A) On the contrary for mix 3 and mix 4 a significant decrease in PPARα activation was observed (Fig. 6A). In the assays testing for PPARγ activation the overall picture was an activating effect of mix 1, mix 2 and the total-mix, with mix 2 being the most potent of the three. The PPARγ activation by mix 1 confirms the previously shown PPARγ activity of genistein and daidzein, the main components of mix 1, at concentrations comparable to those in the current study (Ricketts et al., 2005; Shen et al., 2006). The PPARγ activation by mix 2 points to PPARγ agonistic activities of formononectin and/or biochanin A, the two compounds found in mix 2, which have also previously been shown to activate PPARγ in concentrations from 1 to 90 μM or 100 μM (Shen et al., 2006).

A parallel between the results found on lipid accumulation in the 3T3-L1 cells and the effects found on PPAR activation cannot be drawn as all the different mechanisms underlying the development of adipocytes and adipose tissue, are complex and far from understood. Studies have shown that not only PPARγ but also PPARα could have a role in adipocyte differentiation (Brun et al., 1996; Yu et al., 1995). PPARα is not expressed at significant levels in white fat, but it is expressed in brown fat (Beck et al., 1992; Issemann and Green, 1990). Data by Brun et al., (1996) indicate that while PPARα possesses some adipogenic potential, PPARγ is a more effective inducer of adipocyte differentiation (Brun et al., 1996). As can be seen from the results of the combination of TBT and GW9662, both ours and others (Inadera and Shimomura, 2005), even though PPARγ is thought to play a big role in 3T3-L1 adipocyte differentiation other pathways are involved as well.

Overall, based on the result of the current study, the different PE mixtures had more effect on PPARγ than PPARα. Our conclusion is that many PEs as well as dietary relevant mixtures of PEs have a decreasing effect on lipid accumulation *in vitro*. However, the role of different players in adipogenesis and lipolysis, including the different PPAR isoforms, as well as ER and RXR, is not yet understood. Therefore, based on the results of the current study the influence of PEs on adipogenisis in *in vivo* is unclear. To our knowledge little is known regarding the interaction between different PEs and RXR, and such information would be relevant to help with the interpretation of the observed effects of the PEs on adipocyte differentiation and lipid accumulation. Nevertheless, combined with future experiments the current result may add to our knowledge on what effects various EDCs can exert on different pathways playing a role in the development obesity..

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References

Auwerx, J. (1999) PPARgamma, the ultimate thrifty gene. *Diabetologia*, 42, 1033-1049.

Barak, Y., Nelson, M.C., Ong, E.S., Jones, Y.Z., Ruiz-Lozano, P., Chien, K.R., Koder, A. and Evans, R.M. (1999) PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol Cell*, 4, 585-595.

Beck, F., Plummer, S., Senior, P.V., Byrne, S., Green, S. and Brammar, W.J. (1992) The ontogeny of peroxisome-proliferator-activated receptor gene expression in the mouse and rat. *Proc Biol Sci*, 247, 83-87.

Blanck, H.M., Marcus, M., Rubin, C., Tolbert, P.E., Hertzberg, V.S., Henderson, A.K. and Zhang, R.H. (2002) Growth in girls exposed in utero and postnatally to polybrominated biphenyls and polychlorinated biphenyls. *Epidemiology*, 13, 205-210.

Boberg, J., Metzdorff, S., Wortziger, R., Axelstad, M., Brokken, L., Vinggaard, A.M., Dalgaard, M. and Nellemann, C. (2008) Impact of diisobutyl phthalate and other PPAR agonists on steroidogenesis and plasma insulin and leptin levels in fetal rats. *Toxicology*, 250, 75-81.

Brun, R.P., Tontonoz, P., Forman, B.M., Ellis, R., Chen, J., Evans, R.M. and Spiegelman, B.M. (1996) Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev*, 10, 974-984.

Cho, K.W., Lee, O.-H., Banz, W.J., Moustaid-Moussa, N., Shay, N.F. and Kim, Y.-C. (2010) Daidzein and the daidzein metabolite, equol, enhance adipocyte differentiation and PPAR[gamma] transcriptional activity. *The Journal of Nutritional Biochemistry*, 21, 841-847.

Collins, S. (2005) Overview of clinical perspectives and mechanisms of obesity. *Birth Defects Res A Clin Mol Teratol*, 73, 470-471.

Cooke, P.S. and Naaz, A. (2005) Effects of estrogens and the phytoestrogen genistein on adipogenesis and lipogenesis in males and females. *Birth Defects Res A Clin Mol Teratol*, 73, 472-473.

Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T. and Gore, A.C. (2009) Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev*, 30, 293-342.

Ferre, P. (2004) The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. *Diabetes*, 53 Suppl 1, S43-50.

Gladen, B.C., Klebanoff, M.A., Hediger, M.L., Katz, S.H., Barr, D.B., Davis, M.D. and Longnecker, M.P. (2004) Prenatal DDT exposure in relation to anthropometric and pubertal measures in adolescent males. *Environ Health Perspect*, 112, 1761-1767.

Gladen, B.C., Ragan, N.B. and Rogan, W.J. (2000) Pubertal growth and development and prenatal and lactational exposure to polychlorinated biphenyls and dichlorodiphenyl dichloroethene. *J Pediatr*, 136, 490-496.

Gregoire, F.M., Smas, C.M. and Sul, H.S. (1998) Understanding adipocyte differentiation. *Physiol Rev*, 78, 783-809.

Grun, F., Watanabe, H., Zamanian, Z., Maeda, L., Arima, K., Cubacha, R., Gardiner, D.M., Kanno, J., Iguchi, T. and Blumberg, B. (2006) Endocrine-disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. *Mol Endocrinol*, 20, 2141-2155.

Guerre-Millo, M., Gervois, P., Raspe, E., Madsen, L., Poulain, P., Derudas, B., Herbert, J.M., Winegar, D.A., Willson, T.M., Fruchart, J.C., Berge, R.K. and Staels, B. (2000) Peroxisome proliferator-activated receptor alpha activators improve insulin sensitivity and reduce adiposity. *J Biol Chem*, 275, 16638-16642.

Harmon, A.W. and Harp, J.B. (2001) Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. *Am J Physiol Cell Physiol*, 280, C807-813.

Hedelin, M., Klint, A., Chang, E.T., Bellocco, R., Johansson, J.E., Andersson, S.O., Heinonen, S.M., Adlercreutz, H., Adami, H.O., Gronberg, H. and Balter, K.A. (2006) Dietary phytoestrogen, serum enterolactone and risk of prostate cancer: the cancer prostate Sweden study (Sweden). *Cancer Causes Control*, 17, 169-180.

Hiromori, Y., Nishikawa, J.-i., Yoshida, I., Nagase, H. and Nakanishi, T. (2009) Structure-dependent activation of peroxisome proliferator-activated receptor (PPAR) [gamma] by organotin compounds. *Chemico-Biological Interactions*, 180, 238-244.

Homma, H., Kurachi, H., Nishio, Y., Takeda, T., Yamamoto, T., Adachi, K., Morishige, K., Ohmichi, M., Matsuzawa, Y. and Murata, Y. (2000) Estrogen suppresses transcription of lipoprotein lipase gene. Existence of a unique estrogen response element on the lipoprotein lipase promoter. *J Biol Chem*, 275, 11404-11411.

Howdeshell, K.L., Hotchkiss, A.K., Thayer, K.A., Vandenbergh, J.G. and vom Saal, F.S. (1999) Exposure to bisphenol A advances puberty. *Nature*, 401, 763-764.

Hu, E., Tontonoz, P. and Spiegelman, B.M. (1995) Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. *Proc Natl Acad Sci U S A*, 92, 9856-9860.

Inadera, H. and Shimomura, A. (2005) Environmental chemical tributyltin augments adipocyte differentiation. *Toxicol Lett*, 159, 226-234.

Issemann, I. and Green, S. (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*, 347, 645-650.

Karmaus, W., Osuch, J.R., Eneli, I., Mudd, L.M., Zhang, J., Mikucki, D., Haan, P. and Davis, S. (2009) Maternal levels of dichlorodiphenyl-dichloroethylene (DDE) may increase weight and body mass index in adult female offspring. *Occup Environ Med*, 66, 143-149.

Kirchner, S., Kieu, T., Chow, C., Casey, S. and Blumberg, B. (2010) Prenatal exposure to the environmental obesogen tributyltin predisposes multipotent stem cells to become adipocytes. *Mol Endocrinol*, 24, 526-539.

Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., Satoh, S., Nakano, R., Ishii, C., Sugiyama, T., Eto, K., Tsubamoto, Y., Okuno, A., Murakami, K., Sekihara, H., Hasegawa, G., Naito, M., Toyoshima, Y., Tanaka, S., Shiota, K., Kitamura, T., Fujita, T., Ezaki, O., Aizawa, S., Kadowaki, T. and et al. (1999) PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell*, 4, 597-609.

Le Bail, J.C., Champavier, Y., Chulia, A.J. and Habrioux, G. (2000) Effects of phytoestrogens on aromatase, 3beta and 17beta-hydroxysteroid dehydrogenase activities and human breast cancer cells. *Life Sci*, 66, 1281-1291.

le Maire, A., Grimaldi, M., Roecklin, D., Dagnino, S., Vivat-Hannah, V., Balaguer, P. and Bourguet, W. (2009) Activation of RXR-PPAR heterodimers by organotin environmental endocrine disruptors. *EMBO Rep*, 10, 367-373.

Liao, Q.C., Li, Y.L., Qin, Y.F., Quarles, L.D., Xu, K.K., Li, R., Zhou, H.H. and Xiao, Z.S. (2008) Inhibition of adipocyte differentiation by phytoestrogen genistein through a potential downregulation of extracellular signal-regulated kinases 1/2 activity. *J Cell Biochem*, 104, 1853-1864.

Longnecker, M.P. and Michalek, J.E. (2000) Serum dioxin level in relation to diabetes mellitus among Air Force veterans with background levels of exposure. *Epidemiology*, 11, 44-48.

Newbold, R.R., Padilla-Banks, E., Snyder, R.J. and Jefferson, W.N. (2005) Developmental exposure to estrogenic compounds and obesity. *Birth Defects Res A Clin Mol Teratol*, 73, 478-480.

Park, H.J., Yang, J.Y., Ambati, S., Della-Fera, M.A., Hausman, D.B., Rayalam, S. and Baile, C.A. (2008) Combined effects of genistein, quercetin, and resveratrol in human and 3T3-L1 adipocytes. *J Med Food*, 11, 773-783.

Rachon, D., Vortherms, T., Seidlova-Wuttke, D. and Wuttke, W. (2007) Effects of dietary equol on body weight gain, intra-abdominal fat accumulation, plasma lipids, and glucose tolerance in ovariectomized Sprague-Dawley rats. *Menopause*, 14, 925-932.

Rayalam, S., Della-Fera, M.A. and Baile, C.A. (2008) Phytochemicals and regulation of the adipocyte life cycle. *J Nutr Biochem*, 19, 717-726.

Rayalam, S., Della-Fera, M.A., Yang, J.-Y., Park, H.J., Ambati, S. and Baile, C.A. (2007) Resveratrol Potentiates Genistein's Antiadipogenic and Proapoptotic Effects in 3T3-L1 Adipocytes. *J. Nutr.*, 137, 2668-2673.

Relic, B., Zeddou, M., Desoroux, A., Beguin, Y., de Seny, D. and Malaise, M.G. (2009) Genistein induces adipogenesis but inhibits leptin induction in human synovial fibroblasts. *Lab Invest*, 89, 811-822.

Ribas-Fito, N., Gladen, B.C., Brock, J.W., Klebanoff, M.A. and Longnecker, M.P. (2006) Prenatal exposure to 1,1-dichloro-2,2-bis (p-chlorophenyl)ethylene (p,p'-DDE) in relation to child growth. *Int J Epidemiol*, 35, 853-858.

Ricketts, M.L., Moore, D.D., Banz, W.J., Mezei, O. and Shay, N.F. (2005) Molecular mechanisms of action of the soy isoflavones includes activation of promiscuous nuclear receptors. A review. *J Nutr Biochem*, 16, 321-330.

Rosen, E.D., Sarraf, P., Troy, A.E., Bradwin, G., Moore, K., Milstone, D.S., Spiegelman, B.M. and Mortensen, R.M. (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell*, 4, 611-617.

Rubin, B.S. and Soto, A.M. (2009) Bisphenol A: Perinatal exposure and body weight. *Mol Cell Endocrinol*, 304, 55-62.

Shen, P., Liu, M.H., Ng, T.Y., Chan, Y.H. and Yong, E.L. (2006) Differential effects of isoflavones, from Astragalus membranaceus and Pueraria thomsonii, on the activation of PPARalpha, PPARgamma, and adipocyte differentiation in vitro. *J Nutr*, 136, 899-905.

Taxvig, C., Elleby, A., Sonne-Hansen, K., Bonefeld-Jorgensen, E.C., Vinggaard, A.M., Lykkesfeldt, A.E. and Nellemann, C. (2010) Effects of nutrition relevant mixtures of phytoestrogens on steroidogenesis, aromatase, estrogen, and androgen activity. *Nutr Cancer*, 62, 122-131.

Tontonoz, P., Hu, E. and Spiegelman, B.M. (1994) Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell*, 79, 1147-1156.

Verhulst, S.L., Nelen, V., Hond, E.D., Koppen, G., Beunckens, C., Vael, C., Schoeters, G. and Desager, K. (2009) Intrauterine exposure to environmental pollutants and body mass index during the first 3 years of life. *Environ Health Perspect*, 117, 122-126.

Ying, C., Hsu, J.-T., Yang, C.-D., Hsung, S.-J., Wang, L.-K., Lin, C.-M. and Chen, L.-F.O. (2005) The Combination Effects of Phytoestrogens on Cell Proliferation, Cell Cycle Progression and Estrogen Receptor Alpha. *Journal of Food and Drug Analysis*, 13, 30-37.

Yoon, M. (2009) The role of PPARalpha in lipid metabolism and obesity: focusing on the effects of estrogen on PPARalpha actions. *Pharmacol Res*, 60, 151-159.

Yu, K., Bayona, W., Kallen, C.B., Harding, H.P., Ravera, C.P., McMahon, G., Brown, M. and Lazar, M.A. (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *J Biol Chem*, 270, 23975-23983.

Figure legends

Fig 1. - Overview of the different phytoestrogens and mixtures tested.
The selected PEs consists of both mother compounds (biochanin A, formononetin, secoisolariciresinol, metairesinol, lariciresinol, pinoresinol, and coumestrol) and metabolites (genistein, daidzein, equol, enterodiol and enterolactone). The different mixtures and the total mixture are presented in black boxes, and the compounds, which were (also) tested alone, are represented in light gray boxes.

Fig. 2 - Effect of the individual tested PEs and mixtures on lipid accumulation in 3T3-L1 maturing preadipocytes.
The 3T3-L1 preadipocytes were grown to confluence and induced to differentiate using standard MDI treatment as described in the material and method section. The control was cells supplemented with 0.1% DMSO instead of test compound. After exposing the cells to test compound for 6 days during the differentiation period the adipocytes were stained with Oil Red O, and the amount of lipid accumulation was analyzed by microscopic inspection and several phototropic images from each well were taken including five to twelve random fields images from each well photographed under phase contrast (magnification, x10). Images were analyzed in ImageJ and data are shown as the mean ± SD from one representative experiment out of three independent experiments.
* Statistically significantly different from control. ($P< 0.05$).

Fig. 3 - Effect of rosiglitazone and the PEs on rosiglitazone induced adipocyte differentiation.
The 3T3-L1 preadipocytes were induced to differentiate by standard MDI treatment in the presence of 0.5 μM rosiglitazone (rosi). The 3T3-L1 cells were exposed to 1 μM and 5 μM rosi (A) or to coumestrol, daidzein, genistein or mix 1 (0 -75 μM). The control was cells supplemented with 0.1% DMSO instead of test compound. After exposing the cells to test compound for 6 days during the differentiation period the adipocytes were stained with Oil Red O, and the amount of lipid accumulation was analyzed by microscopic inspection and several phototropic images from each well were taken including five to twelve random fields images from each well photographed under phase contrast (magnification, x10). Images were analyzed in ImageJ and data are shown as the mean ± SD from one representative experiment out of two independent experiments.
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Fig. 4 –Effect of the PPARγ antagonist GW9662 on the effect of TBT and genistein.

The 3T3-L1 preadipocytes were treated with 10 nM and 100 nM TBT (A) or different concentrations (μM) of genistein (B) either alone or in the presence of 1μM GW9662 as described in the material and method section. The control was cells supplemented with 0.1% DMSO or 0.1% DMSO plus 1μM GW9662, respectively. After 6 days exposure during the differentiation period the adipocytes were stained with Oil Red O and the amount of lipid accumulation was analyzed as previously described. Images were analyzed in ImageJ and data are shown as the mean ± SD from one representative experiment out of three independent experiments.

Statistically significantly different from control. ($P< 0.05$).

Fig.5 – Effect of genistein, rosiglitazone and TBT on 3T3-L1 differentiation

The 3T3-L1 cells were cultured in the 6-well plates to confluence and exposed to standard MDI induction media, and then during differentiation they were exposed to 100 nM TBT, 5 μM rosiglitazone or 25 and 75 μM of genistein. In the lowest panel the 3T3-L1 cells were exposed to standard MDI induction media in the presence of 0.5 μM rosiglitazone (+ 0.5 μM Rosi), and then exposed to 25 or 75 μM genistein during differentiation. The control was cells supplemented with DMSO instead of test compound. The assay was ended by staining the cells with Oil Red O and photomicrographs were taken at 10x magnification.

Fig. 6 – The effect of the PE mixtures and Coumestrol on PPAR α and γ transactivation .

To examine the effect of the different mixtures and coumestrol on ligand-induced PPAR transactication, NIH-3T3 cells were transiently transfected with expression plasmid for the ligand binding domain of either mouse PPARα or mouse PPARγ coupled to Gal4 and a plasmid containing an UAS linked luciferase reporter gene (UAS-TK-luc). Results are shown as activity obtained relative to the activity is wells containing media only (control = 100%).

Data represent mean ± SD of three independent experiments.

C = cytotoxic. * Statistically significantly different from control. ($P< 0.05$).

Table 1 – Information on test compounds – The phytoestrogens

Compounds	Mixture	MW	Purity (%)	Daily intake* (μg/day)	Proportion in mix	Proportion in the total-mix
Genistein	1	270,2	96	205	775	12385
Daidzein		254,2	98	133	533	8520
Equol		242,2	99	1	1	16
Formononetin	2	268,3	99	2	1	19
Biochanin A		284,3	99	1	1	18
Enterolactone	3	298,3	95	16	159	159
Enterodiol		301,2	95	0.06	1	1
Secoisolarici resinol	4	362,4	95	1871	252	81912
Matairesinol		358,4	95	49	1	325
Lariciresinol		360	95	621	6	1906
Pinoresinol		358,4	95	268	3	908
Coumestrol	coumestan	267	95	0.1	1	5

The table list the 12 phytoestrogens investigated in the current study with information on their molecular weight as well as their proportions in each of their mixtures.

* The daily intake is based on the amounts reported in the study by (Hedelin et al., 2006)

Table 2 – Overview of the effects found on PPAR activation and lipid accumulation

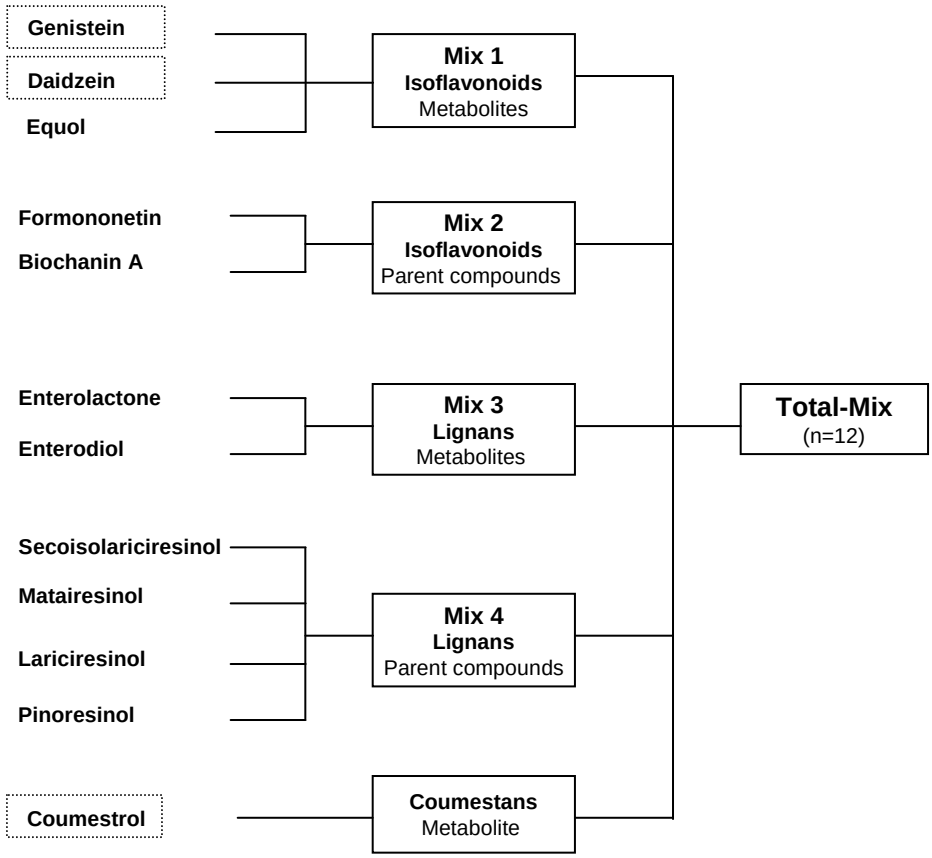
Compounds	PPARα transactivation	PPARγ transactivation	Effect on lipid accumulation
Mix 1	-	↑	↓
Mix 2 #	-	↑	↓
Mix 3	↓	-	↓
Mix 4	↓	-	-
Totalmix	-	↑	-
Coumestrol	-	-	↓
Genistein	ND(↑*)	ND (↑*)	↓
Daidzein	ND (↑*)	ND (↑*)	↓

- : no effect.

ND: not determined

cytotoxic at the highest (100 μM) concentration tested.

* Shown in Rickett et al., (2005)



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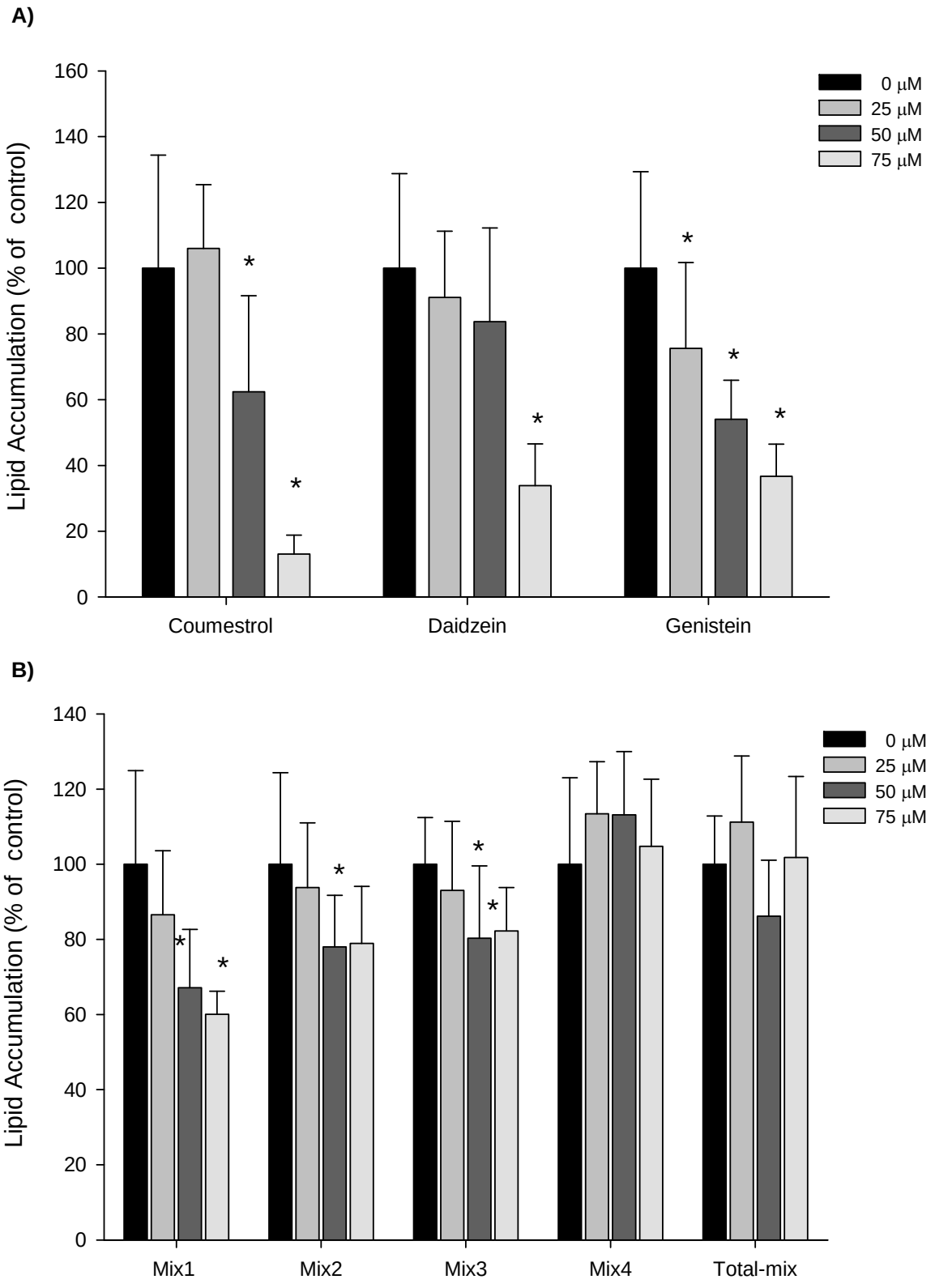

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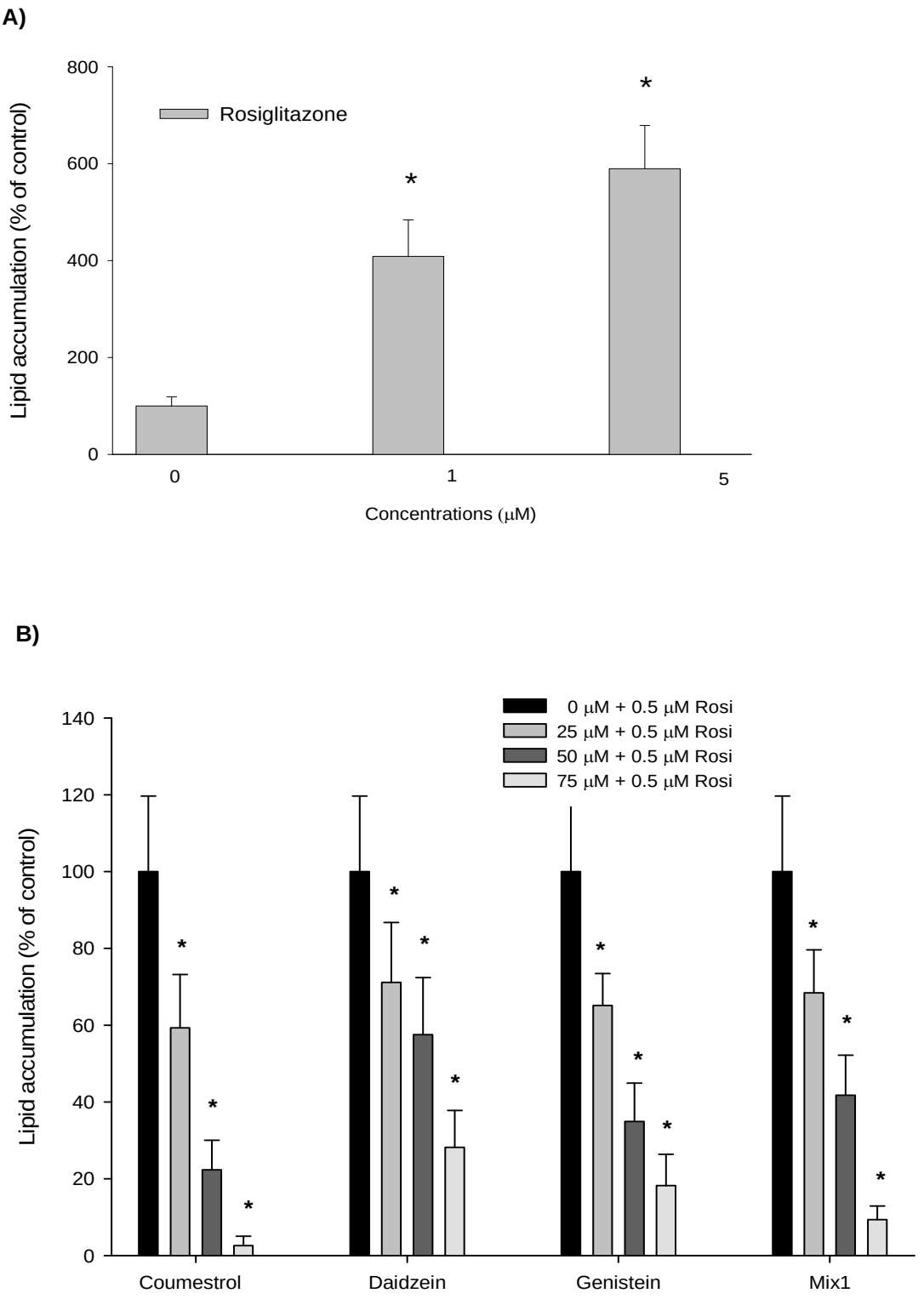

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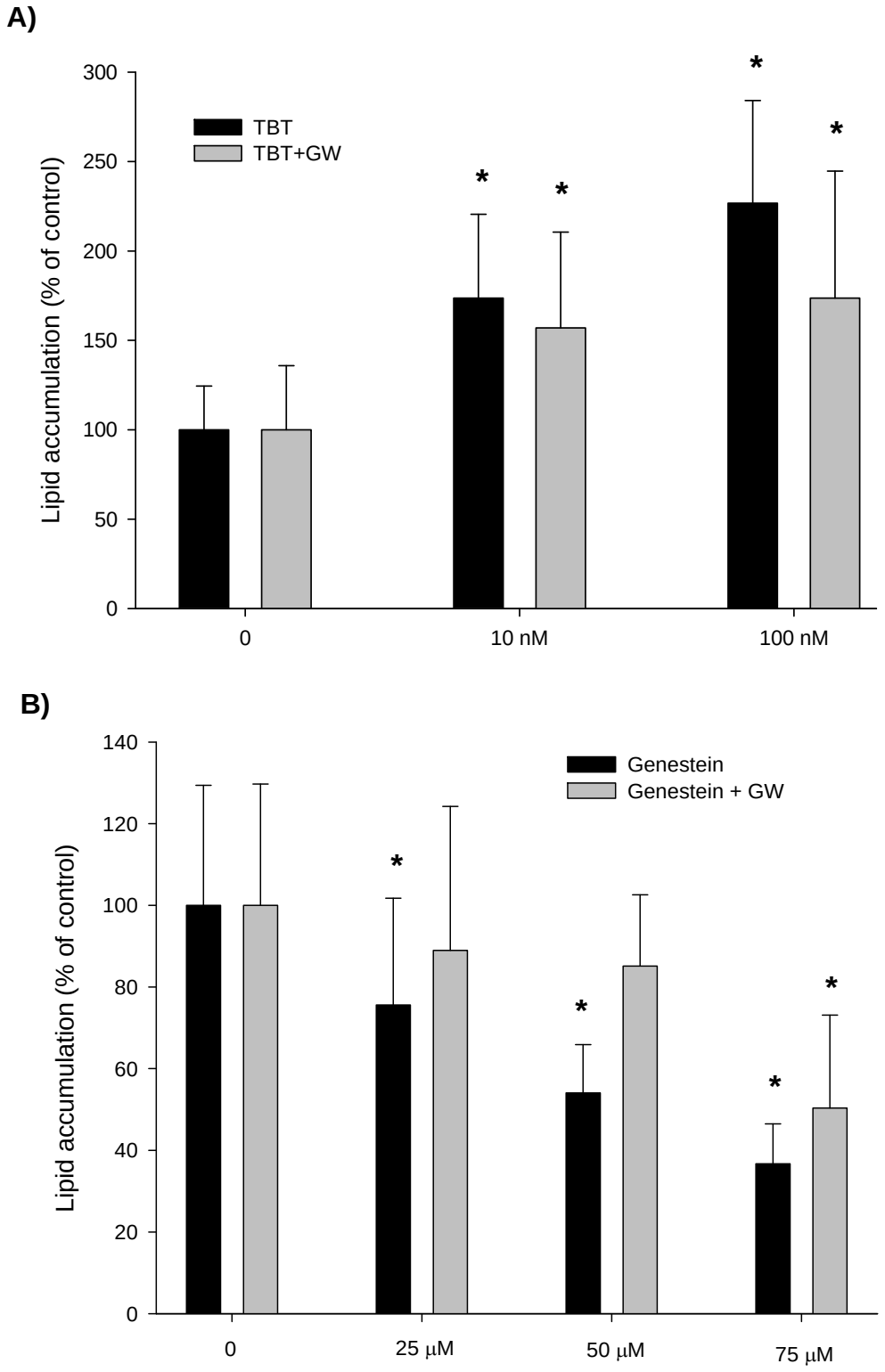

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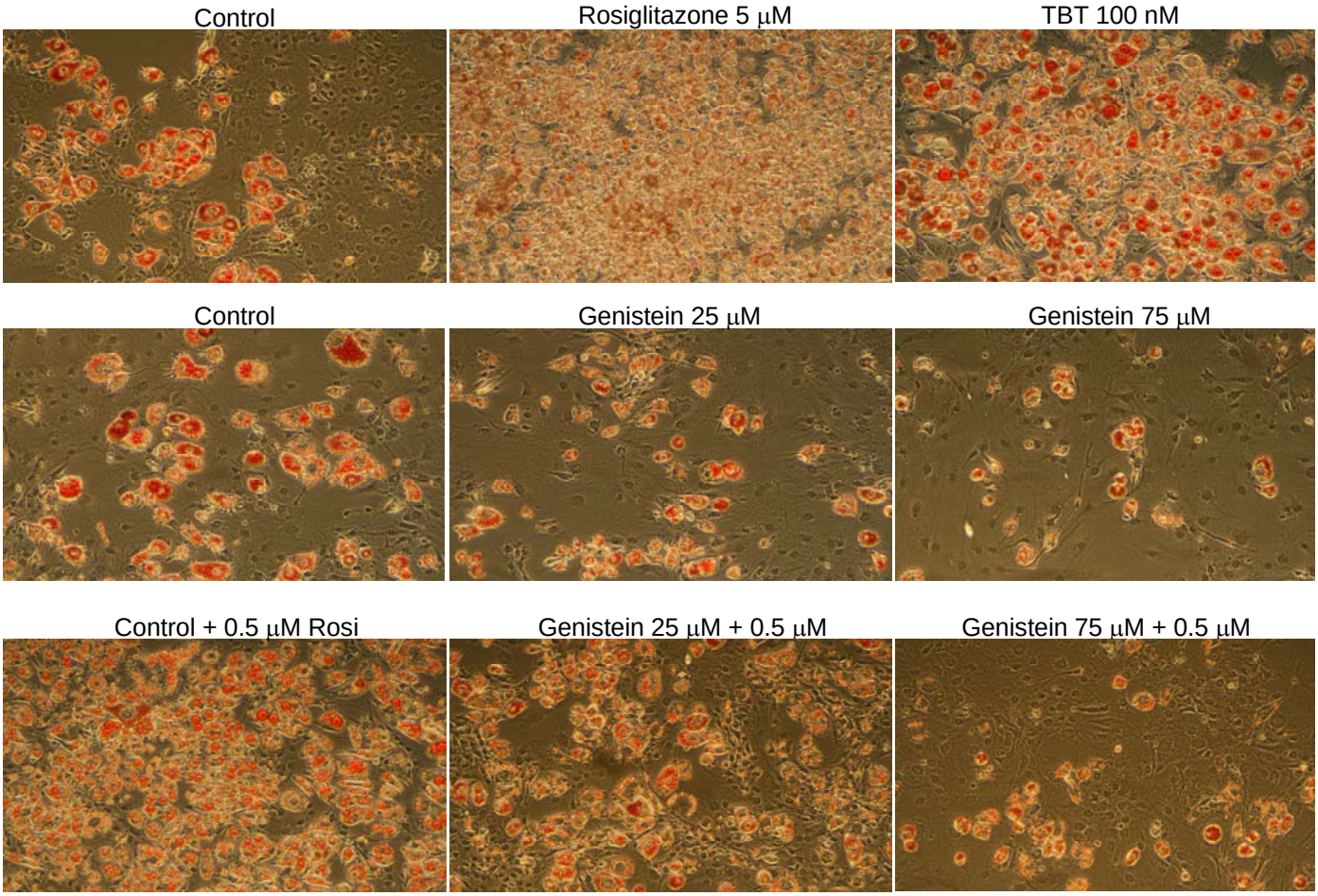

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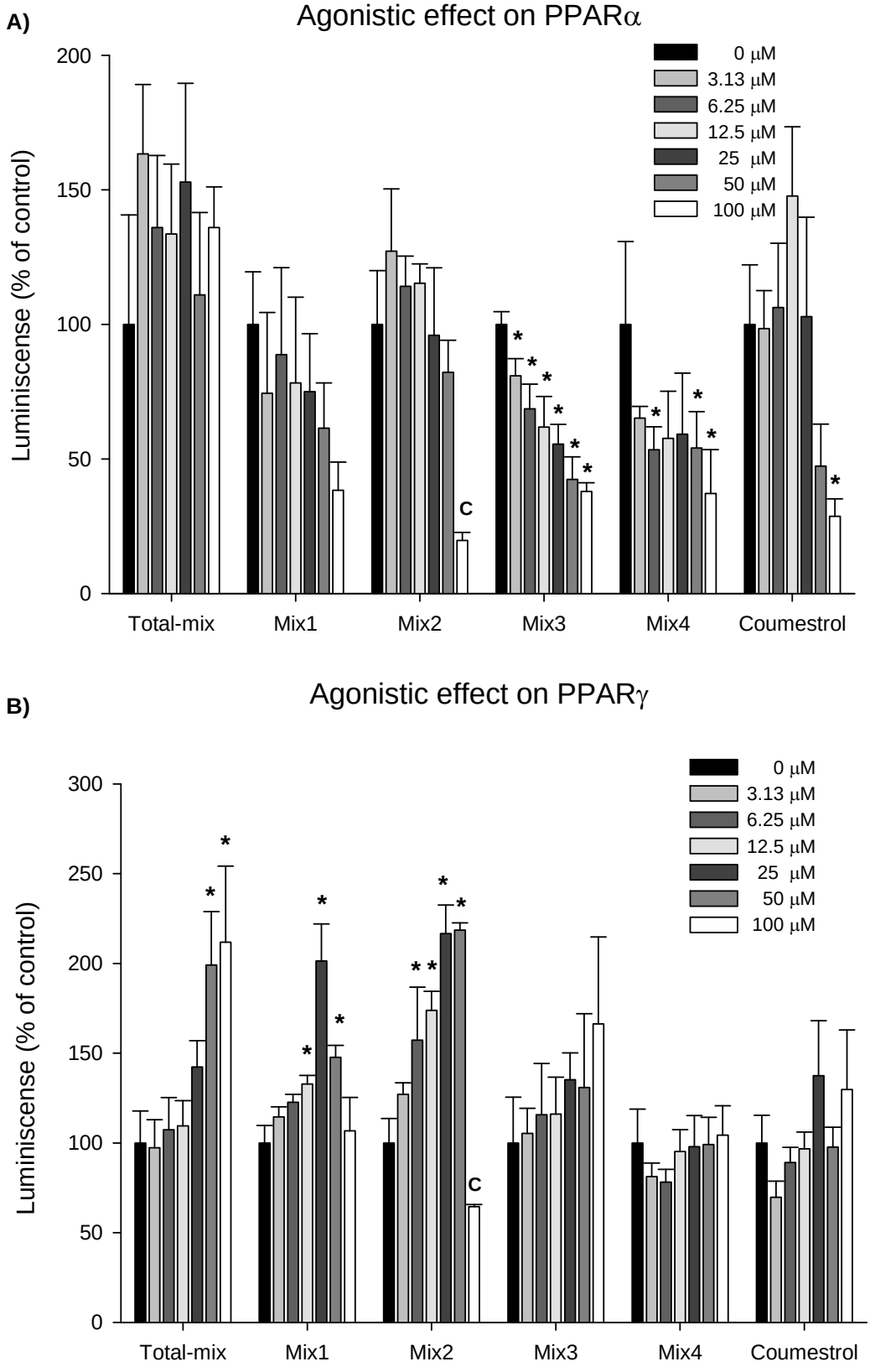

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Appendix 2 – Additional published papers not part of this thesis

Kusk KO, Krüger T, Long M, **Taxvig C**, Lykkesfeldt AE, Frederiksen H, Andersson AM, Andersen HR, Hansen KM, Nellemann C, Bonefeld-Jørgensen EC. Endocrine potency of wastewater: Contents of endocrine disrupting chemicals and effects measured by in vivo and in vitro assays. *Environ Toxicol Chem*; **2011** 30:413-26

Mia Birkhøj Kjærstad, **Camilla Taxvig**, Christine Nellemann, Anne Marie Vinggaard and Helle Raun Andersen. Endocrine disruptive effects in vitro of conazole antifungals used as pesticides and pharmaceuticals. *Reproductive Toxicology* **2010** Dec;30(4):573-82. .

Julie Boberg, **Camilla Taxvig**, Sofie Christiansen, and Ulla Hass. Possible endocrine disrupting effects of parabens and their metabolites. *Reproductive Toxicology* **2010**. 30(2), 301-312.

Mia Birkhøj Kjærstad, **Camilla Taxvig**, Helle Raun Andersen, and Christine Nellemann. Mixture effects of endocrine disrupting compounds *in vitro*. *Int J Androl*. **2010** Apr;33(2):425-33.

Pernille Reimer Hansen, **Camilla Taxvig**, Sofie Christiansen, Marta Axelstad, Julie Boberg Maria Kristina Kiersgaard, Christine Nellemann and Ulla Hass **(2009)**. Evaluation of endocrine disrupting effects of nitrate after in utero exposure in rats and of nitrate and nitrite in the H295R and T-screen assay. *Toxicol Sci* 108, 437-444.

Frederiksen, H., **Taxvig, C**., Hass, U., Vinggaard, A.M., and Nellemann, C. **(2008)**. Higher levels of ethyl paraben and butyl paraben in rat amniotic fluid than in maternal plasma after subcutaneous administration. *Toxicol Sci* 106, 376-383.

Camilla Taxvig, Anne Marie Vinggaard, Ulla Hass, Marta Axelstad, Julie Boberg, Pernille Reimer Hanseen, Hanne Frederiksen, and Christine Nellemann **(2008)**. Do parabens have the ability to interfere with steroidogenesis? *Toxicol Sci* 106, 206-213.

Karin S Hougaard, Keld A Jensen, Pernille Nordly, **Camilla Taxvig**, Ulla Vogel, Anne T Saber and Haakan Wallin **(2008)**. Effects of prenatal exposure to diesel exhaust particles on postnatal development, behavior, genotoxicity and inflammation in mice. *Part Fibre Toxicol* 5, 3.

Camilla Taxvig, Anne Marie Vinggaard, Ulla Hass, Marta Axelstad, Stine Metzdorff and Christine Nellemann **(2008)**. Endocrine-disrupting properties in vivo of widely used azole fungicides. *Int J Androl* 31, 170-177.

Camilla Taxvig, Ulla Hass, Marta Axelstad, Majken Dalgaard, Julie Boberg, Helle Raun Andersen and Anne Marie Vinggaard **(2007)**. Endocrine-disrupting activities in vivo of the fungicides tebuconazole and epoxiconazole. *Toxicol Sci* 100, 464-473