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ASSESSMENT OF ENDOCRINE ACTIVE SUBSTANCES USING IN SILICO AND REPORTER ASSAYS IN CELL AND MICE

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Riassunto

Negli ultimi decenni, sono aumentate le preoccupazioni scientifiche, il dibattito pubblico e l'attenzione dei media sui possibili effetti deleteri nell' uomo e nell' ambiente che possono derivare dall' esposizione ad alcune sostanze in grado di interferire con il sistema endocrino. I composti perturbatori, o interferenti, endocrini (EDC) comprendono una varietà di classi di sostanze, tra cui ormoni naturali e sintetici, costituenti vegetali, pesticidi, sostanze utilizzate nell'industria e nei prodotti di consumo, sostanze inquinanti. È ben documentato che i target degli EDC sono principalmente i recettori nucleari (NR) come i recettori estrogenici (ER) tipici degli ormoni sessuali. Questo recettore ER è coinvolto sia in un ampio spettro di processi fisiologici che avvengono in diversi organi sia in diverse malattie, come il cancro della mammella e dell'endometrio, l'osteoporosi e l'ipertrofia della prostata, le malattie neurodegenerative o l'attivazione del sistema immunitario. Le agenzie deputate alla protezione della salute umana e dell'ambiente stanno affrontando la necessità di indagare e chiarire le modalità di azione di questi EDC mediante lo sviluppo di metodi tossicologici alternativi, come il modello *in silico* ed i saggi in vitro utili col fine di prevedere la tossicità di questi EDC. Il mio progetto di ricerca ha avuto l'ambizioso obiettivo di sviluppare una strategia tossicologica integrata basata sulla combinazione delle informazioni disponibili sull' interferenza endocrina presenti in letteratura, sui saggi *in silico*, sulle metodologie di "imaging" applicate a sistemi di attivazione recettoriale in vitro, in vivo ed ex vivo con l'obiettivo di caratterizzare all'interno di un set di molecole quelle sostanze chimiche con attività di interferenza endocrina ma anche capaci di attivare altri percorsi tossicologici come l'infiammazione e lo stress ossidativo misurati nell'area

genitale maschile ed addominale di topi maschi. Le molecole selezionate variano da interferenti endocrini noti (DES) a sospetti (BPA) e comprendono composti sia sintetici (DEHP) che naturali (genisteina).

Il primo approccio utilizzato è stato l'analisi in *silico* con valutazione del possibile legame di queste sostanze al recettore alfa estrogenico per supportare l'ipotesi iniziale che l'attività ormonale di queste sostanze avviene attraverso un meccanismo recettoriale. Questo approccio è ampiamente utilizzato e fa anche parte del primo livello di indagine suggerito dall'EFSA / ECHA nel riconoscimento degli interferenti endocrini. La metodologia computazionale ha stimato diversi valori di affinità di ciascun ligando con il suo sito di legame nel recettore. L'uso di due diversi approcci (XP GLIDE SCORE e MMGBSA dG Bind) ha anche permesso di considerare la solvatazione. Ciò significa che il protocollo MMGBSA ha considerato l'interazione di alcune molecole di acqua con il sito di legame recettoriale ed anche l'interazione solvente-ligando. L'estradiolo ha mostrato i migliori valori di affinità in entrambi gli approcci, poiché essendo l'ormone endogeno, è stato in grado di contrastare l'effetto di solvatazione.

I valori della costante di dissociazione (K_i) calcolati a partire da XP GLIDE SCORE si sono adattati bene con il saggio di legame determinato sperimentalmente in vitro da altri gruppi di ricerca. Pertanto, il K_i calcolato è stato scelto come parametro per la predizione della possibile attività di perturbazione (o interferenza) endocrina (IE). Tuttavia, la mancanza di correlazione tra il K_i (calcolato e sperimentale) e l'attività IE osservata sperimentalmente in vivo (dati di letteratura) per tutte le sostanze chimiche (solo estradiolo e zearalenone hanno affinità simili a ER ed evidenze bibliografiche di attività di IE; genisteina ha buoni valori Ki ma non attività IE come BPA e metossicloro), non ha consentito la definizione di una priorità per l'attività di interferenza endocrina delle sostanze chimiche studiate attraverso il calcolo della loro affinità.

Nel passo successivo per verificare se il legame del recettore fosse ben correlato con l'attività ormonale, è stato eseguito il test in vitro di attivazione del recettore estrogenico, basato sulla capacità di un composto di stimolare l'attività trascrizionale estrogeno-dipendente nelle cellule di mammifero geneticamente modificate. Le linee cellulari usate sono cellule MCF-7 che esprimono il recettore estrogenico endogeno. Le cellule sono trasformate (transfettate) introducendo vettori contenenti sequenze di DNA per il recettore, insieme agli ERE (elementi che

rispondono all'estrogeno) legati a un gene "reporter" ed il gene "reporter" stesso. Il gene "reporter" utilizzato nelle cellule tumorali umane di solito codifica per l'enzima luciferasi (CALUX, espressione della luciferasi chimicamente attivata). Nel test di transattivazione gli EDC hanno mostrato la loro potenza estrogenica calcolata come EC50, rispetto al controllo positivo, il 17β-estradiolo. Questo sistema ci ha permesso di valutare la cinetica e le conseguenze biologiche dell'attivazione cellulare nello stesso strato di cellule mediante la cattura di immagini di bioluminescenza di emissioni di fotoni dell'attivazione recettoriale a 6 e 48 ore dai trattamenti iniziali. Da questo saggio in vitro sono stati presi in considerazione tre fattori quali potenza, efficacia e tendenza nel tempo. L'attivazione dinamica in vitro del recettore estrogenico ha mostrato che per alcune sostanze chimiche (genisteina, BPA, metossicloro), la potenza (EC50) e l'efficacia (induzione delle pieghe) sono cambiate nel tempo, ma non per altri (estradiolo, zearalenone e DES). Considerando che 17β-estradiolo, zearalenone e DES hanno certamente un'attività nell'animale e nell'uomo come interferenti endocrini, la durata del parametro effetto combinato con la potenza e l'efficacia si è mostrato utile nel predire l'attività ormonale. Insieme all'affinità del recettore e alla capacità di indurre una risposta biologica, esso è sembrato anche importante determinare quanto la risposta potesse essere sostenuta nel tempo. Di conseguenza, la combinazione della variazione della potenza e dell'efficacia, "normalizzata" rispetto ai valori di efficacia quantificati a 48 ore, è stata utilizzata con successo nella discriminazione di composti positivi e negativi per la loro attività di interferente endocrino. Per mezzo di questa analisi estradiolo, DES e zearalenone sono stati messi in cima alla lista (supportati anche dalla loro nota attività di IE), la genisteina è risultata rappresentare una presunta soglia di non-preoccupazione per l'effetto IE, in supporto dei dai di letteratura), mentre il metossicloro e il BPA non sono stati considerati priorità in termini di attività di IE. Questa classifica in vitro ha mostrato una correlazione con i risultati del modello in *silico*, poiché i composti più forti nel legarsi al recettore estrogenico sono stati classificati nelle prime posizioni (estradiolo, DES e zearalenone). Inoltre, non è stato possibile calcolare i valori di EC50s per il 4-nonilfenolo, DEHP e vinclozolin, di conseguenza non è stato possibile classificarli come IE, che totalmente in accordo con i risultati *in silico* ed in linea con i dati di letteratura (vinclozolin è principalmente un'antagonista androgenico). Il terzo approccio della nostra procedura è stato condotto per

verificare in vivo l'interazione delle sostanze chimiche selezionate con il recettore degli estrogeni ma anche l'attivazione di altri percorsi che innescano effetti primari nocivi. Abbiamo utilizzato tre topi transgenici progettati per valutare l'effetto dei composti nell'attivare il recettore estrogenico e/o causare stress ossidativo ed infiammazione. Abbiamo scelto di testare lo zearalenone (noto interferente endocrino e chiaramente identificato come tale dal nostro approccio in silico-in vitro) e il BPA per il quale esistono dati controversi in letteratura e che il nostro approccio ha già classificato come non IE. Nei nostri esperimenti, lo zearalenone ha dimostrato di essere attivo sulla via estrogenica nella zona addominale e ha attivato in modo significativo la via infiammatoria nei genitali (in questo caso specifico nella prostata, risultato di analisi di bioluminescenza ex vivo). Questi risultati sono risultati perfettamente in linea con i dati della letteratura, in cui l'infiammazione e metaplasia della prostata sono state rilevate sia nei topi che nei ratti. Il bisfenolo A non ha prodotto un'attivazione significativa in entrambe le aree e nell'analisi ex vivo, sempre in accordo con i nostri risultati in silico / in vitro.

La mancanza dell'attivazione del recettore estrogenico dell'area genitale da parte dell'estradiolo è stata probabilmente dovuta alla somministrazione orale del composto tramite l'acqua potabile. Questo composto è noto per esercitare potenti effetti avversi negli studi tossicologici quando somministrato per iniezione, ma la nostra intenzione è stata quella di indagare gli effetti IE per via orale che è considerata rilevante per l'esposizione umana. Quindi abbiamo usato una combinazione di approcci innovativi che hanno portato alla conclusione che lo screening in *silico* non può essere usato come procedura autonoma a causa della sua intrinseca mancanza di significato biologico, sebbene possa essere usato con successo come primo passo per la definizione delle priorità in un approccio di livello. Il secondo controllo obbligatorio per i risultati positivi in *silico* dovrebbe essere una procedura di valutazione in vitro, in cui l'affinità dei composti positivi viene misurata attraverso un test cellulare di riferimento. I nostri risultati hanno mostrato che integrando la variabile temporale nella valutazione della potenza e dell'efficacia, i composti testati possono essere classificati come IE o no-IE. Mentre l'esperimento in vivo ha evidenziato che un potente composto estrogenico, come lo zearalenone, potrebbe anche destare preoccupazione per l'attivazione di altre vie tossicologiche come quelle infiammatorie.

Siamo consapevoli che questa proposta di procedura debba essere valutata e validata su dozzine di molecole la cui attività in vivo è già nota, prima di arrivare al suo utilizzo per predire la possibile attività di IE di molecole sconosciute, ma è importante che questo approccio meriti di essere implementato.

Abstract

In the last two decades, there have been growing scientific concern, public debate, and media attention over the possible deleterious effects in humans and wildlife that may result from exposure to substances that have the potential to interfere with the endocrine system. Endocrine disrupting compounds (EDCs) encompass a variety of substance classes, including natural and synthetic hormones, plant constituents, pesticides, substances used in industry and in consumer products, pollutant. It is well documented that EDCs targets are mainly the nuclear receptors (NRs) such as the sexual hormones estrogen receptors ERs. ER is involved in a broad spectrum of physiological processes in different organs and tissues as well as in several diseases, such as breast and endometrial cancer, osteoporosis, and prostate hypertrophy, neurodegenerative diseases or in immune system activation. The regulatory agencies for the protection of human health and wildlife have been issuing the necessity to investigate and clarify the mode of action of these exogenous substances by the development of alternative toxicological methods such as in silico model and in vitro testing in order to predict the toxicity of these EDCs. This research had the ambitious aim to develop an integrated toxicological strategy based on the combination of available information of endocrine disrupting activity retrieved from the scientific literature, in silico model, imaging methodologies applied to reporter systems in vitro and in vivo and ex vivo to predict among a set of chemicals those with an endocrine disrupting activity or ability to activate other toxicological pathways such as inflammation and oxidative stress measured in the male reproductive organs and in the genital and abdominal area of mice. The

selected molecules range from known (DES) to suspected (BPA) endocrine disruptors and included both synthetic (DEHP) and natural (genistein) compounds. The first step used was in silico analysis with evaluation of the possible binding of selected substances to the estrogen alpha receptor to support the hypothesis that their hormonal activity occurred through a receptorial mechanism. This approach is commonly used and is also part of the first level of investigation suggested by EFSA/ECHA in the recognition of EDCs. The computational methodology estimated different values of affinity of each ligand to hER Ligand Binding Domain (LBD). The use of two different approaches (XP GLIDE SCORE and MMGBSA dG Bind) also allowed for solvation to be taken into account. That meant that MMGBSA protocol considered both the interactions of some water molecules with the LBD and the solvent-ligand ones. The estradiol showed the best affinity values in both approaches as being the endogenous hormone was able to contrast the solvation effect. The dissociation constant (K_i) values calculated from the XP GLIDE SCORE fitted well with the K_i experimentally determined in vitro binding assay by other research groups. Thus, the computed K_i has been chosen as parameter for the prediction of putative endocrine disruptor activity. However, the lack of correlation between the (computed and experimental) K_i and in vivo experimental observed ED activity (from literature data) for all chemicals (only estradiol and zearalenone have similar affinity to ER and literature evidences of ED activity; genistein has a good K_i values but not ED activity such as BPA and methoxychlor), did not allow a prioritization of the investigated chemicals for ED activity through the results of their affinity. In the next step, to check if the receptor binding well correlated with the hormonal activity, the ER Reporter gene assay was performed, based on the ability of a compound to stimulate ER-dependent transcriptional activity in genetically engineered mammalian cells. The cell lines are MCF-7 cells which express human endogenous ERα. The cells are transformed (transfected) by introducing vectors containing DNA sequences for the receptor, along with EREs linked to a reporter gene, and the reporter gene itself. The reporter gene used in human cancer cells usually codes for luciferase (CALUX, chemically activated luciferase expression). In the transactivation the EDCs show their estrogenic potency calculated as EC50, in respect to the positive control, 17β-estradiol. This system has enabled us to evaluate the kinetic and the biological consequences of cellular activation in the same cell monolayer by bioluminescence

imaging of photon emissions that were pictures of activated ER status at 6 and 48 hours after the initial treatments. From this in vitro assay three factors were taken into consideration, power, efficacy and trend over time. The in vitro dynamic ER activation showed that for some chemicals (genistein, BPA, methoxychlor), the potency (EC50) and the efficacy (fold induction) changed over time, but not for others (estradiol, zearalenone and DES).

Considering that estradiol, zearalenone and DES certainly have an activity in the animal and in man as endocrine disruptors, the duration of effect parameter combined with power and efficacy were likely to be associated in predicting the activity. Together with receptor affinity and the ability to induce a biological response, it also seemed relevant how long the response was lasting. By consequence, the combination of the variation of the potency response and the efficacy, "normalized" respect to the efficacy values quantify at 48 hours, was successfully used in discriminating positive and negative compounds for their endocrine disrupting activity. By means of this analysis 17β-estradiol, DES and zearalenone were put at the top of the list (also supported by their known ED activity), genistein resulted to represent a putative threshold of no-concern for ED effect, in supporting published data, while methoxychlor and BPA were definitely not considered a priority in terms of ED activity. This in vitro classification fitted well with the *in silico* outcomes, since the strongest estrogen receptor binders were ranked in the first positions (17β-estradiol, DES and zearalenone). Besides, was no possible to calculate EC50s for 4-nonylphenol, DEHP and vinclozolin, not making possible to classify them as ED, totally in agreement with in silico results and in line with literature data (vinclozolin is mainly an androgen antagonist). The third step of our stepwise approach was intended to verify in vivo the interaction of selected chemicals with the estrogen receptor and in addition the activation of their pathways triggering primary harmful effects. We used three reporter mice designed in order to evaluate the effect of selected compounds to activate ER and causing oxidative stress and inflammation. We have chosen to test zearalenone (well-known endocrine disruptor and clearly identified as such by our in silico-in vitro approach), and BPA for which there are controversial data in the literature and that our approach has negatively classified as ED. In our experiments zearalenone showed to be active on ER pathway in the abdominal area and significantly activated the inflammatory pathway in the genitals (in this specific case in the prostate, result of

ex vivo bioluminescence analysis). These results were perfectly in line with the literature reports, in which prostate inflammation and metaphase were detected in both mice and rats. Bisphenol A did not produce a significant activation in both the areas and in the ex vivo analysis, again in agreement with in silico/in vitro results.

We used a combination of innovative approaches that led to a conclusion that in silico screening cannot be used as a stand-alone procedure due to its intrinsic lack of biological meaning, although it can be successfully used as a first prioritizing step in a tier approach. The second mandatory check for the in silico positive hits should be an in vitro evaluation procedure, in which the affinity of the positive hits is measured through a reference cellular assay. Our results showed that integrating the time variable in the evaluation of the potency, the tested compounds could be classified as ED or no-ED. The in vivo experiment highlighted that a potent estrogenic compound, as zearalenone, could also raise concern for the activation of other toxicological pathway such as the inflammatory ones.

We are aware that this indication of procedure must be evaluated and validated on dozens of molecules whose in vivo activity is already known before arriving at its use to predict the possible activity of ED of unknown molecules, but we think that this approach deserves to be implemented.

Chapter 1

1.1 Introduction

In the last two decades, there have been growing scientific concern, public debate, and media attention over the possible deleterious effects in humans and wildlife that may result from exposure to substances that have the potential to interfere with the endocrine system. The endocrine system is a communication system that maintains normal physiological balance across multiple organ systems. It accomplishes this by modulating or regulating the activity of almost everybody system in reaction to variations in body temperature, activity level, stress, and circulating levels of nutrients and hormones required for growth, reproduction, and metabolism. Substances which interfere with the endocrine system are endocrine modulators or endocrine disruptors, depending on the final effect on the whole organism. The latter term has been defined in Weybridge in the 1996 and has been slightly modified by WHO in the 2002: ‘‘An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations’’. Endocrine disrupting compounds (EDCs) encompass a variety of substance classes, including natural and synthetic hormones, plant constituents, pesticides, substances used in industry and in consumer products, and other industrial by-products and pollutants [1, 2]. The target of EDCs is mainly the nuclear receptors (NRs) such as the sexual hormones receptors (estrogen receptors, ERs and androgen receptors, ARs). These are ligand-activated transcriptional factors belonging to the super family of nuclear receptors. ERs are present as two receptor subtypes, ERα and ERβ [3]. ERs mediate a broad spectrum of physiological processes in different organs and tissues as well as a range of diseases, such as breast and endometrial cancer, osteoporosis, and prostate hypertrophy. Recently, estrogens and their receptors have also been implicated in cardiovascular and central nervous system disorders [4] or in immune system activation [5]. The regulatory agencies for the protection of human health and wildlife have been issuing the necessity to investigate and clarify the mode of action of these exogenous substances by the development of

alternative toxicological methods such as in silico model and in vitro testing in order to predict the toxicity of these EDCs to human health and wildlife.

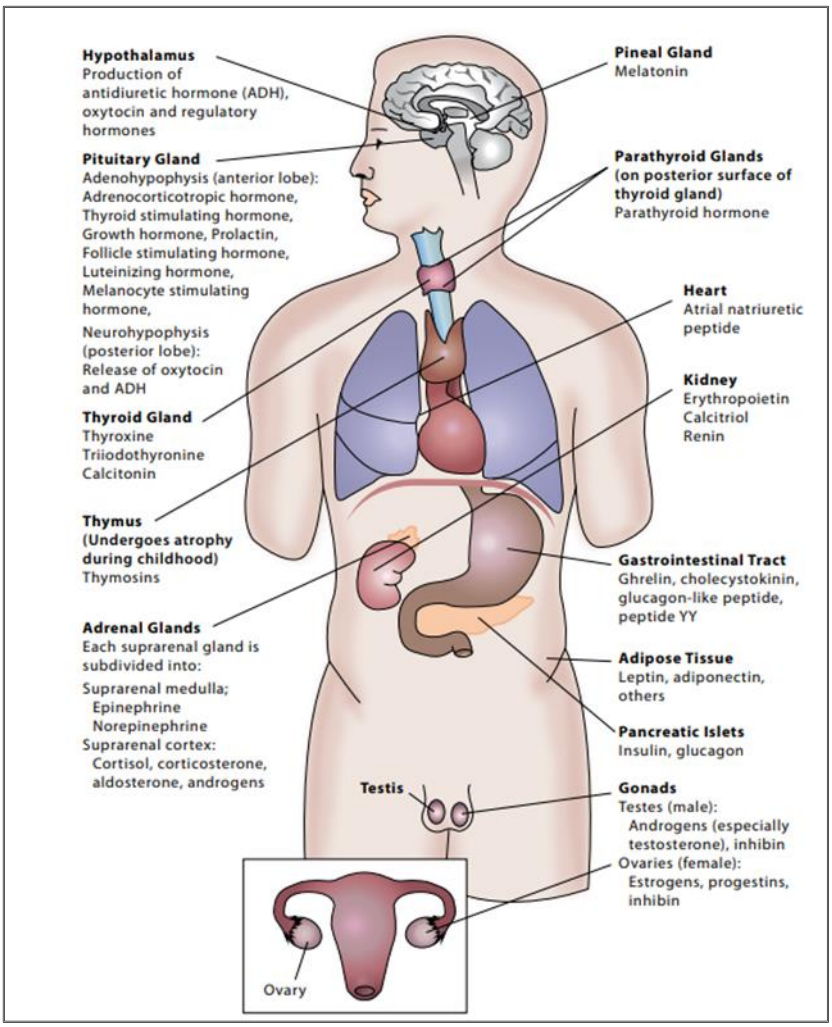
1.2 Endocrine system: hormones & receptors

The endocrine system represents a complex network system of glands, hormones and receptors, which play a critical function by maintaining, for example, the normal physiological balance, growth, reproduction, development, metabolism [2]. It acts through the actions of molecules called hormones which are produced by endocrine glands such as pituitary gland at the base of the brain, the thyroid gland in the neck, the adrenal glands in the abdominal next to the kidneys, the gonads and certain parts of the pancreas (Figure 1). The hormones are grouped in four structural classes (Table 1): protein and peptide hormones such insulin; steroids that derive from cholesterol (Figure 2) as estrogen, testosterone, mineralocorticoids, vitamin D; tyrosine or tryptophan derivates such catecholamines, thyroid hormones, serotonin; eicosanoid as prostaglandins, prostacyclin, thromboxane [6].

These hormones (Table 1) are chemical messengers that travel through the bloodstream to produce effects on distant cells and tissues.

For example, reproductive hormones, steroids (estrogens, androgens, progestins) and proteins (LH and FSH) control the complex reproduction physiological processes, the thyroid hormones (TSH, T3 and T4) are involved in the metabolic processes then in the appetite and body weight regulation and brain development. In addition to their actions on these physiological processes, all hormone systems are governed in such way so that hormone levels are at the appropriate concentration in blood to be effective at physiological process [2].

Figure 1. Overview of endocrine system and hormones. Figure from the report "State of the Science of Endocrine Disrupting Chemicals – 2012" [2]



Hormones exert their activities by specific interaction with some proteins called receptors which have a widespread expression in all body tissues or outside - inside the cells.

Generally, hormones act at very low concentrations because of its specificity and selectivity to bind to receptor while at higher concentration could cause a downregulation of receptor number [2; 7]. In addition to this mechanism, higher concentration of hormones can cause increased of cytotoxicity of receptor expression with the consequence of low hormonal response. For example, the MCF7 breast cancer cell lines proliferate in response to estrogen until the high doses (10^{-5} – 10^{-4} M) produce cytotoxicity. The same toxicity has been observed in a

subpopulation of MCF7 cells that no longer express the estrogen receptor, suggesting that 17β-estradiol (the natural endogenous estrogen) has not an endocrine effect at high doses, but it has a general toxicity [2].

Most of these receptors belongs to nuclear receptor family. Nuclear receptors (NRs) are members of a large superfamily of evolutionarily related transcription factors that control a plethora of biological processes. are modular proteins organized into three major functional domains, namely (i) a variable and intrinsically unfolded N-terminal A/B domain harboring the transcriptional activation function 1 (AF-1), (ii) a conserved DNA-binding domain (DBD), and (iii) a C-terminal ligand-binding domain (LBD) hosting the activation function 2 (AF-2) (Figure 3) [3; 8].

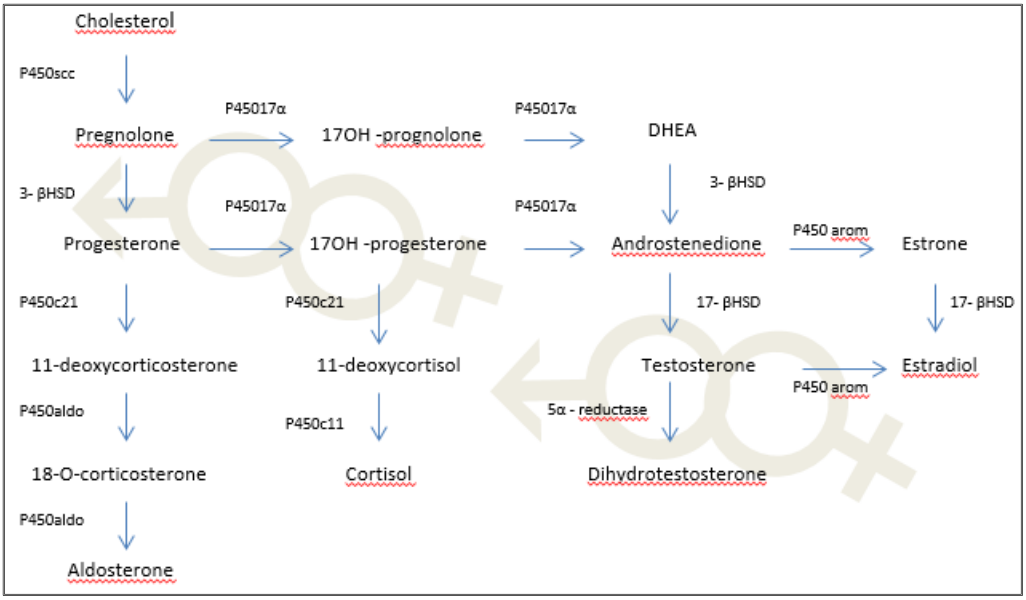


Figure 2. Pathways for the synthesis of human sex and adrenal steroids under the specific enzyme regulation common to both sexes. P450scc = Cholesterol side-chain cleavage enzyme; 3-βHSD = 3β-Hydroxysteroid dehydrogenase/Δ5-4 isomerase; P45017α = 17 alpha-hydroxylase = 17, 20 lyase; 17- β HSD = 17β-Hydroxysteroid dehydrogenases; P450c21 = 21-hydroxylase; P450aldo = aldosterone synthase; P450c11 = Steroid 11β-hydroxylase; P450arom = aromatase; 5α-reductase.

1.3 Estrogen receptor

Estrogens like other steroid hormones, are naturally occurring cyclopentanophenanthrene compounds whose synthesis begins with cholesterol. The most potent and dominant estrogen in humans is 17β-estradiol (E2), but lower levels of the estrogens estrone and estriol are also present. 17β-Estradiol (E2) controls many aspects of human physiology, including development, reproduction and homeostasis, through regulation of the transcriptional activity of its cognate receptors (ERs) [4; 9]. The estrogen receptors exist in two subtypes, ERα and ERβ (Figure 4). ERs mediate a broad spectrum of physiological effects in different organs and tissues and are involved in a range of diseases, such as breast and endometrial cancer, osteoporosis, and prostate hypertrophy. Recently, estrogens and their receptors have also been implicated in cardiovascular and central nervous system disorders [4] or in immune system activation [5]. The biological cascade of events reacted by interaction with a ligand (xenobiotics or endogenous substances) causes that NRs undergoes dimerization and translocation to the respective HRE in the nucleus. Then transcriptional activity follows, as modulated by co-regulators, through recruitment of transcription factors and RNA polymerase to the initiation site. Thus, there is the synthesis of mRNA which released into cytoplasm (Figure 3) [8].

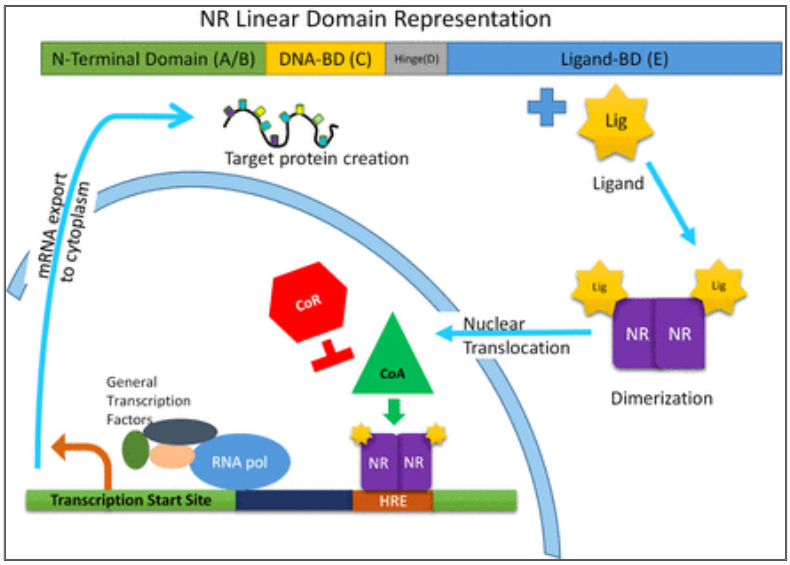


Figure 3. Depicted schema representing structural domains generally harbored by NRs, in addition to their canonical downstream functionality. Upon binding to activating ligand, NRs undergo dimerization and translocation to the respective HRE in the nucleus. Transcriptional activity follows, as modulated by co-regulators, through recruitment of transcription factors and RNA polymerase to the initiation site. CoR, corepressor; Lig, activating ligand; RNA pol, RNA polymerase; general regions shared by all NRs labeled in parentheses (A–E) within domains (from El Hokayem et al., 2017 [8]).

ERα and ERβ synthesis are under the transcriptional activity of two separate genes, ESR1 and ESR2, respectively, present on distinct chromosomes: locus 6q25.1 and locus 14q23-24.1, respectively (Figure 4). Both receptors have a different widespread tissue distribution. ER α is mainly expressed in uterus, ovary, prostate, testis (Leydig cells), epididymis, brain, thymus, bladder, kidney, liver. ER β is predominantly distributed colon, prostate (epithelium), testis, ovary (granulosa cells), bone marrow, salivary gland, vascular endothelium, and certain regions of the brain [4; 9; 10].

The ERα subunit is largely studied as pharmaceutical target [11; 12] and in toxicological field to understand the mechanism of action of endocrine disruptor compound (EDCs) [2; 10; 13].

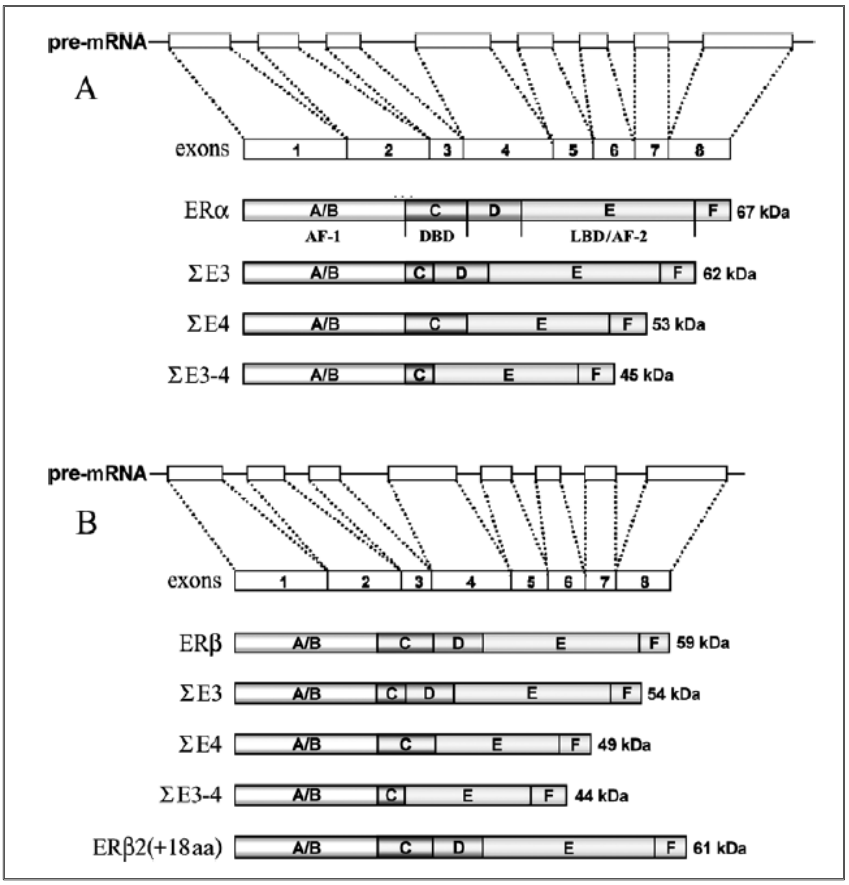


Figure 4. Genomic format, domain structure, and common splice variants of human ERα (A) and ERβ(B). Both ERα and ERβ gene are comprised of eight exons interrupted by long introns. The common splice variants of both ERa and ERb exhibit in-frame deletion of exon 3 (R E3), exon 4 (R E4) or both (R E3-4), with an exception of intron coding insertion, ERb2, which bears 18 amino acids insertion in the LBD compared to full-length ERb (from Ascanzi et al., 2006 [9]).

1.4 Endocrine disruptors: definition

Interaction of chemicals with endocrine system may lead to changes that are transient, where the control mechanisms of endocrine systems will compensate for chemically-induced effects; or may cause permanent changes that may then lead to adverse effects. In the latter situation, a chemical may be an endocrine disruptor (ED). By consequence, substances which interfere with the endocrine system are endocrine modulators or endocrine disruptors, depending on the final effect on the whole organism. The latter term has been defined in Weybridge in the 1996 [1] and has been slightly modified by WHO in the 2002: ‘‘An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or

(sub)populations'' [2]. This definition refers to different group of chemicals such as home-made substance, pesticide, synthetic hormones, plant constituents, pesticides, pollutants.

EDCs can affect the endocrine systems and cause adverse effects by several mechanisms:

- by mimicking the biological activity of an endogenous hormone by binding to a cellular receptor, leading to an unwanted response by initiating the cell's normal response to the naturally occurring hormone at the wrong time or to an excessive extent (agonistic effect);
- by binding to the receptor without any activation and blocking the binding of the natural hormone (antagonistic effect);
- by binding to transport proteins in the blood, thus altering the amounts of natural hormones that are present in the bloodstream;
- by interfering with the metabolic processes in the body, affecting the synthesis or breakdown rates and release of the natural hormones [14]; EDCs exert mainly their activity towards the interaction with nuclear receptor [2].

1.5 Biological implication of endocrine disruptor compounds

The EDCs show several characteristics:

- act at low doses
- bioaccumulate
- produce a non-linear dose response curve, high dose effects are not same as low dose.

The effect is always dependent on mechanism of action, the target tissue and the specific life - stage of exposure (Figure 5) [2].

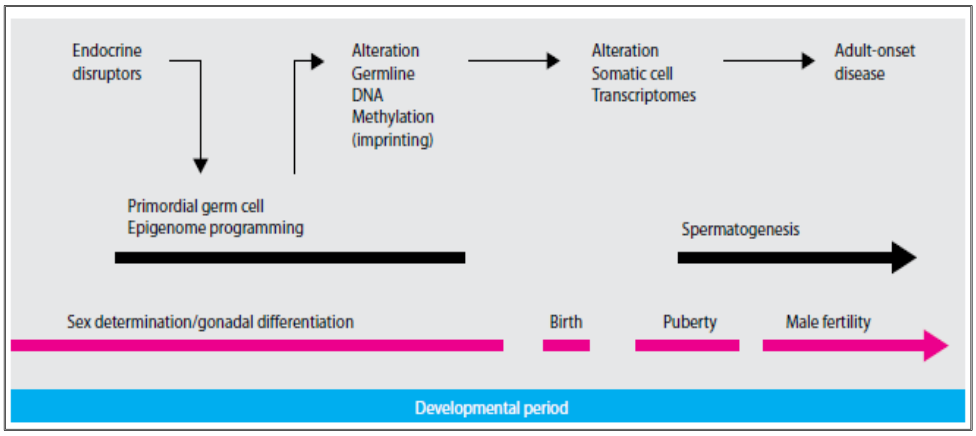


Figure 5. Example of EDCs mechanism and exposure window (figure from [2])

It is well documented that animals and humans exposed in utero or perinatally to the potent synthetic estrogen diethylstilbestrol (DES), developed reproductive adversity (e.g., reduced sperm counts, reproductive tract malformations), tumours to reproductive tract, and other endocrine-related endpoints [15; 16].

Chemicals that modulate endocrine signaling pathways are widespread in the environment or they occur naturally (e.g., phytoestrogens), and they are synthetically produced for a variety of uses, including pesticides in agriculture, polymers used in food packaging materials, etc... Despite the evidence of correlation between the exposure to pharmacological doses of DES during the critical developmental time points and the adverse effects in humans has been clearly defined, the evidence between the exposure to low concentration of a weaker hormonally active chemical and the toxicity in humans has not been clearly documented [17].

1.6 Regulatory perspective and toxicological characterization (in silico, in vitro and in vivo testing)

Regulatory bodies for the protection and safety of consumer health and environment such as ECHA, EFSA, EPA etc. have been facing the endocrine disruptor problem by issuing severe regulations. Indeed, according to Regulation EC No. 1107/2009 relative to Plant Protection Product, no PPP could be place on the market if "An active substance, safener or synergist shall only be approved if, on the basis of the assessment of Community or internationally agreed test guidelines or other available data and information, including a review of the scientific literature,

reviewed by the Authority, it is not considered to have endocrine disrupting properties that may cause adverse effect in humans, unless the exposure of humans to that active substance, safener or synergist in a plant protection product, under realistic proposed conditions of use, is negligible, that is, the product is used in closed systems or in other conditions excluding contact with humans and where residues of the active substance, safener or synergist concerned on food and feed do not exceed the default value set in accordance with point (b) of Article 18(1) of Regulation (EC) No 396/2005".

Under the Regulations No 1907/2006 REACH (Registration, Evaluation and Authorization of Chemicals) a chemical identified as endocrine disruptor activity is ruled out by the article 57 (f) of SVHC.

To identify this toxicological profile ECHA and EFSA have issued a guidance "Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009" based also on OECD (Organization for Economic Co-operation and Development) framework "Guidance Document 150 on Standardized Test Guidelines for Evaluating Chemicals for Endocrine Disruption, revised 2018" [18;19] (Table 1).

Table 1. Conceptual framework for testing and assessment of endocrine disrupters [19]

Mammalian and Non-Mammalian Toxicology	
Level 1 **Existing data** • **Physical & chemical properties, e.g., MW reactivity, volatility, biodegradability.** • **All available (eco)toxicological data from standardized or non-standardized tests.** • **Read across, chemical categories, QSARs and other *in silico* predictions, and ADME model predictions.**	
Level 2 **In vitro assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non-mammalian methods)** • **Estrogen or androgen receptor binding affinity (OECD TG 493).** • **Estrogen receptor transactivation (OECD TG 455 & TG 457).** • **Androgen transactivation assay (OECD TG 458).** • **Steroidogenesis in vitro (OECD TG 456).** • **MCF-7 cell proliferation assays (ER ant/agonist).** • **Other assays as appropriate.**	
Mammalian and Non-Mammalian Toxicology	**Mammalian and Non-Mammalian Toxicology**
Level 3	

In vivo assays providing data about selected endocrine mechanism(s) / pathway(s)

- **Uterotrophic assay (OECD TG 440).**
- **Hershberger assay (OECD TG 441)**
- **Xenopus embryo thyroid signaling assay (When/if TG is available).**
- **Amphibian metamorphosis assay (OECD TG 231).**
- **Fish Reproductive Screening Assay (OECD TG 229).**
- **Fish Screening Assay (OECD TG 230).**

Androgenized female stickleback screen (OECD).

Level 4

In vivo assays providing data on adverse effects on endocrine relevant endpoints

- **Repeated dose 28-day study (OECD TG 407).**
- **Repeated dose 90-day study (OECD TG 408).**
- **1-generation reproduction toxicity study (OECD TG 415).**
- **Male pubertal assay (see GD 150, Chapter C4.3).**
- **Female pubertal assay (see GD 150, Chapter C4.4).**
- **Intact adult male endocrine screening assay (see GD 150, Chapter Annex 2.5).**
- **Prenatal developmental toxicity study (OECD TG 414).**
- **Chronic toxicity and carcinogenicity studies (OECD TG 451-3).**
- **Reproductive screening test (OECD TG 421).**
- **Combined 28-day/reproductive screening assay (OECD TG 422).**
- **Developmental neurotoxicity (OECD TG 426).**
- **Fish sexual development test (OECD TG 234).**
- **Fish Reproduction Partial Lifecycle Test (when/If TG is Available).**
- **Larval Amphibian Growth & Development Assay (OECD TG 241).**
- **Avian Reproduction Assay (OECD TG 206).**
- **Mollusc Partial Lifecycle Assays (OECD TG 242 & TG 243).**
- **Chironomid Toxicity Test (TG 218 & TG 219).**
- **Daphnia Reproduction Test (with male induction) (OECD TG 211).**
- **Earthworm Reproduction Test (OECD TG 222).**
- **Enchytraeid Reproduction Test (OECD TG 220).**
- **Sediment Water Lumbriculus Toxicity Test Using Spiked Sediment (OECD TG 225).**
- **Predatory mite reproduction test in soil (OECD TG 226).**
- **Collembolan Reproduction Test in Soil (OECD TG 232).**

Level 5

In vivo assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism

- **Extended one-generation reproductive toxicity study (OECD TG 443).**
- **2-Generation reproduction toxicity study (OECD TG 416).**
- **FLCTT (Fish LifeCycle Toxicity Test) (when TG is available).**
- **Medaka Extended One Generation Reproduction Toxicity Study Test (OECD TG 240).**
- **Avian 2 generation reproductive toxicity assay (when TG is available).**
- **Mysid Life Cycle Toxicity Test (when TG is available).**
- **Copepod Reproduction and Development Test (when TG is available).**
- **Sediment Water Chironomid Life Cycle Toxicity Test (OECD TG 233);**
- **Mollusc Full Lifecycle Assays (when TG is available).**
- **Daphnia Multigeneration Assay (if TG is available).**

1.7 3Rs in hazard assessment

Furthermore, Regulatory agencies worldwide and scientific research stakeholders as European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) or Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) have also supporting the development of new strategies in the chemical risk assessment based on alternative toxicological testing methods to laboratory animals. According to that it was created the Adverse Outcome Pathways (AOP) that is an analytical construct that describes a sequential chain of causally linked events at different levels of biological organization that lead to an adverse health or ecotoxicological effect (Fig.6). AOPs are the central element of a toxicological knowledge framework being built to support chemical risk assessment based on mechanistic reasoning [20; 21].

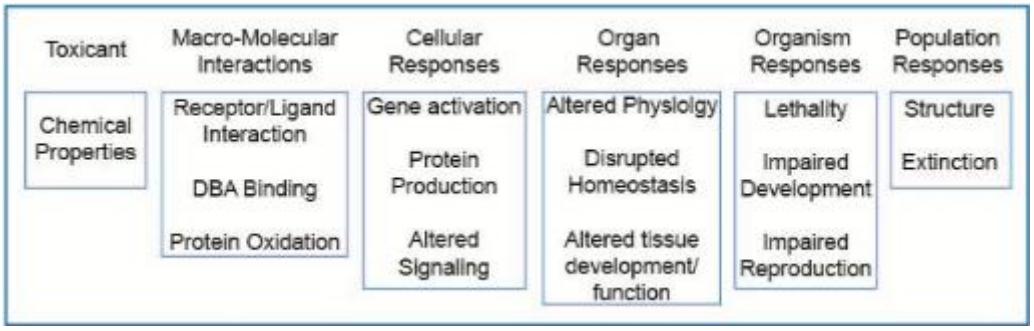


Figure 6. Adverse Outcome Pathway conceptual framework [20].

In addition to have a quickly and reduced testing cost characterization of chemical profilers, also for endocrine disruptor toxicity, several computational or in silico methods have been developed. Benfenati et al [22] drafted a report where they explain what those new approaches are: "*In the broad sense of the term, in silico models refer to computer tools and models available to scientists to simulate biological processes for a range of applications, species and level of biological organization (cellular, molecular, species, population, ecosystem, landscape etc.). In toxicology and risk assessment, in silico tools often aim to predict toxicity of chemicals and cover a wide range of methodologies that would also comprise molecular modelling approaches and general computational toxicology tools, including theoretical models based on the intrinsic structural and physicochemical properties of chemicals and rule-based expert systems. Structure-Activity*

Relationships (SAR) and Quantitative Structure Activity Relationships (QSAR) models are often collectively referred to as (Q)SAR as mathematical models that relate the structure of chemicals to their biological activities. The term 'quantitative' refers to the fact that the molecular descriptors are quantifiable on a continuous scale and thus provide a quantitative relationship with toxicity (which may itself be expressed in quantitative or categorical terms). Molecular descriptors of chemicals include their inherent physicochemical properties (i.e. atomic composition, structure, sub-structures, hydrophobicity, surface area charge, and molecular volume). ….. SAR and QSAR models can provide a fast method for the toxicity screening of untested substances, for identifying emerging chemicals in the food chain that have not yet been tested for their safety to human health or the environment. They are typically used in combination with other non-testing (e.g. read-across) and testing (e.g. in vitro) methods in the context of integrated testing strategies (ITS) and Weight-of-Evidence assessments (EFSA, 2014). Read-across represents 'a technique for predicting endpoint information for one substance (target substance), by using data from the same endpoint from (an)other substance(s), (source substance(s)' as defined by the European Chemical Agency (ECHA) (ECHA, 2008). ECHA used two key approaches for read across: 1. Analogue approach for which read-across is applied within a group of a very limited number of substances e.g. simplest read-across from a single source substance to a target substance; 2.Category approach for which compounds can be grouped in the case of a high number of substances and comprehensive guidance on grouping and read-across has been published by the OECD (OECD, 2007) and ECHA (ECHA, 2008). Examples of criteria to group chemicals include physic-chemical properties, functional/mechanistic/structural alert groups, chemical similarity …. The development of QSAR and read-across approaches for predicting toxicity of chemicals ideally involves quantitative understanding and data relating both toxicokinetic and toxicodynamic processes and some of the underlying parameters as predictor variables consider TK (e.g. partitioning coefficients) or TD (e.g. electronic properties) in the QSARs modelling. Key databases for QSAR and read-across have been described elsewhere (EFSA, 2014) and include: -The OECD QSAR Toolbox (http://www.qsartoolbox.org/) as a hazard identification tool which contains QSAR relationship methodologies to group chemicals into categories sharing the same structural characteristics and/or MoA. -The VEGA

platform (www.vega-qsar.eu) includes many models including physicochemical properties, ecotoxicological and toxicological properties ….. Key QSAR software and models used by international and national organizations including the Toxicity Estimation Software Tool (TEST), the OECD QSAR toolbox models and High-throughput Virtual Molecular Docking (HTVMD), MetaCore, DEMETRA, CAESAR, DEREK, METEOR, Multicase, PASS, OASIS Times" [22].

In addition to QSAR models, new computational methods have generated to better investigate the behaviour of ligand (toxicant or drug) at the target level (e.g. proteins or nucleic acids). Then two computational structural biology techniques have developed as the molecular docking of ligands (e.g. toxicants) with their biological receptors or targets (e.g. proteins or nucleic acids); and the molecular dynamics (MD) simulations of ligand–receptor complexes compared with receptors alone. Molecular docking tool enable us to determine the best pose, in term of orientation and unique conformations, of ligand at the binding site and to assess the value of affinity of this specific and unique interaction. The best pose is computed by docking algorithms which employ distinct sampling techniques to identify energetically favourable ligand poses. The most commonly used sampling techniques are genetic algorithms, Monte Carlo techniques, and matching algorithms. While the assessment of affinity is generated by application of scoring function which comprises mathematical models that approximate the non-covalent binding energy of a ligand pose within the binding cavity of the receptor.

This approach allows us to screen receptor–ligand interactions. These models have resulted in some advantages such as they are firstly inexpensive; it is possible to high throughput screen wide number of chemicals against a single target receptor (classical docking) or multiple target receptors against a single ligand (inverse docking); also to rapidly identify new ligand-binding sites within a receptor; the identified ligand poses can be scored in vacuo or in implicit solvent to obtain theoretical binding affinities; the results can be combined with pharmacophore mapping, shape-matching and molecular dynamic simulations to further investigate the nature and structural basis for a ligand– receptor interaction. While the possible disadvantages are the potential identification of incorrect binding site or target especially in the case of novel or previously uncharacterized xenobiotics; unreliable or misleading outcome when used with ligands that covalently modify their target receptor(s) [23].

The biological mechanisms of EDCs have been broadly characterized by using cell culture system. OECD (reported Table 1, Level 2 above) and ICCVAM [13] have issued some guidelines for testing in a good reliability way such chemicals for endocrine disrupting activity while a large number of works have been showing the action of EDCs at cellular level, mainly towards nuclear receptors, estrogen and androgen ones [24; 25].

Considering the family of estrogen receptor Kuiper and co-workers investigated for the first time the ligand binding affinity of ERα and ERβ separately. In their work they found it out that for the physiological estrogens, the order of competition was 17β-estradiol > estrone, 17a-estradiol (ERα) > estriol > catechol estrogens, 17α-estradiol (ERβ)> estrone-3-sulfate. While different orders of competition were found: diethylstilbestrol > hexestrol > dienestrol > (E2) for ERα and dienestrol > diethylstilbestrol > hexestrol > (E2) for ERβ. In the same research of Kuiper, it was shown that genistein and coumestrol, two plant-derived nonsteroidal compounds, had a higher estrogenic affinity to ERβ, previously discovered by Kuiper [26], than ERα [10].

Different in vitro test have been developed and used to characterize the estrogenic like property of chemicals including competitive ER binding assay that measure the ability of test compound to compete with radiolabelled 17β-estradiol for binding to ER [10]; the E-screen assay that is a cell proliferation assay done with MCF-7 breast cancer cell lines, where it is quantified the increased cell number after treatment with the chemical [27] but it showed a disadvantage because of its lack of estrogen specificity; ER Reporter gene assays which is based on the ability of a compound to stimulate ER-dependent transcriptional activity in genetically engineered mammalian cells. The cell lines are T47D cells or MCF-7 cells which express endogenous ER or yeast cells or HeLa cells which have not endogenous ER. In both case the cells are transformed (transfected) by introducing vectors containing DNA sequences for the receptor, along with EREs linked to a reporter gene, and the reporter gene itself. The reporter gene used in human cancer cells usually codes for luciferase (CALUX, chemically activated luciferase expression) and the reporter gene used in yeast cells usually codes for β-galactosidase [28]. In the transactivation the EDCs show their estrogenic potency calculated as EC50, respect to the positive control, 17β-estradiol. Indeed, a water environmental contaminant such a mixture of 4n-nonylphenol showed a significant activation of ER in both transfected firefly

luciferase reporter cell, MCF-7 and HeLa cell [28]. Another transactivation assay using MELN cells (derived from MCF-7) stable transfected with the estrogen responsive gene ERE-βGlob-Luc-SVNeo, showed robustness and reproducibility in the screening of estrogenic potency of a panel of chemicals selected from a European project ReProTect. In this study test compounds ranked from highest to lowest of estrogenic potency, then 17α-ethynylestradiol > 17β-estradiol > hexestrol > diethylstilbestrol > norethynodrel > nonylphenol > n-butylparaben in the range of 10^{-12}-10^{-10} M; three chemicals showed a moderate ER activation with a ranking as follow: genistein > equol > o,p_-DDT having EC_{50}-values in a very narrow range from 0.99×10^{-6} to 3.35×10^{-6} M [29]. However, the in vitro assays have not the same sensitivity to detect estrogenic potency. Indeed, Gutendorf and Westendorf reported a decrease of EC_{50} value for 17β-estradiol into different cell assays as follows: MVLN-cells=E-Screen > HGELN-cells (HGELN cells (derived from HeLa cells) > binding to ER- α binding to ER β. While a good correlation was registered for the other tested chemicals such as Bisphenol A but not for genistein which its relative potency ranked in the range of 10^{-4}–10^{-5} M [24].

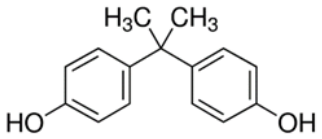
In vivo evidence of estrogenic like behaviour of EDCs has been investigated in animal models (mammalian and other species). Nowadays the use of the classical toxicological strategy is limited to the case when there is not a strong evidence from existing data of the scientific literature or from the in silico and in vitro testing based on OECD, ECHA, EFSA requirements, to reduce the number of laboratory animals as well as to follow the 3R principles, replacement, reduction and refinement. [30]. The reported Table 1, level 3 above shows the state of science to determine the biological effects in vivo.

Several works in the literature made a correlation between in vitro and in vivo experiments. Sonneveld et al, established a good correlation between the in vitro test ERα-CALUX and the in vivo ovariectomized test in young rats by scoring vaginal cornification (Allen-Doisy test) [31]. Nevertheless, the plausibility of the in vivo model are still some limitations such as a lack of certainty on the suitability of animal species treated and its life stages, a lack of correlation between the mechanistic of actions (which are predictive of the endocrine disrupting concern) and the apical response observed in vivo, i.e. the adverse outcomes [32]. In the last period new in vivo approach based on transgenic mouse model have been generated

to measure estrogen receptor (ER) transcriptional activity in living organisms [33] (further detail in the vivo paragraph of this thesis).

1.8 Chemicals of interest

I focused on few compounds of industrial and regulatory relevance such as:

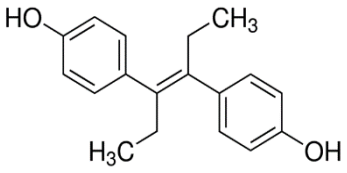


4,4'-isopropylidenediphenol, Bisphenol A, (BPA) (CAS No. 85-05-7) is a synthetic chemical widely produced and used in the manufacturing of polycarbonate plastics and epoxy resins. This substance is manufactured and/or imported in the European Economic Area in 1000000 - 10000000 tons per year [34]. This substance has a broadly application in the production of consumer products such plastic food containers (e.g. bottles, baby bottles, dishes), food packaging (canned food), thermal paper, articles made of PVC, also toys and medical equipment's contain BPA, and others consumer and professional use.
Migration from those products represents the major routes of human exposure even if there are accountable environmental sources such as surface water (during swimming) and outdoor air (inhalation of aerosols). In addition, the release of BPA from epoxy-based floorings, adhesives, paints, electronic equipment and printed circuit boards is reported to be a source of contamination of indoor air (including airborne dust) and dust [35]. Indeed it was estimated different BPA concentration if food matrices as consequence of a migration process, e.g. in some canned food categories have an average (MB) BPA concentration above 30 μg/kg, such as grain and grain-based products, legumes, nuts and oilseeds, meat and meat products, fish and other seafood; other canned food categories have average BPA concentrations (MB) between 2.7 and 23.5 μg/kg, such as vegetables and vegetable products, fruit and fruit products, fruit and vegetable juices, milk and dairy products. While from

the environmental sources, e.g. at the urban traffic site, the BPA concentrations in the particulate phase ranged from 0.06 to 18.6 ng/m3 (average 6.78 ng/m3); at the industrial site the BPA concentrations ranged from LOD to 47.3 ng/m3 (average 13.2 ng/m3).

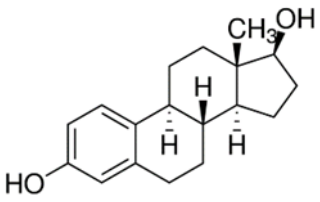
The estimated BPA dietary intake was highest in infants and toddlers, up to 0.875 µg/kg body way (bw) for day. Women of childbearing age had dietary exposures comparable to men of the same age (up to 0.388 µg/kg bw day). The highest aggregated exposure of 1.449 µg/kg bw day was estimated for adolescents [35].

BPA has raised concern for its high production volume and for the evidence of toxicity in laboratory animal studies mainly on reproductive and developmental systems, liver, kidney some evidence of alteration and cancer in the mammary gland [16,36-39]. So according to the strong evidence of animal toxicity, ECHA has classified the BPA as toxic for reproduction category 1B (according to art. 57 of Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP Regulation) [34]. Although on 2015 EFSA has issued a scientific opinion that BPA does not pose no health risk to all consumers (including infant and pregnant women) because the current exposure to the chemical is too low to cause harm. In addiction EFSA has established a temporary TDI (tolerable daily intake) of 4 micrograms per kilogram of body weight per day based on the toxicological effect on mean relative kidney weight in a mouse study [35].



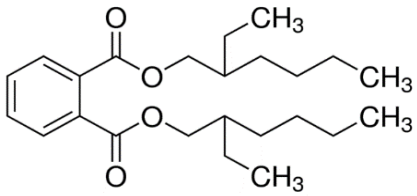
3,4-Bis(4-hydroxyphenyl)-hex-3-ene or Diethylstilbestrol, DES, (CAS No. 56-53-1) is a synthetic nonsteroidal estrogen. It was widely prescribed in the United States from the early 1940s until 1971, primarily as a treatment to prevent miscarriages or premature deliveries, control of menstrual disorders, relief or prevention of postpartum breast engorgement, palliative therapy for cancer of the prostate in men and breast cancer in postmenopausal women, and as a post-coital contraceptive. Diethylstilbestrol has also been used in veterinary medicine and as a growth promoter (as a feed supplement or subcutaneous implant) in cattle, sheep, and poultry (IARC 1979). Its use as a growth promoter was banned in 1979. Its use as human therapy was stopped

for the first time in the 1978 when the U.S. Food and Drug Administration (FDA) withdrawn the approval of any estrogen-containing drug product (including diethylstilbestrol). Then this substance went under evaluation the International Agency for Research on Cancer (IARC) that in the 1978 and 1987 confirmed that diethylstilbestrol was a human carcinogen (category 1A) based on sufficient evidence of carcinogenicity from studies in humans. The evidence of carcinogenicity in human has been discovered from epidemiological studies of 1970 where it was determined the association of women exposed to diethylstilbestrol in utero ("diethylstilbestrol daughters"), to clear-cell adenocarcinoma of the vagina or cervix. While in another cohort study it has been reported an increased risk of testicular cancer among diethylstilbestrol sons. Also, the evidence of carcinogenicity has been demonstrated in experimental animals by several routes of administration (e.g. oral, subcutaneous implantation). Prenatal exposure to diethylstilbestrol caused benign cervical and vaginal tumours (epidermoid tumours) in female mice, benign and malignant cervical and vaginal tumours (polyps, squamous-cell papilloma, and myosarcoma) in female hamsters, and benign and malignant testicular tumours (granuloma, adenoma, and leiomyosarcoma) in male hamsters. Also, in adult animal study DES caused carcinogenicity [15; 16; 40].



17β-estradiol (CAS No: 50-28-2) is a potent mammalian estrogenic hormone; it is produced in the ovary, placenta, testis, adrenal cortex. It is also produced by the adipose tissue of men and postmenopausal women. Estradiol has been used in estrogenic hormone therapy for the treatment of menopausal and postmenopausal symptoms, metastatic prostate cancer, & breast cancer [41]. Because of its natural affinity and potency to Estrogen receptors [10] 17β-estradiol has been using as positive reference compound in toxicological studies such as in the reproductive and developmental testing or in the mechanistic studies [13; 31; 42; 43] and pharmacological research [11]. Estradiol is to be considered as a carcinogenic chemical in woman in post-menopausal estrogen therapy. There is a strong evidence of carcinogenicity in several organs in animal studies. Mammary, pituitary, uterine, cervical, vaginal, testicular, lymphoid

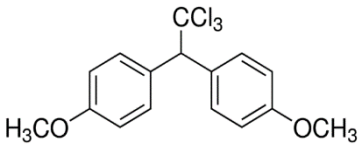
and bone tumours were observed in mice. Also, β-estradiol induced mammary and/ or pituitary tumours in rats. In hamsters and in guinea pigs malignant kidney tumours were noted. It was also reported the evidence of genotoxicity of 17β-estradiol [44].



Di-2-ethylhexyl phthalate, DEHP, (CAS No: 117-81-7) is a high production volume chemical used as a plasticizer of polyvinyl chloride in the manufacture of a wide variety of consumer goods, such as building products, car products, clothing, food packaging, children's products, and in medical devices made of polyvinyl chloride [45]. Because of its widespread use, it also represents a widely environmental pollutant. DEHP is expected to be strongly adsorbed to organic matter. DEHP is therefore expected to be found in the solid organic phase in the environment. DEHP has the property to bioaccumulate in aquatic organisms [46]. Human can be subjected to combined routes of exposure such as ingestion, inhalation and dermal exposure. It is estimated that the general population of the United States is exposed to DEHP levels ranging from 1 to 30 μg/kg bw day) [45]. Concentrations of DEHP and its metabolites detected at levels above the LOD have been characterized in urine samples from of mothers and their sons [47]. DEHP has been raising concern for its predominantly anti-androgenic activity elicited both in vitro and in vivo studies. While it is considered a weaker estrogenic compound [48]. In vitro DEHP caused cytotoxicity to MVNL cells line and displayed weak estrogenic potency in the MVLN transactivation assays. it decreased testosterone concentration in the H295R Steroidogenesis assay [49]. In a reproductive study (in utero and lactational exposure), it was reported a reduced daily sperm production and sperm quality and tract abnormalities (e.g., cryptorchidism) in adult male offspring. In this study the authors established a lowest observed adverse effect levels (LOAELs) for these effects were 15 and 5 mg/kg/day, respectively. Therefore, the no observed adverse effect level (NOAEL) for this study was set at 1.215 mg/kg/day [50].

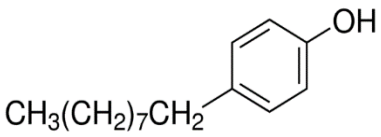
The EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) in 2005 determined a TDI of 0.05 mg/kg bw

day, based on the NOAEL of 5 mg/kg bw day for testicular toxicity observed in a feed multigeneration reproductive assessment in rats [51].



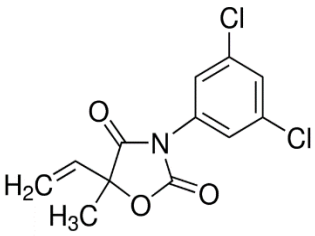
Methoxychlor (CAS No. 72-43-5) is a synthetic organochlorine insecticide used in the crop protection of ornamentals, livestock against a wide range of insects that attack fruits, vegetables, shade trees, home gardens, forage crops, and livestock. It is also used for the spray treatment of barns, grain storage bins, mushroom houses, dairies [52]. So, the agriculture activities, food and water contamination represented the mainly route of exposure to famers or people living close to area where methoxychlor was used.

The toxicity of methoxychlor was clear in the animal rather than human. Exposure to high concentrations of methoxychlor caused adverse effects to the animal nervous system. Some breakdown products of methoxychlor produced adverse effects similar to those produced by estrogen such as alteration in ovaries, uterus, and mating cycle in females, and the testes and prostate in males. Also, infertility occurred in both female and male animals. Those effects were seen both in adult animals and in developing animals exposed prenatally or shortly after birth [53]. In another study methoxychlor caused a decrease of weight of testis, prostate, seminal vesicles in adult rats feed at 50 and 100mg/kg bw day [54].

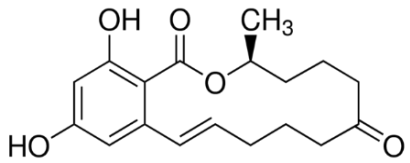


4-nonylphenol (CAS No. 104-40-5) is an environmental pollutant arising from the degradation of non-ionic surfactants in sewage, Nonoxynol-9, one of the APEs (alkylphenol polyethoxylates), used as a surfactant in cleaning, in cosmetic products, as a spermicide in contraceptives. Nonylphenol is an organic compound which belongs to the wide family of alkylphenols. It is a product of industrial synthesis formed during the alkylation process of phenols, particularly in the synthesis of polyethoxylate detergents. Because of their man-made origins, nonylphenols are classified as xenobiotics [55]. This alkylphenol raised concern for its ability to affect the reproductive organs in female and male laboratory animals. Most alkylphenols showed an affinity to the

estrogen receptors in a concentration-dependent manner and thus potentially act as activators (agonists) or inhibitors (antagonists) of cellular responses mediated through the estrogen receptor. The binding activity of alkylphenols is associated with the presence of substituted alkyl group [56]. Law and co-workers found it out the 4-nonylphenol induced a significant increase in uterine weight in the prepubertal rats after oral administration. Also, the age of vaginal opening was advanced following oral exposure from postnatal days 21–35 [57].



Vinclozolin (CAS No. 50471-44-8) was previously used as a fungicide on some fruits, nuts, vines, vegetables, ornamentals and wood preservative in the US. After 2004, its use in the US was restricted to only include use on canola and on turf used on golf courses and industrial sites. The only food import allowed following use of vinclozolin is wine grapes. Some other countries continue to use this fungicide [58]. Human data are very limited. It has low toxicity following single exposures via the oral, inhalation, or dermal route. The most sensitive targets to the toxicity of vinclozolin are the male reproductive organs. At low dose levels (>3 mg/kg/day), vinclozolin produced a decrease of prostate weight, reduction of nipple/areolas development, and decrease of ano-genital distance in male rats. At higher dose levels, the reduction of weight of male sexes organs is exacerbated, and sex organ malformations are seen, such as reduced penis size, ectopic testes, vaginal pouches, hypospadias, and additional ambiguities of the urogenital system [59]. This anti-androgenic activity is due to the binding to androgen receptor (AR) [60] or to other receptors such as the progesterone (PR), glucocorticoid (GR), mineralocorticoid (MR) or estrogen receptors (ERα and ERβ) [61].



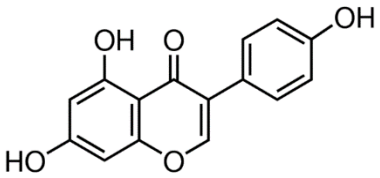
Zearalenone (CAS No. 17924-92-4) is a phenolic resorcyclic acid lactone mycotoxin produced by several Fusarium species. It is commonly found in maize, but it can be found also in other crops such as wheat, barley, sorghum and rye. People are exposed mainly through the diet based on the

consumption of a breakfast cereal. It is estimated a chronic dietary exposure in the range concentration from 2.4 ng/kg bw day to 54 ng/kg bw for high consumers. Infant and toddler are the most exposed consumer.

EFSA Contam Panel has derived a TDI of 0.25 μg/kg bw day based on the NOEL (no observed effect level) of 10 μg/kg bw. per day derived from a reproductive and development toxicity study in immature female pigs, considered as the most sensitive species to the estrogenic action of the zearalenone and its metabolites such as α-zearalenol. The estrogenic effects associate to this mycotoxin were the alterations of the oestrous cycle, ovulation, conception and implantation, embryonic death, reduced fetal weight, reduced litter size and impaired neonatal survival.

The toxicity of the zearalenone was considered relevant for the human health because the reproductive tract of female pigs was like the woman ones [62]. After the ingestion, zearalenone is metabolized to a secondary product with a higher estrogenic activity than the parent compound, by the CYP450 or other enzymes as 3α- and 3β-hydroxysteroid dehydrogenases (HSDs) family. Among these metabolites, α-zearalenol was linked to an alteration of steroidogenesis production as the parent compound does, in Leydig cells of mice [63] and in the H295R steroidogenesis assay where increased the production of progesterone, estradiol, testosterone and cortisol hormones [65]. Oral administration of zearalenone to adult male mice produced only adverse effects on the sperm parameters such as the decrease of sperm concentration and the increase of morphologically abnormal spermatozoa [65]. In the subchronic and chronic toxicity studies of NTP (technical report 235, 1982) [66] zearalenone caused atrophy of testis and seminal vesicles in both male rats F344/N and mice B6C3F1. These effects were observed also in the 90 days toxicity studies in rats at 27 – 270 mg/kg bw per day while in the 104 weeks toxicity study only the testes were sensitive to low doses (1.25-2.5 mg/kg per day). In the subchronic and carcinogenicity studies, male mice B6C3F1 treated at different doses, 9 and 150 and 450 mg/kg bw per day, developed a testicular atrophy. In NTP studies also the prostate was altered. Indeed, the rats developed a hyperplasia at 27 mg/kg bw per day in the subchronic study, while in carcinogenicity study was observed an increase of inflammation at both doses tested (1 and 2 mg/kg bw per day). In mice there was an incidence of squamous metaplasia at 450 mg/kg bw per day in the 13 weeks exposure study, while no adverse effect was detected in the carcinogenicity test.

Zearalenone was also defined as ER agonist [13]; it was determined a high value of half maximal inhibitory concentration (IC_{50}) in estrogen receptor competitive binding assay [67], while different transactivation assays (as in Hela cells) determined that zearalenone was a potent activator of estrogen receptor [68; 69].



Genistein, is an isoflavone that occurs in soy products. Human exposure to genistein comes is predominantly through the consumption of soy products, including soy-based infant formula and dietary supplements. The consumption of soy and thus of genistein, has been associated with a variety of beneficial effects in animals and humans. Nevertheless, some concerns have also been raised regarding the putative toxicity of genistein, particularly regarding on the reproductive system or the induction or potentiation of carcinogenesis, due to its weak estrogenic activity [70]. Indeed, in in vitro ER binding assay genistein showed a lower relative binding affinity towards ERα as reported by Kuiper [10] and by Gutendorf et al. [24].

In addition, the estrogenic potency of genistein was assessed to be several orders of magnitude lower than 17β-estradiol one in different in vitro assays [24]. In the animal testing, this isoflavone was linked to an alteration of both female and male reproductive organs. In a subchronic study where adult female rats were exposed to high concentration (500 mg/kg bw per day) of genistein, was observed an increase of ovarian and uterine weights, while in a carcinogenicity assay there was an increase of metaplasia and squamous metaplasia of uterine cervix, increase of uterine weight, uterine horn dilation at the same dose [71]. Also, male species were affected by the exposure to genistein mainly during the most sensible window as in utero exposure. In the multigenerational study done by NTP the male rats exposed during all pregnancy till to the adult age had significant increased weight of prostate [72].

However, even there is some evidence of toxicity of genistein from animal studies, maybe through the ER activation, EFSA opinion stated the safety of isoflavone consumption from soy-products [73].

1.9 Aim

The general aim of the project was to develop an integrated strategy of testing based on the combination of scientific literature review of in vivo data, in silico method, cellular and animal bioluminescence imaging methodologies to predict among a set of chemicals those with an endocrine disrupting activity or ability to activate other toxicological pathways such as inflammation and oxidative stress measured in the male reproductive organs and in the genital and abdominal area of mice. The selected molecules range from known (DES), to suspected (BPA), endocrine disruptors and included both synthetic (DEHP) and natural (genistein) compounds. Therefore, the assessment of ED activity/mechanism and the activation of inflammatory and oxidative stress pathways (as indirect toxicological mechanisms) of selected chemicals is based on the final observation of the correlation between the computed binding affinity values (binding free energies of chemicals towards human estrogen receptor, see Material and Methods), the following determinations of the dynamic ER activations quantified by in vitro and in vivo bioluminescence imaging methodologies and the available toxicological information from the literature.

The in vitro testing is a ERE-Luc reporter system where that is the cell transfection assay in ERE-Luc B17 cells, a clone of the breast cancer cell line MCF-7 stably transfected with a luciferase reporter system of estrogenic activity. The in vivo imaging was conducted in 3 different male transgenic mice carrying a firefly luciferase reporter system: one type under the control of estrogen-responsive promoter (ERE-Luc), ones under the control of inflammation pathway (NFkB-Luc), and the last was responsive to oxidative stress signaling (ARE-Luc).

Because of the nature of endocrine disruptors to alter the reproductive tract we have restricted our investigation on the ability of specific compound to activate the luciferase systems (ERE-Luc, NFkB-Luc and ARE-Luc) in the genital area of the transgenic mice. The abdominal area and intestine were integrated into the analysis as controls because of the high expression of the estrogen receptor.

The activities have been scheduled as reported below:

1° year	Literature search to retrieve toxicological data inherent to the reproductive alterations of our selected set of chemicals. Application of in silico model to human ERα to prioritize the chemicals based on their different ability to bind the receptor
2° year	Gathering of information on available in vitro systems that elucidate the mechanism of action of estrogen like compound. Then focus on reporter cells system such as that used in this project activity ERE-Luc B17 cells, a clone of the breast cancer cell line MCF-7. Imaging of ER activity in the reporter ERE-Luc cell to measure the EC50 of the compounds to be prioritized for the in vivo assay.
3° year	In vivo imaging of ER activity in reporter mice of compounds which were prioritized in the in vitro assay. Integration of all results in a rationale flow to be use in the toxicological risk assessment.

Chapter 2

2.1 Materials and Methods

2.2 Chemicals used in the luciferase reporter systems

Reagents: 17β-estradiol (CAS Number 50-28-2, purity 100%), Diethylstilbestrol (CAS Number 56-53-1, purity≥ 99%), Zearalenone (CAS Number 17924-92-4, purity ≥ 99%, stored condition at -20 °C), Bisphenol A (CAS Number 80-05-7, purity ≥ 99%), Genistein (CAS Number 446-72-0, purity ≥ 98%), Methoxychlor (CAS Number 72-43-5, purity ≤ 100%), Vinclozolin (CAS Number 50471-44-8, purity ≤ 100%), Bis(2-ethylhexyl) phthalate (CAS Number 117-81-7, purity ≤ 100%), 4-nonylphenol (CAS Number 104-40-5, purity ≤ 100%), Dimethyl

sulfoxide DMSO (CAS Number 67-68-5), were purchased form Sigma Aldrich S.r.l. The cell culture media RMPI 1640 supplemented was from LIFE Technologies Europe BV.

2.3 Literature search of endocrine disruptor endpoints

The existing toxicological data on the substances tested in the project, where collected from available research works where the apical effects on endocrine system ware investigated after oral (diet > drinking water > gavage) administration of the compounds. Only the toxicological data from rodent species (both sexes of mice and rats) were collected, except a study showing adverse effects of zearalenone on the reproductive system of female pig. The endpoints of interest were based on the ECHA and EFSA "Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009" and on the OECD (Organization for Economic Co-operation and Development) framework "Guidance Document 150 on Standardized Test Guidelines for Evaluating Chemicals for Endocrine Disruption, revised 2018" [18] (Table 1, previously reported). The checked database were first that of National Toxicological Programs Database where the studies reports are considered of high quality and reliability, then the others were: Toxnet, eChemPortal by the OECD which allows simultaneous searching of reports/datasets through the chemical name and number, the chemical properties and it also provides a direct link to collections of use, exposure, hazard and risk information from government chemical review programs at national, regional and international levels; Chembase, ChemIDplus, ChemSpider, Carcinogenic Potency Database, DSSTox, European chemical Substances Information System, PubChem BioAssay Database that contains bioactivity screens of chemical substances described in PubChem Substance. It provides searchable descriptions of each bioassay, including descriptions of the conditions and readouts specific to that screening procedure.

2.4 In silico methodology

All the computational procedures were carried out by the Schrödinger Small-Molecule Drug Discovery Suite 2018-32. The crystallographic structure of the ligand binding domain of human estrogen alpha bound to estradiol was downloaded from the RCSB PDB (code: 3UUD, chain A) [74]. The Schrödinger Protein Preparation Wizard was used for locating and fixing structural defects or missing information in ERα structure and preparing it for use with Schrödinger Glide for molecular docking. Tested ligands were built by the Schrödinger Maestro Build Toolbar and prepared for docking by the Schrödinger Ligand Preparation. The molecular docking procedure was carried out by the Schrödinger Glide Docking in "extra precision, XP" mode, which is an estimation of the binding free energy, in order to evaluate the ability of the tested ligand to bind the ERα ligand binding domain.

The top-scoring solution for each ligand was submitted to Schrödinger Prime MM-GBSA, which integrates molecular mechanics energies combined with the generalized Born and surface area continuum solvation [75] in order to calculate ligand binding and ligand strain energies for a set of ligands and a single receptor.

The dissociation constant (Ki) was computed starting from empirical binding free energy values, according to the following equation:

$$\Delta G = RT \ln(K_i)$$

where R represents the gas constant and T the temperature. Ki was computed starting from the binding free energy values at a fixed temperature (300 K)

2.5 In vitro bioluminescence imaging assay

The ability of the compounds to transcriptionally activate ERα was tested in ERE-Luc B17 cells, a clone of the breast cancer cell line MCF-7 stably transfected with a reporter constituted by the luciferase gene driven by an estrogen-regulated synthetic promoter previously generated and tested in laboratory of Professor Ciana [76] and already successfully used in other researches [77; 78].

Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Euroclone, UK), 50 U/mL penicillin G, 50 μg/mL streptomycin sulfate, 2 g/L sodium carbonate, and 0.11 g/L sodium pyruvate at 37 °C at 99% humidity and 5% CO2. Cells were split twice a week by seeding 2 × 106 cells in 100 mm diameter Petri (Corning, MA) dishes. For transactivation studies, 105 cells/well were seeded in 96 well plate in phenol red-free RPMI 1640 medium (Sigma-Aldrich, MO) supplemented with 10% dextran-coated charcoal stripped FBS, 1% essential amino acid, 1% vitamin mixture, 50 U/mL penicillin G, 50 μg/mL streptomycin sulfate, 2 g/L sodium carbonate, and 0.11 g/L sodium pyruvate and kept at 37 °C in a humidified incubator for 24 h.

Next, culture medium was replaced with RPMI 1640 with 1% stripped FBS, and cells were incubated for a minimum of 4 h before adding 17β-estradiol or selected EDCs at increasing concentrations (6-10 depending on the in vitro toxicity) of each compound.

After 6h and 48h of incubation the treated cells were rinsed once with PBS before preparing the bioluminescence detection by CCD camera in presence of luciferin substrate. Then the bioluminescence imaging sessions were carried out on the same treated cells after 6h and 48h of treatments. As a control, all compounds were run in parallel with 17β-estradiol. The in vitro assay was repeated two times, with three replicates at each tested concentration (see Figure 7 as an example).

With this system the activity of ER following activation can be easily directly detected by a bioluminescence imaging simply after adding the substrate luciferin to the culture medium of the reporter cells. The ERα activations are then quantified by the bioluminescence imaging of photon emissions from the treated cells (with different concentrations of tested chemicals (in the range of micromolar to picomolar) by using a CCD camera, also used in approach routinely applied for in vivo imaging experiment [11; 12], which takes a picture of photon emission at 6 and 48 hours of incubation (Figure 7).

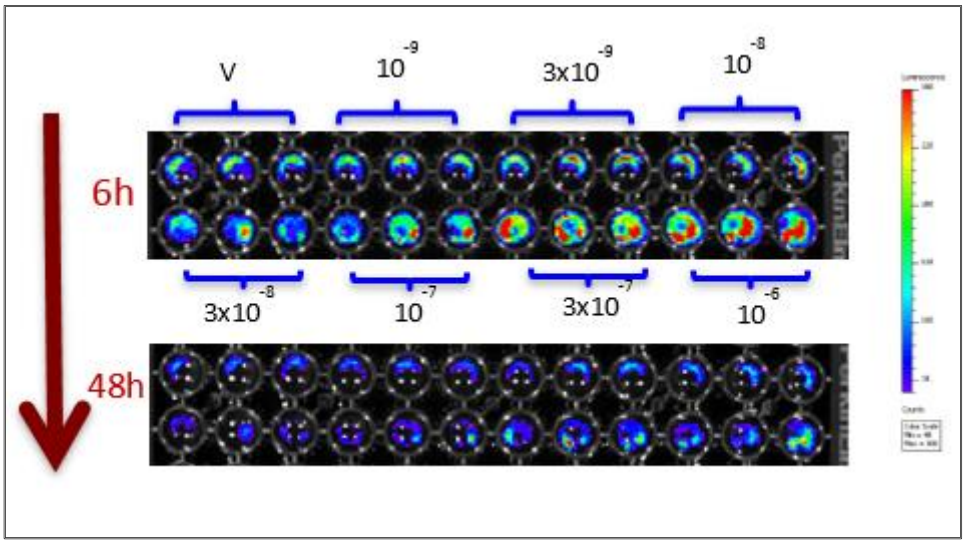


Figure 7. Example of bioluminescence imaging o of photon emission after treatment with different concentrations (three replicates at each tested concentrations) of Bisphenol A at 6h and 48h.

2.6 In vivo bioluminescence imaging experiments

In the in vivo bioluminescence imaging, the selected compounds plus a positive control and the vehicle were orally administered to three different reporter mice. The ERE-Luc mouse model is a transgenic mouse previously generated by prof. Ciana [76] to measure the ER activity in the mouse tissues. Briefly, the construct used for transgenesis consisted of the reporter gene (firefly luciferase) driven by a dimerized ERE and a minimal promoter. Insulator sequences, the matrix attachment region from chicken lysozyme, were used to flank the reporter system in the transgenesis construct to achieve a generalized, hormone-responsive reporter expression [12]. This model was already successfully and routinely used in other works, demonstrating that in this animal model luciferase activity is strictly related with the state of ER transcriptional activity [11; 12; 33; 79; 80].

The insulator technology was successfully applied to the generation of the other model of reporter mice used in this step of the project. The transgenic mice were transfected with a reporter system for the inflammatory (NFkB-Luc) and oxidative stress (ARE-Luc) pathways.

The in vivo bioluminescence imaging represents a newly non-invasive procedure to quantify the interactions of the xenobiotics, in this case estrogen like compounds,

with the reporter luciferase constructs expressed in transgenic mouse, by the charge-couple device (CCD) camera detection which takes a "picture" of the photon emissions arising from the mouse tissues as a consequence of ER activation [11; 12; 33; 79; 80] or inflammation or oxidative stress pathways stimulations.

Also, this non-invasive approach allowed us to quantify by bioluminescence imaging the biological events (e.g. the activations of mentioned pathways) occurring in the same mouse during the entire time of experiment. Thus, the mouse has become itself both its "control group" for the quantification of the baseline bioluminescence values (background noise) and the "experimental group" during the quantifications of the bioluminescence in response to the treatments. So, any biological variation observed are normalized on the basic (baseline bioluminescence) values of the same individual, reducing the component of variability of the measurements among different individuals.

At least this methodology allows also to reduce the number of experimental groups thanks to a limited variability of less than 10% of the standard deviation. Our knowledge of the imaging models used in the project on which preliminary observations were made allowed us to make a hypothetical estimation of the noise, the signal (effect size) and the signal to noise ratio (S / N). Thanks to this estimate, we were able to determine the number of animals per experimental group to be used in our study based on the following parameters:

- significance level of 5%
- power equal to 80%
- signal / noise ratio of 1.2

Based on these parameters and referring to what was suggested on the website http://www.3rs-reduction.co.uk, we have therefore established the number of our samples as N=4 mice for each treatment as also reported in previously works using this non-invasive methodology both in male and female transgenic mice [81]. The following protocol used in my research describes how to run an in vivo imaging experiment:

4 heterozygous young male mice C57BL/6 were enrolled for each treatment. Before the beginning of the experimental study, the baseline luciferase activity was measured in all animals by in vivo imaging (D0 or baseline). The anaesthetized untreated mice were doped with substrate and the introduced inside the CCD camera for 5 minutes to get the baseline luciferase activity. The day after the

compounds Bisphenol A (10 and 100 mg/kg/day, BPA10 and BPA100), Zearalenone (10 and 150 mg/kg/day, ZEA10 and ZEA150), 17β-estradiol, positive control, (10 and 100 μg/kg/day, E10 and E100) and vehicle (2-hydroxypropyl-β-cyclodextrin) were daily administered by drinking water for 21 days to transgenic mice.

The bioluminescence imaging session was done every morning at 10:00. The animals were injected i.p. with 80 mg/kg of luciferin (Beetle Luciferin Potassium Salt; Promega, Madison, WI, USA) 15 minutes prior bioluminescence quantification, to obtain a uniform biodistribution of the substrate. For the imaging, mice were anaesthetized using isoflurane (Isoflurane-Vet; Merial, Lyon, France) and kept under anaesthesia during the 5 minutes of the session carried out with a CCD camera which took a picture of bioluminescence emissions. Photon emission in selected body areas was measured using the Living Image Software (Caliper, PerkinElmer company). The analysis was done in ventral view: whole body, head, thymus, chest, abdominal, genital area, tail, paw and expressed as photon/second/cm2/sr radiant (p/s/cm2/sr). At the end of treatment On day 21, terminal study, , the animals were sacrificed by the intraperitoneal injection of lethal mixtures (ketamine >280.8 and xylazine >21.6 mg/Kg), then the selected organs were excised and placed into the CCD camera for the ex vivo bioluminescence imaging procedure in order to directly quantify the photon emissions of our pathways of interest (hormonal, inflammation and oxidative stress) from localized tissue such as intestine, testis, seminal vesicles and prostate.

The measurements of photon emissions could be quantitatively analysed from the selected and specific body areas are by called regions of interest (ROIs) which can be also manually selected. The software of CCD camera takes the image of the whole animal body and thus we can modify and select the area, the size of ROI (highlighted in red borders) (Figure 8).

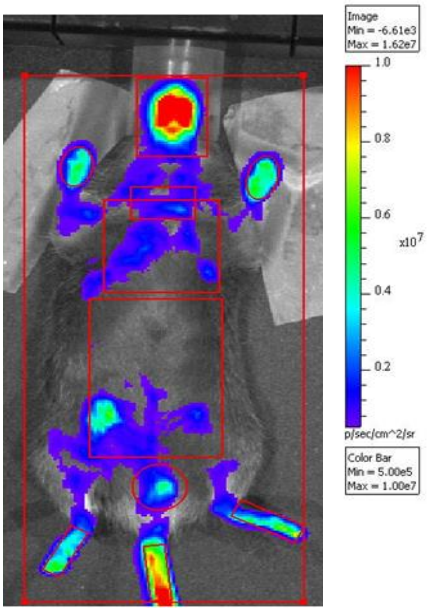


Figure 8: selection of regions of interest (ROI) on the bioluminescence image taken by CCD camera, for whole body, genital area, head, chest, abdominal, paw, tail.

2.7 Statistical analysis

The in vitro bioluminescence imaging assay was carried out in triplicates at each concentration tested (figure 7). The analysis of transactivation data and calculation of EC_{50} values were performed by means of sigmoidal dose-response (variable slope) using GraphPad Prism v. 7 software (GraphPad Software Inc.).

In vivo bioluminescence imaging experiment with reporter mice allowed us to quantify by bioluminescence imaging the biological events (e.g. the activations of mentioned pathways) occurring in the same mouse during the entire time of experiment. Thus, the mouse has become itself both its "control group" for the quantification of the baseline bioluminescence values (background noise) and the "experimental group" during the quantifications of the bioluminescence as response to the treatments. So, any biological variation observed are normalized on the basic (baseline bioluminescence) values of the same individual, reducing the component of variability of the measurements among different individuals.

Imaging allows to reduce the number of experimental groups thanks to a limited variability of less than 10% of the standard deviation. Our knowledge of the imaging models used in the project on which preliminary observations were made allowed us to make a hypothetical estimation of the noise, the signal (effect size) and the signal to noise ratio (S / N). Thanks to this estimate, we were able to determine the number of animals per experimental group to be used in our study based on the following parameters:

- significance level of 5%
- power equal to 80%
- signal / noise ratio of 1.2

Based on these parameters and referring to what was suggested on the website http://www.3rs-reduction.co.uk, we have therefore established the number of our samples as N=4 mice for each treatment.

The analysis of statistical significance was done by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test with $p < 0.05$ versus control.

Chapter 3

3.1 Results

3.2 Literature search of endocrine disruptor endpoints

Before the programming of experimental part of the project, an extensive bibliographic analysis of the available toxicological data was carried out. Those data related to a possible endocrine disrupting activity of the selected molecules described in the paragraph 1.7. The endpoints of interest focused on the parameters described in the "Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009" based also on OECD (Organization for Economic Co-operation and Development) framework "Guidance Document 150 on Standardized Test Guidelines for Evaluating Chemicals for Endocrine Disruption, revised 2018" [18; 19]. The aim was to obtain any information on the active dose ranges and the potency of each molecule with the regard to specific effects in organs targets which are representatives of a hormonal activity. The analysis was not able to show any particular correlations because of many experiments with different protocols, except few cases, using different species, strains, sex, treatment duration, doses etc. Then the attention was exclusively focused on data obtained from reliable toxicological studies carried out by NTP, except few cases. Thus, the data were restricted to the effects observed in the male reproductive organs. An extract of these data is reported in the following table 2.

Table 2. Extract of data from literature: adverse effects of interest for endocrine disrupting activity on testis, seminal vesicles, prostate and liver of male rodent species

- **Testis**

COMPOUND	SPECIES	STRAINS	ROUTE	PROTOCOL	TREATMENT (DAYS)	DOSE (mg/kg bw day)	EFFECT	REFERENCE
17β-estradiol	Rats	Crl:CD BR	diet	Subchronic toxicity	90	0.003; 0.139 - 0.173; 0.5 - 0.7; 3 - 4	↓ weight, size, Degeneration/atrophy, seminiferous tubule (0.5, 3)	Biegel et al., 1998 [42]
17β-estradiol	Rats	Crl:CD BR	diet	Extended One-Generation Reproductive Toxicity Study	in utero + lactation + 77 days (Post weaning - 77)	0.004 - 0.005; 0.225 - 0.273	↓ weight (0.225)	Biegel et al., 1998 [42]
Zearalenone	Rats	F344/N	diet	Subchronic toxicity	90	0; 2.7; 9; 27; 90; 270	↑Atrophy (27; 90; 270)	NTP tr 235, 1982 [66]
Zearalenone	Rats	F344/N	diet	Carcinogenicity	728	1.25; 2.5	↑Atrophy (1.25>2.5)	NTP tr 235, 1982 [66]
Zearalenone	Mice	B6C3F1	diet	Subchronic toxicity	90	0; 4.5; 15; 45; 150; 450	↑Atrophy (150; 450)	NTP tr 235, 1982 [66]
Zearalenone	Mice	B6C3F2	diet	Carcinogenicity	728	m: 7; 14; f: 10; 20	↑Atrophy (9> 17)	NTP tr 235, 1982 [66]
Zearalenone	Mice	CD1	drinking water	One-Generation Reproduction Toxicity Study	90 (GD1-PND70, only male pups)	0.000025; 0.025	no effect	Zatecka et al., 2014] [65]
Genistein	Rats	SD	diet	Extended One-Generation Reproductive Toxicity Study	64 (GD7-PND49) (only pups examination)	0.3; 1.7; 6.4; 16; 38; 72 Pregnant dams (GD7-parturition) 0.6; 3.5; 14; 37; 84; 167 Lactating dams (Pup PND 1 - PND 14) 0.6; 3; 11; 29; 69 ; 166 F1 male ; PND21-PND50 0.6; 3; 12; 31; 73; 166 F1 female, PND 21 - PND 50	no effect	NTP tr 79, 2007 [82]

Genistein	**Rats**	**SD**	**diet**	**Multigeneration reproductive toxicology**	**F0: From PND 42 to PND 140 (98 days)** **F1: From conception to PND 140 (161 days)** **F2: From conception to PND 140 (161 days)** **F3: From conception to PND 21, fed control feed from PND 21 to PND 140 (161 days total; 42 days on dosed feed)** **F4: No exposure; control feed from conception to PND 140 (161 days total; no dosed feed)** **F5: No exposure; control feed from conception to PND 21 (42 days total; no dosed feed)**	**m: 0; 0.03; 7; 35** **f: 0.5; 10; 50**	**↑ weight (abs, rel.) (F0, 35); ↑ age at testicular descent (F3, trend to 35)**	**NTP tr 539, 2008 [83]**
Genistein	**Rats**	**SD**	**diet**	**Carcinogenicity**	**F0: From PND 42 until F1 weaning (77 days)** **F1 (F1C): From conception to 2 years (756 days)** **F1 (F1T140): From conception to PND 140 (161 days), then fed control diet to 2 years** **F3 (F3T21): From conception to PND 21**	**pregnant dams: 0; 0.5; 9; 45; lacting dams: 0.7; 15; 75;** **period prior to PND 140; f: 0.4; 8; 44;** **m: 0.4; 7; 37; period between PND 140 and the end; f: 0.3; 5; 29; m: 0.2; 4; 20**	**no effect**	**NTP tr 545, 2007 [70]**

					(42 days); then fed control diet to 2 years			
Genistein	Rats	Long Evans	diet	One-Generation Reproduction Toxicity Study	35 (GD1-PND21)	Prenatal: 0.1-0.2; 6.4-9.1; Postnatal: 0.2-0.4; 12-23	↓ size (PND40, 0.4;23)	Wisniewski et al., 2003 [84]
Genistein	Rats	SD	diet	Reproductive and development toxicity	14 (PND21-35)	22; 90	no effect	Fritz et al., 2003 [85]
Genistein	Rats	Wistar	diet	Subchronic toxicity	28	0.5; 5; 50; 500	no effect	Mc Clain et al.; 2006 [71]
Genistein	Rats	Wistar	diet	Subchronic toxicity	90	5; 50; 500	↑ rel. weigh (500); ↑tubular degeneration/atrophy (500, reversible after 4 weeks)	Mc Clain et al., 2006 [71]
Genistein	Rats	Wistar	diet	Chronic toxicity	364	5; 50; 500	↑ rel. weigh (500)	Mc Clain et al., 2006 [71]
Bisphenol A	Rats	SD	gavage	Prenatal and Chronic toxicity	In utero + PND1-21 (no dosing to 365)	0; 0.0025; 0.025; 0.25; 2.5; 27	no effect	NTP RR 09; sept [86]
Bisphenol A	Rats	SD	gavage	Prenatal and Chronic toxicity	In utero + PND1-365	0; 0.0025; 0.025; 0.25; 2.5; 25	no effect	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	SD	gavage	Prenatal and Chronic toxicity	In utero + PND1-21 (no dosing to 730)	0; 0.0025; 0.025; 0.25; 2.5; 28	↑testes polyarteritis (2.5)	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	SD	gavage	Prenatal and Chronic toxicity	In utero + PND1-730	0; 0.0025; 0.025; 0.25; 2.5; 26	no effect	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	F344	diet	Carcinogenicity	730	50; 100	↑ interstitial-cell tumours (50; 100)	NTP tr 215, 1982 [66]

Bisphenol A	Mice	B6C3F1	diet	Carcinogenicity	730	750; 1500	no effect	NTP tr 215, 1982 [66]
Bisphenol A	Rats	Wistar	gavage	Reproductive and development toxicity	GD7-PND22 (30)	0.025; 0.250; 5	no effect	Christiansen et al., 2014 [87]
Bisphenol A	Rats	SD	gavage	One-Generation Reproduction Toxicity Study	GD6-PND90	0.0025; 0.008; 0.025; 0.08; 0.26; 0.840; 2.7; 100; 300	↓ size (PND90; F1, 0.26; 300)	Delclos et al., 2016 [39]
Bisphenol A	Rats	SD	diet	Multigenerational reproductive toxicology	130 (F0 through F3)	0.0007– 0.003; 0.015– 0.062; 0.22– 0.73; 4.1– 15.4; 37.6 – 167.2; 434 –1823	no effect	Tyl et al., 2002 [36]
Bisphenol A	Mice	CD-1	diet	Multigeneratio reproductive toxicology	112	0.003; 0.03; 0.3; 5; 50; 600	↑ testes weights (F1; F2; 600); ↑ increased incidence of minimal to mild hypoplasia of the seminiferous tubule (F1; F2; 600)	Tyl et al., 2008 [37]

- **Seminal vesicles**

COMPOUND	SPECIES	STRAINS	ROUTE	PROTOCOL	TREATMENT (DAYS)	DOSE (mg/kg bw day)	EFFECT	REFERENCE
17β-estradiol	Rats	SD	gavage	Reproductive and development toxicity	38 (GD6-PND20)	0.01	↓weight (0.01)	Kang et al., 2002 [88]
17β-estradiol	Rats	Crl:CD BR	diet	Subchronic toxicity	90	0.003; 0.139 - 0.173; 0.5 - 0.7; 3 - 4	↓weight, ↑ atrophy (0.5, 3)	Biegel et al., 1998 [42]
17β-estradiol	Rats	Crl:CD BR	diet	One-Generation Reproduction Toxicity Study	in utero + lactation + 77 days (Post weaning - 77)	0.004 - 0.005; 0.225 - 0.273	↓weight (0.225)	Biegel et al., 1998 [42]
Zearalenone	Rats	F344/N	diet	Subchronic toxicity	90	0; 2.7; 9; 27; 90; 270	↑Atrophy (9; 27; 90; 270)	NTP tr 235, 1982 [66]
Zearalenone	Rats	F344/N	diet	Carcinogenicity	728	1; 2	no effect	NTP tr 235, 1982 [66]
Zearalenone	Mice	B6C3F1	diet	Subchronic toxicity	90	0; 4.5; 15; 45; 150; 450	↑Atrophy (150; 450)	NTP tr 235, 1982 [66]
Zearalenone	Mice	B6C3F2	diet	Carcinogenicity	728	9; 17; f: 10; 20	no effect	NTP tr 235, 1982 [66]

Zearalenone	Mice	CD1	drinking water	One-Generation Reproduction Toxicity Study	90 (GD1-PND70, only male pups)	0.000025; 0.025	no effect	Zatecka et al., 2014 [65]
Genistein	Rats	SD	diet	One-Generation Reproduction Toxicity Study	64 (GD7-PND49) (only pups examination)	0.3;1.7; 6.4; 16; 38; 72 Pregnant dams (GD7-parturition) 0.6; 3.5; 14; 37; 84; 167 Lactating dams (Pup PND 1 - PND 14) 0.6; 3; 11; 29; 69; 166 F1 male; PND21-PND50	no effect	NTP tr 79, 2007 [82]
Genistein	Rats	SD	diet	Multigeneration reproductive toxicology	F0: From PND 42 to PND 140 (98 days) F1: From conception to PND 140 (161 days) F2: From conception to PND 140 (161 days) F3: From conception to PND 21, fed control feed from PND 21 to PND 140 (161 days total, 42 days on dosed feed) F4: No exposure; control feed from conception to PND 140 (161 days total, no dosed feed) F5: No exposure; control feed from conception to PND 21 (42 days	0; 0.03; 5; 25 f: 0.5; 10; 50	no effect	NTP tr 539, 2008 [83]

					total; no dosed feed)			
Genistein	Rats	SD	diet	Carcinogenicity	F0: From PND 42 until F1 weaning (77 days) F1 (F1C): From conception to 2 years (756 days) F1 (F1T140): From conception to PND 140 (161 days), then fed control diet to 2 years F3 (F3T21): From conception to PND 21 (42 days), then fed control diet to 2 years	pregnant dams: 0; 0.5; 9; 45; lacting dams: 0.7; 15; 75; period prior to PND 140: 0.4; 7; 37; period between PND 140 and the end: 0.2; 4; 20	no effect	NTP tr 545, 2007 [70]
Genistein	Rats	Long Evans	diet	One-Generation Reproduction Toxicity Study	35 (GD1-PND21)	Prenatal: 0.1-0.2; 6.4-9.1; Postnatal: 0.2-0.4; 12-23	no effect	Wisniewski et al., 2003 [84]
Genistein	Rats	Wistar	diet	Subchronic toxicity	28	0.5; 5; 50; 500	↓size (500)	Mc Clain et al., 2006 [71]
Genistein	Rats	Wistar	diet	Subchronic toxicity	90	5; 50; 500	congestion, ↓ colloide (500)	Mc Clain et al., 2006 [71]
Genistein	Rats	Wistar	diet	Chronic toxicity	182 - 364	5; 50; 500	no effect	Mc Clain et al., 2006 [71]
Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-21 (no dosing to 365)	0; 0.0025; 0.025; 0.25; 2.5; 25	no effect	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-365	0; 0.0025; 0.025; 0.25; 2.5; 25	no effect	NTP RR 09; sept. 2018 [86]

Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-21 (no dosing to 730)	0; 0.0025; 0.025; 0.25; 2.5; 25	no effect	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-730	0; 0.0025; 0.025; 0.25; 2.5; 25	no effect	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	F344	diet	Carcinogenicity	730	50; 100	no effect	NTP tr 215, 1982 [66]
Bisphenol A	Mice	B6C3F1	diet	Carcinogenicity	730	750; 1500	no effect	NTP tr 215, 1982 [66]
Bisphenol A	Rats	Wistar	gavage	Reproductive and development toxicity	GD7-PND22 (30)	0.025; 0.250; 5	no effect	Christiansen et al., 2014 [87]
Bisphenol A	Rats	SD	gavage	One-Generation Reproduction Toxicity Study	GD6-PND90	0.0025; 0.008; 0.025; 0.08; 0.26; 0.840; 2.7; 100; 300	no effect	Delclos et al., 2016 [39]

- **Prostate**

COMPOUND	SPECIES	STRAINS	ROUTE	PROTOCOL	TREATMENT (DAYS)	DOSE (mg/kg bw day)	EFFECT	REFERENCE
17β-estradiol	Rats	SD	gavage	Reproductive and development toxicity	38 (GD6-PND20)	0.01	no effect	Kang et al., 2002 [88]
17β-estradiol	Rats	Crl:CD BR	diet	Subchronic toxicity	90	0.003; 0.139 - 0.173; 0.5 - 0.7; 3 - 4	↓weight, ↑ atrophy (0.5, 3)	Biegel et al., 1998 [42]
17β-estradiol	Rats	Crl:CD BR	diet	One-Generation Reproduction Toxicity Study	in utero + lactation + 77 days (Post weaning - 77)	0.004 - 0.005; 0.225 - 0.273	↓weight (0.225)	Biegel et al., 1998 [42]
Zearalenone	Rats	F344/N	diet	Subchronic toxicity	90	0; 2.7; 9; 27; 90; 270	↑ Hyperplasia (27; 90; 270)	NTP tr 235, 1982 [66]
Zearalenone	Rats	F344/N	diet	Carcinogenicity	728	1; 2	↑ inflammation (1;2)	NTP tr 235, 1982 [66]
Zearalenone	Mice	B6C3F1	diet	Subchronic toxicity	90	0; 4.5; 15; 45; 150; 450	↑ squamous metaplasia (450>150)	NTP tr 235, 1982 [66]
Zearalenone	mice	B6C3F2	diet	Carcinogenicity	728	m: 9; 17; f: 10; 20	no effect	NTP tr 235, 1982 [66]

Zearalenone	mice	CD1	drinking water	One-Generation Reproduction Toxicity Study	90 (GD1-PND70, only male pups)	0.000025; 0.025	no effect	Zatecka et al., 2014 [65]
Genistein	Rats	SD	diet	One-Generation Reproduction Toxicity Study	64 (GD7-PND49) (only pups examination)	0.3; 1.7; 6.4; 16; 38; 72 Pregnant dams (GD7-parturition) 0.6; 3.5; 14; 37; 84; 167 Lactating dams (Pup PND 1 - PND 14) 0.6; 3; 11; 29; 69; 166 F1 male; PND21-PND50	↓ ventral prostate weight (F1; 166); ↑ Dorsolateral Prostate Gland Inflammation Ventral Depletion of Secretory Fluids (F1; 166)	NTP tr 79, 2007 [82]
Genistein	Rats	SD	diet	Multigeneration reproductive toxicology	F0: From PND 42 to PND 140 (98 days) F1: From conception to PND 140 (161 days) F2: From conception to PND 140 (161 days) F3: From conception to PND 21, fed control feed from PND 21 to PND 140 (161 days total, 42 days on dosed feed) F4: No exposure; control feed from conception to PND 140 (161 days total, no dosed feed) F5: No exposure; control feed from conception to PND 21 (42 days	m: 0; 0.03; 5; 25	no effect	NTP tr 539, 2008 [83]

					total, no dosed feed)			
Genistein	Rats	SD	diet	Carcinogenicity	F0: From PND 42 until F1 weaning (77 days) F1 (F1C): From conception to 2 years (756 days) F1 (F1T140): From conception to PND 140 (161 days), then fed control diet to 2 years F3 (F3T21): From conception to PND 21 (42 days), then fed control diet to 2 years	pregnant dams: 0; 0.5; 9; 45; lacting dams: 0.7; 15; 75; period prior to PND 140: 0.4; 7; 37; period between PND 140 and the end: 0.2; 4; 20	↑ lateral- dorsal prostate gland weight (F1C; 20 - 37)	NTP tr 545, 2007 [70]
Genistein	Rats	Long Evans	diet	One-Generation Reproduction Toxicity Study	35 (GD1-PND21)	Prenatal: 0.1-0.2; 6.4-9.1; Postnatal: 0.2-0.4; 12-23	↓ size (PND70, 23)	Wisniewski et al., 2003 [84]
Genistein	Rats	SD	gavage	Reproductive and development toxicity	5 (PND1-5)	12.5; 25; 50; 100	no effect	Nagao et al., 2001 [89]
Genistein	Rats	Wistar	diet	Subchronic toxicity	28	0.5; 5; 50; 500	no effect	Mc Clain et al., 2006 [71]
Genistein	Rats	Wistar	diet	Subchronic toxicity	90	5; 50; 500	↑ inflammation (500)	Mc Clain et al., 2006 [71]
Genistein	Rats	Wistar	diet	Chronic toxicity	182 - 364	5; 50; 500	↑ weight (500); ↑ inflammation (50; 500)	Mc Clain et al., 2006 [71]
Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-21 (no dosing to 365)	0; 0.0025; 0.025; 0.25; 2.5; 27	no effect	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-365	0; 0.0025; 0.025; 0.25; 2.5; 25	↑dorsal/lateral prostate lobes inflammation (0.0025)	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-21 (no dosing to 730)	0; 0.0025; 0.025; 0.25; 2.5; 25	↑ventral prostate lobes adenocarcinoma (0.025)	NTP RR 09; sept. 2018 [86]

Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-730	0; 0.0025; 0.025; 0.25; 2.5; 25	↑dorsal/lateral prostate lobes inflammation (0.0025); ventral lobe prostate epithelium hyperplasia (0.25); ↑ventral prostate lobes adenoma (25)	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	F344	diet	Carcinogenicity	730	50; 100	no effect	NTP tr 215, 1982 [66]
Bisphenol A	Mice	B6C3F1	diet	Carcinogenicity	730	750; 1500	no effect	NTP tr 215, 1982 [66]
Bisphenol A	Rats	Wistar	gavage	Reproductive and development toxicity	GD7-PND22 (30)	0.025; 0.250; 5	no effect	Christiansen et al., 2014 [87]
Bisphenol A	Rats	SD	gavage	One-Generation Reproduction Toxicity Study	GD6-PND90	0.0025; 0.008; 0.025; 0.08; 0.26; 0.840; 2.7; 100; 300	no effect	Delclos et al., 2016 [39]
Bisphenol A	Rats	SD	diet	Multigeneration reproductive toxicology	130 (F0 through F3)	0.0007– 0.003; 0.015– 0.062; 0.22– 0.73; 4.1– 15.4; 37.6 – 167.2; 434 –1823	↓weight (434)	Tyl et al., 2002 [36]
Bisphenol A	Mice	CD-1	diet	Multigeneration reproductive toxicology	112	0.003; 0.03; 0.3; 5; 50; 600	no effect	Tyl et al., 2008 [37]

- **Liver**

COMPOUND	SPECIES	STRAINS	ROUTE	PROTOCOL	TREATMENT (DAYS)	DOSE (mg/kg bw day)	EFFECT	REFERENCE
17β-estradiol	Rats	Crl:CD BR	diet	Subchronic toxicity	90	0.003; 0.139 - 0.173; 0.5 - 0.7; 3 - 4	↑ relative weight; ↑ slight to mild centrilobular hepatocellular hypertrophy (F0, ♂, 0.5)	Biegel et al., 1998 [42]
Zearalenone	Rats	F344/N	diet	Subchronic toxicity	90	0; 2.7; 9; 27; 90; 270	no effect	NTP tr 235, 1982 [66]
Zearalenone	Rats	F344/N	diet	Carcinogenicity	728	1.25; 2.5	↑ Cytoplasmic vacuolization (♂, 1.25; 2.5)	NTP tr 235, 1982 [66]
Zearalenone	mice	B6C3F1	diet	Subchronic toxicity	90	0; 4.5; 15; 45; 150; 450	no effect	NTP tr 235, 1982 [66]

Genistein	**Rats**	**SD**	**diet**	**One-Generation Reproduction Toxicity Study**	**64 (GD7-PND49) (only pups examination)**	**0.3; 1.7; 6.4; 16; 38; 72 Pregnant dams (GD7-parturition)** **0.6; 3.5; 14; 37; 84; 167** **Lactating dams (Pup PND 1 - PND 14)** **0.6; 3; 11; 29; 69; 166 F1 male; PND21-PND50**	**↑ weight (F1♂, 29)**	**NTP tr 79, 2007 [82]**
Genistein	**Rats**	**SD**	**diet**	**Multigenerational reproductive toxicology**	**F0: From PND 42 to PND 140 (98 days)** **F1: From conception to PND 140 (161 days) F2: From conception to PND 140 (161 days) F3: From conception to PND 21, fed control feed from PND 21 to PND 140 (161 days total, 42 days on dosed feed)** **F4: No exposure; control feed from conception to PND 140 (161 days total, no dosed feed)** **F5: No exposure; control feed from conception to PND 21 (42 days total, no dosed feed)**	**m: 0; 0.03; 5; 25**	**↓weight (F4♂, from parent exposed to 25-50);**	**NTP tr 539, 2008 [83]**

Genistein	Rats	SD	diet	Carcinogenicity	F0: From PND 42 until F1 weaning (77 days) F1 (F1C): From conception to 2 years (756 days) F1 (F1T140): From conception to PND 140 (161 days), then fed control diet to 2 years F3 (F3T21): From conception to PND 21 (42 days), then fed control diet to 2 years	pregnant dams: 0; 0.5; 9; 45; lacting dams: 0.7; 15; 75; period prior to PND 140: 0.4;7; 37; period between PND 140 and the end: 0.2; 4; 20	↑ rel. Weight (F1C♂, 20-37): ↑weight (F3T21♂, 45-75); ↓ rel. Weight (F3T21♂, 9-15)	NTP tr 545, 2007 [70]
Genistein	Rats	Wistar	diet	Chronic toxicity	364	5; 50; 500	↓ rel weight (♂, 500 at 132 and 364)	Mc Clain et al., 2006 [71]
Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-21 (no dosing to 365)	0; 0.0025; 0.025; 0.25; 2.5; 27	↑ liver mononuclear cell infiltration (F1♀, 0.0025; 25)	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-365	0; 0.0025; 0.025; 0.25; 2.5; 25	↑liver hepato-diaphragmatic nodule (F1♂,2.5); ↑ liver mononuclear cell infiltration (F1♂ 0.25; 2.5)	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-21 (no dosing to 730)	0; 0.0025; 0.025; 0.25; 2.5; 28	no effect	NTP RR 09; sept. 2018 [86]
Bisphenol A	Rats	SD	gavage	Perinatal and Chronic toxicity	In utero + PND1-730	0; 0.0025; 0.025; 0.25; 2.5; 26	↑liver angiectasia (F1♂; 0.025)	NTP RR 09; sept. 2018 [86]
Bisphenol A	Mice	B6C3F1	diet	Carcinogenicity	730	500; 750	↑multinucleated giant hepatocytes (F♂, 500; 750)	NTP tr 215, 1982 [66]
Bisphenol A	Rats	SD	gavage	One-Generation Reproduction Toxicity Study	GD6-PND90	0.0025; 0.008; 0.025; 0.08; 0.26; 0.840; 2.7; 100; 300	↑ weigh (F1 ♀, 300, PND90); ↓ abs weight (F1♂	Delclos et al., 2016 [39]
Bisphenol A	Mice	CD-1	diet	Multigeneration reproductive toxicology	112	0.003; 0.03; 0.3; 5; 50; 600	↑liver centrilobular hepatocyte hypertrophy; abs weight (F0♂, 50; 600)	Tyl et al., 2008 [37]

3.3 Results of in silico methodology

From molecular docking to ERα, the molecular poses, the binding free energies of each compound and the MMGBSA values were obtained. These energies ranged from -11.3 to -7.2 kcal/mol and are listed in Table 3. The docking data showed that all tested compounds were able to bind the ligand binding domain (LBD) of ERα. 17β-estradiol, the natural hormone, is the top scoring compound for both bind free energy and MMGBSA binding values. Zearalenone and Diethylstilbestrol can be classified as very competitive ligands with respect to 17β-estradiol, while the other chemicals can be classified as good binders (Genistein, Bisphenol A, DEHP and Methoxychlor) or weak binders (Vinclozolin and 4-nonylphenol).

Table 3. Computed binding free energies of each ligand with hERα

Ligand	***XP GScore****	***MMGBSA dG Bind**#**	***K_i (M)°***
17β –estradiol	-11.3	-78.6	4.6E-09
Zearalenone	-11.3	-57.2	4.8E-09
Diethylstilbestrol	-11.0	-59.1	8.4E-09
Genistein	-9.9	-48.4	4.9E-08
Bisphenol A	-9.7	-47.0	7.6E-08
DEHP	-9.4	-2	1.2E-07
Methoxychlor	-9.0	-37.8	2.5E-07
Vinclozolin	-8.3	-43.4	8.1E-07
4-nonylphenol	-7.2	-46.3	5.4E-06

*** Extra Precision Glide score: approximates a very close and entire systematic search for the conformational, orientational and positional space of the docked ligand. Glide utilizes a series of hierarchical filters to search for possible locations of the ligand in the active-site region of the receptor.**

 # MM-GBSA, molecular mechanics-generalized Born surface area: calculates binding free energies for molecules by combining molecular mechanics calculations and continuum (implicit) solvation models (Implicit solvent models are often used to estimate free energies of solute-solvent interactions and significantly improve the computational speed and reduce errors in statistical averaging that arise from incomplete sampling of solvent conformations.

° Dissociation constant computed starting from empirical binding free energy values (*XP GScore).* The smallest value means that the compound has a larger affinity at lower concentration.

Affinity is inversely proportional to the binding free energy. Smaller is the binding free energy value (the most negative), higher is the affinity.

The dissociation constant (K_i) was computed starting from empirical binding free energy values (XP GScore), according to the following equation: $\Delta G = RT \ln(K_i)$,

where R represents the gas constant and T the temperature. Ki was computed starting from the binding free energy values at a fixed temperature (300 K). The smallest value means that the compound has a larger affinity at lower concentration.

A carefully inspection of the Table 3 has highlighted some interesting differences in the affinity calculated by XP GScore and MMGBSA dG Bind , respectively.
These differences in the affinities values between these two docking protocols were due to that MMGBSA docking protocol take into account also the solvation effect of the solvent (water molecules) towards the ligands, computing both the solvent/LBD and ligand/solvent interactions. In our results (Table 3), 17β-estradiol, as expected, showed the highest affinity values in both approaches as, being the physiological hormone was able to contrast the solvation effect. The other ligands had greater values of MMGBSA scores because they, in the modelling conditions set, were not able to displace the water molecules into the LBD. Indeed, the binding free energy of DEHP (as XP GLIDE SCORE) was in the same range of BPA and Genistein while MMGBSA dG values of DEHP had the highest value (-2 kcal/mol). Thus, DEHP seems to remain in a soluble condition and it is not able to displace the water molecules into the LBD. There is a concordance between *in silico* results and literature data, where DEHP showed a high affinity to androgen receptor [see paragraph 1.7]. Zearalenone showed a binding free energy similar to that of the 17- β-estradiol but the MMGBSA score was less negative than the endogenous hormones because of the solvation effect.
The dissociation constant (Ki) was computed starting from empirical binding free energy values (XP GScore), according to the following equation:

$$\Delta G = RT \ln(K_i)$$

where R represents the gas constant and T the temperature. K_i was computed starting from the binding free energy values at a fixed temperature (300 K). The smallest value means that the compound has a larger affinity at lower concentration. Then I retrieved from the literature the K_i values experimentally determined in vitro binding assay by other research groups (Table 4). These were compared with the computed K_i values to make a validation of the *in silico* molecular modelling approach.

Table 4. in silico calculated Ki and experimental Ki from literature*

Ligand	*Calculated K_i (M)#*	*In vitro experimental K_i (M)#**
17β –estradiol	4.6E-09	1.3E-10
Zearalenone	4.8E-09	8.0E-10
Diethylstilbestrol	8.4E-09	4..0E-11
Genistein	4.9E-08	2..6E-9
Bisphenol A	7.6E-08	1..95E-07
Methoxychlor	2.5E-07	1.174E-06

The smallest value means that the compound has a larger affinity at lower concentration.

*Kuiper et al., 1996, [10]

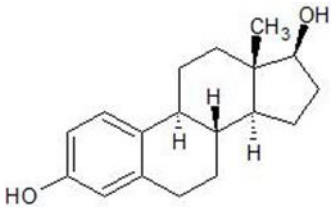
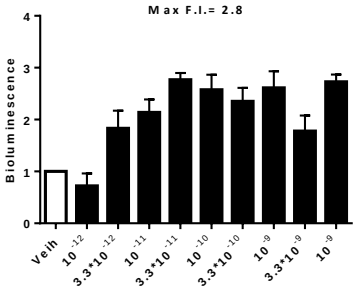
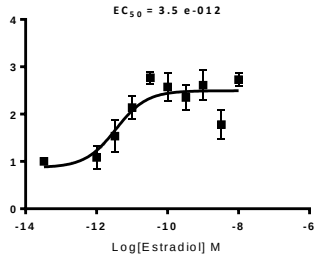
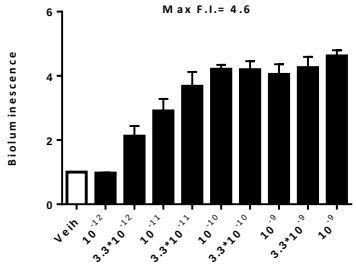
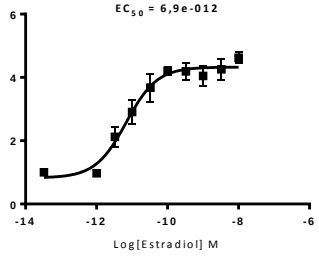
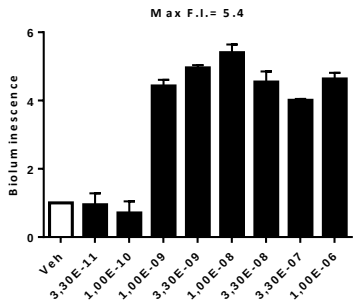
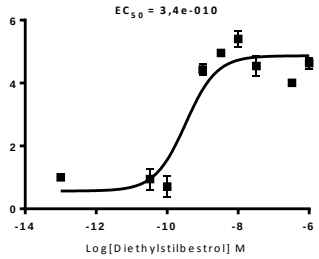
From these comparisons, it could be noted that there is a good concordance between predicted and experimental values. Thus, the *in silico* K_i seems reliable and could be used as a parameter for the prediction of putative endocrine disruptor activity. 17β-estradiol showed a very small K_i value in both experimental assays (data from literature), as well as in the in silico molecular docking. It is also known that 17β-estradiol is a potent endocrine disruptor that causes several toxic effects such as the decreasing of weight of seminal vesicles at very low concentration (see table 2 of apical target of EDCs). For this reason, 17β-estradiol is used a positive control in toxicology. Experimental K_i of genistein was smaller than the computed ones but very close to the experimental K_i value of endogenous hormone, whereas it is similar to the computed K_i of estradiol. Based on this observation, genistein should decrease the weight of seminal vesicles as the 17β-estradiol does. However, from the well conducted and highly reliable NTP studies (reported in table 2 above), genistein does not display any effect on seminal vesicles; thus, even if genistein has a low K_i (computed and experimental) with a potential ED activity, it cannot be considered as 17β-estradiol in term of ED activity, but simply a good binder to ERα. The same observation could be done also for Bisphenol A, whereas the computed K_i is smaller than its experimentally calculated value and it is 1 order of magnitude greater of the computed K_i of estradiol. From the carcinogenesis NTP study of 2 years treatment (see table 2, seminal vesicles) BPA does not alter the physiology of seminal vesicles as estradiol, although that study mice were treated with high doses

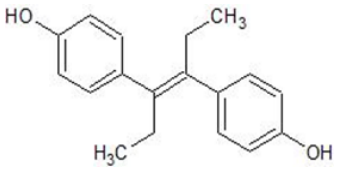
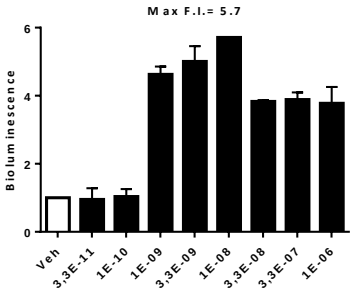
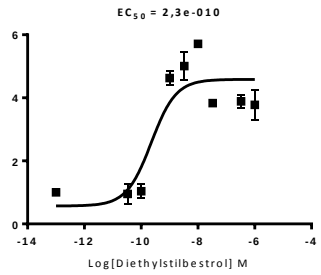
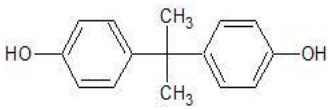
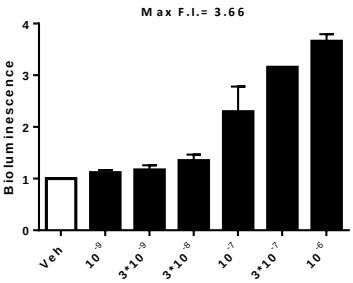
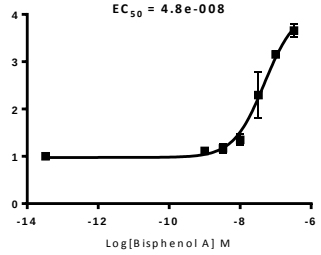
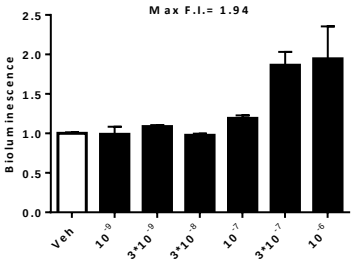
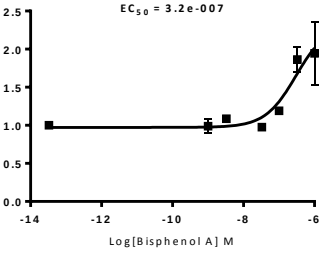
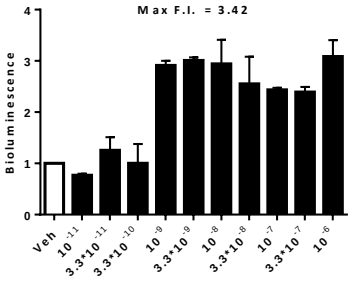
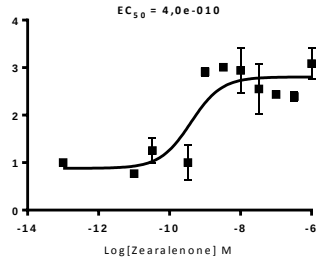
up to 25 mg/kg bw day. Thus, even if BPA binds with a good affinity to ERα, it has not the toxicant effects on the seminal vesicles observed with 17β-estradiol.
On the contrary with what found with BPA, methoxychlor has the highest value of both K_i (calculated in silico and in vitro), thus it is a very weak binder. Nevertheless, Chapin et al., 1997 [54] found out that methoxychlor administered at 50 and 100 mg/kg bw day from GD14 to PND 42 caused a decrease of testis, seminal vesicles and prostate weight leading to conclude that methoxychlor could affect the endocrine organs only at higher concentration compared to Estradiol [54].
Zearalenone is known to be an ED, and its K_i (both experimental and computed) are in the same range of 17β-estradiol. Zearalenone was not able to affect the weight of seminal vesicles but caused other alterations possibly ER-mediated.
The data are indicating that both computed and experimental K_i could be properly used as a parameter to classify the chemicals according to their binding ability to ERα; however, in the light of the evidences regarding the lack of correlation between the binding ability (computed or experimental) and the ED effects observed in vivo, it can be concluded that the Ki parameters do not have a predictive value of the ED activity of the compounds.

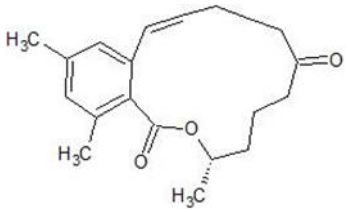
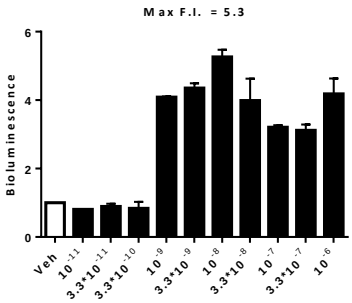
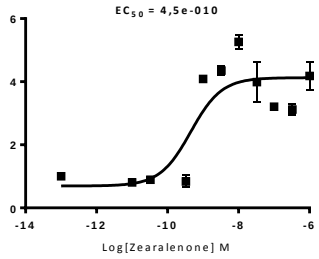
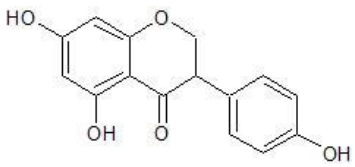
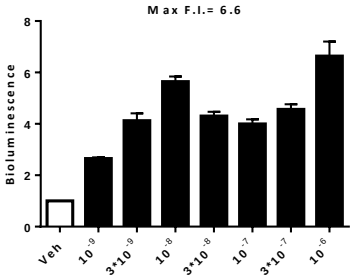
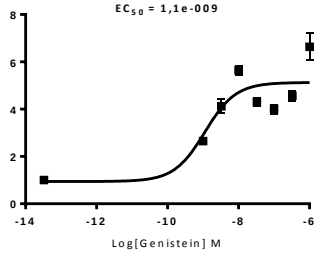
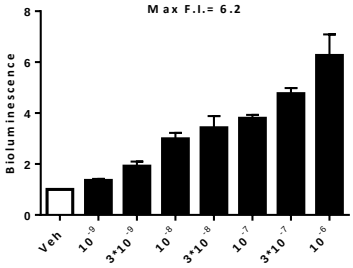
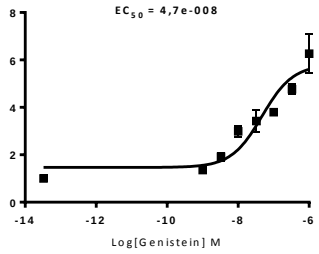
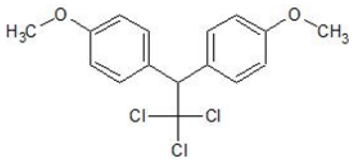
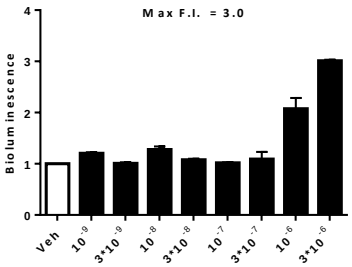
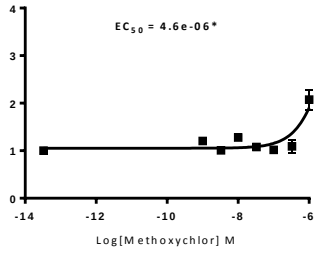
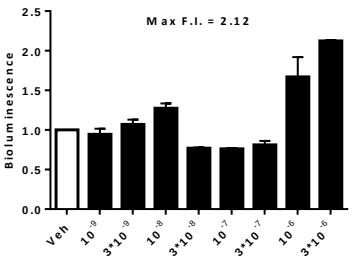
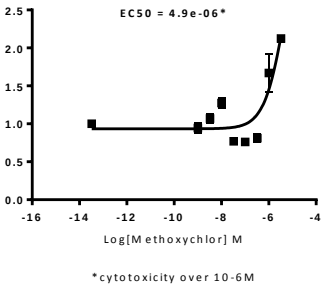
3.4 Results of in vitro bioluminescence imaging assay

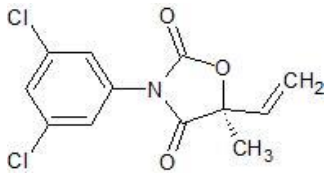
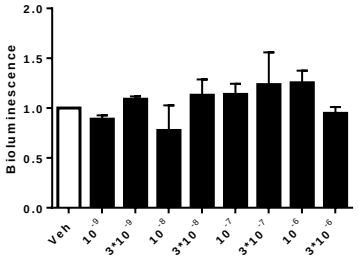
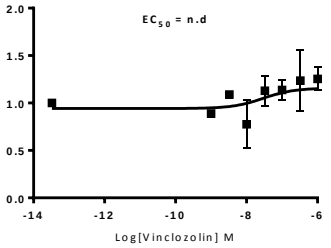
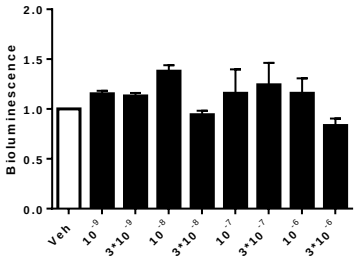
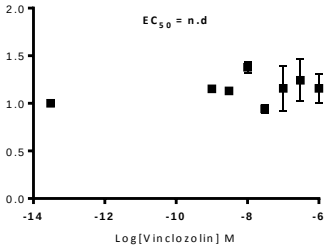
The ability of the selected compounds to transcriptionally activate ERα was tested in ERE-Luc B17 cells, a sub-clone of the breast cancer cell line MCF-7, stably transfected with a luciferase reporter system under control of an estrogen-regulated synthetic promoter [76]. Photon emissions were recorded and quantified by a CCD camera upon adding to the medium the substrate luciferin as described in material and methods. B17 cells were treated with increasing concentrations (6-10 depending on the in vitro toxicity) of each compound. Photon emission values were acquired before treatment and at 6 and 48 hours; values were normalized on the photon emissions measured before treatment (set as 1). The EC50 values of ERα activation were calculated using GraphPad Prism v.7 program and are reported in Figure 9 and summarized in Table 5.

Figure 9. Graphical representation of fold induction (curve dose-response) and potency (EC50) calculated at 6 and 48 hours after treatment with tested compounds.

Compound	Time	Dose-response	EC_{50}(M)
17β-estradiol 	6h		
	48h		
Diethylstilbestrol	6h		

	48h		
Bisphenol A 	6h		
	48h		
Zearalenone	6h		

	48h		
Genistein 	6h		
	48h		
Methoxychlor 	6h		
	48h		

Vinclozolin 	6h		
	48h		

Each data represents the bioluminescence of each doses tested, bars represent the average ± SEM. GraphPad Prism v. 7 was used.

Then the chemicals were ranked for their estrogenic potency at 6 and 48 h treatment. At 6 h the results were as follows: 17β-estradiol > DES > zearalenone > genistein > bisphenol A > methoxychlor (Figure 10a and b). 4-nonylphenol concentrations were cytotoxic at both time of exposure. DEHP and vinclozolin did not activate the ERα (EC50 not detectable at both time of exposure). At 48 hours after treatment the potency rank did not change. The potency data revealed that the estrogenic potencies of 17β-estradiol, diethylstilbestrol, zearalenone did not significantly change in the time course from 6 to 48 hours, while the potency of genistein and bisphenol A decreased of one order of magnitude. EC50 of Methoxychlor, at micromolar concentrations, appeared to be stable at 6 hours and 48 hours. DEHP, Vinclozolin and 4-nonylphenol did not induce any activation of estrogen receptor (Figure 9; Table 5). These last findings are in line with the literature, where it is reported a preferential action on the androgen receptor (AR) signaling by DEHP and Vinclozolin [57-60], and the lack of estrogenic activity, in general, of alkylphenols [61].

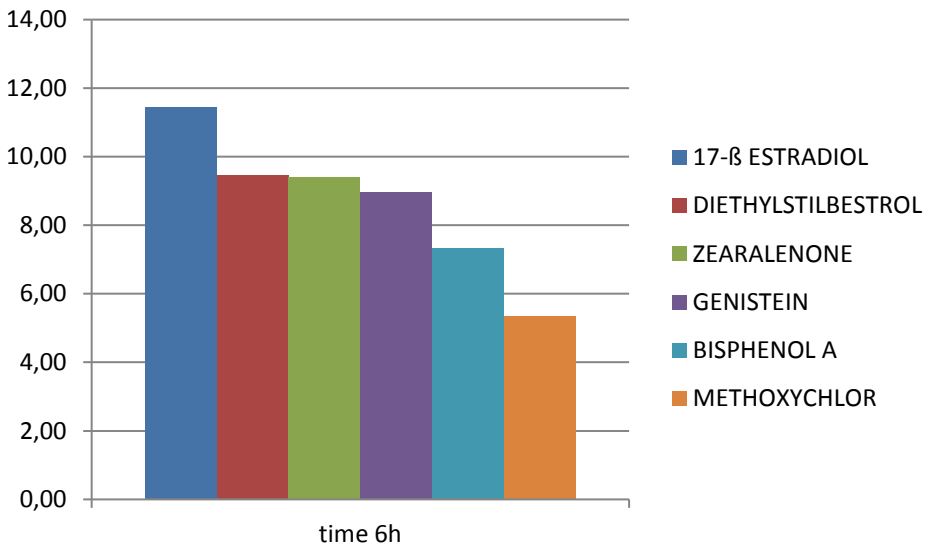


Figure 10 a. Ranking of EC50s values at 6 hours after treatment in B17 clone MCF-7 cell lines of selected compounds. 17β-estradiol (most potent) > DES > zearalenone > genistein > bisphenol A > methoxychlor. DEHP, Vinclozolin and 4-nonylphenol were not ranked because of the missing of estrogen receptor activation or cytotoxicity.

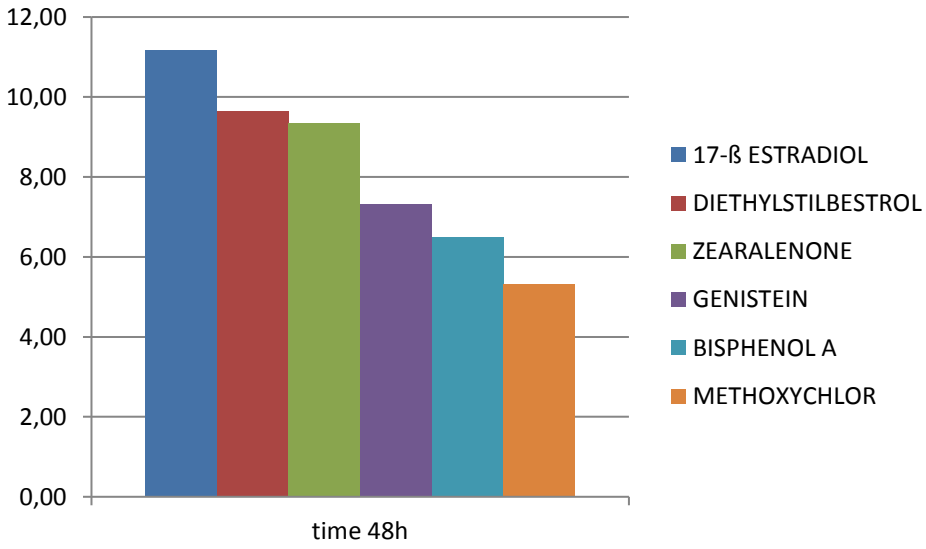


Figure 10 b. Ranking of EC50s values at 48 hours after treatment in B17 clone MCF-7 cell lines with increasing concentrations of selected compounds. 17β-estradiol (most potent) > DES > zearalenone > genistein > bisphenol A > methoxychlor DEHP, Vinclozolin and 4-nonylphenol were not ranked because of the missing of estrogen receptor activation or cytotoxicity.

Table 5) Potency and efficacy values of the selected compounds to activate the ERE-Luc system in B17 MCF-7 cell lines. The values of fold induction represent the maximum activation of our cellular reporter system. 17β-estradiol is the reference positive control.

	POTENCY		EFFICACY	
COMPOUND	EC50 6h	EC50 48h	F.I. 6h	F.I. 48h
17ß - ESTRADIOL	3.50E-12	6.90E-12	2.80	4.60
DIETHYLSTILBESTROL	3.40E-10	2.30E-10	5.40	5.70
ZEARALENONE	4.00E-10	4.50E-10	3.42	5.30
GENISTEIN	1.10E-09	4.70E-08	6.60	6.20
BISPHENOL A	4.80E-08	3.20E-07	3.66	1.94
METHOXYCHLOR	4.60E-06	4.90E-06	3.00	2.12
BIS-(2-ETHYLHEXYL) PHTHALATE	n. d	n. d	n.d	n.d.
4-NONYLPHENOL	n.d	n. d	n.d	n.d.
VINCLOZOLIN	n.d	n. d	n.d.	n. d

EC50 6h = concentration of the chemical that gives half-maximal response detected at 6 hours of treatment; EC50 48h = concentration of the chemical that gives half-maximal response detected at 48 hours of treatment; F.I. 6h = fold induction (maximal luciferase activity) detected at 6 hours of treatment; F.I. 48h = fold induction (maximal luciferase activity) detected at 48 hours of treatment.

This work for the first time analysed the ER activity in time; the results indicated that while the rank of the potency did not change, the potency itself for some compound decreased during time suggesting a depotentiation mechanism is operating for some but not all the compound tested. Initially, it was hypothesized that this observation could be due to the compound metabolism that could be different among the compounds. To test the hypothesis, a LC-MS analysis was performed by the Laboratory of Mass-Spectrometry of Prof.ssa Donatella Caruso. Analysis were done on the medium and on the cellular total extracts of MCF7-B17 treated with Bisphenol A, Zearalenone and Genistein at 6 and 48 hours. Qualification and quantification of the parental compounds by specific analytical condition set up in LC-MS technique (these data are shown in APPENDICES section to this thesis). These analytical data completely ruled out the possibility that the depotentiation mechanism was due to breakdown of the molecules, while it appears that was more likely due to changes at transcriptional level occurring on the receptor in time.

In all previous experiments, the ability of a compound to activate or inhibit ER was considered at a single time point. Taking into account this static observation, the rank of the potency at 6 or at 48 hours (Figure 10 a and b) compared with the ED effects observed in vivo (Table 2) of the different compounds gave similar non-predictive results for ED activity as those obtained with the calculated (and experimental) Ki parameter (see paragraph 3.2). For example, considering the potency of genistein and zearalenone at 6 hours, they are potent ER activators while only zearalenone displays a potent ED effects (Table 2).

The dynamic analysis of ER activation allows to define novel parameters for the compound action through the receptor taking into account the change in potency and efficacy in time. By analysing the single parameter in time (potency or efficacy) and ranking the substances based on the magnitude of decrease observed can already discriminate from active EDs like 17β-estradiol, diethylstilbestrol and zearalenone from those that display weak or none ED effect genistein, bisphenol A and methoxychlor (Table 6a). While the efficacy table (Table 6b) is less prompt to highlight the differences observed as ED effects between genistein and the other disruptors. By combining the dynamic of potency and efficacy parameters these differences were magnified (Table 7) and ranking the compounds with a parameter which take into account the variation of potency and efficacy as well as the absolute value of efficacy after 48 h, it is possible to observe a clear classification of active ED compounds with those that does not have effect (genistein) or have effects only at very high doses (e.g. methoxychlor).

Table 6a. Ranking of selected chemicals based on the magnitude of the potencies (EC50s) variations. The right column reported the magnitude of the potencies variations during the time course from 6 to 48 hours. The colours represent the relationship between the ability to activate the estrogen receptor and the endocrine disrupting activity (ED) observed in vivo on male reproductive organs. Red = very high ED; Orange = high ED; Green = no-ED

COMPOUND	-log potency 6h	-log potency 48h	potency decreasing/increasing
DIETHYLSTILBESTROL	9.47	9.64	0.17 ↑
ZEARALENONE	9.40	9.35	-0.05 ↓
17ß-ESTRADIOL	11.46	11.16	-0.29↓
GENISTEIN	8.96	7.33	-1.63↓↓
BISPHENOL A	7.32	6.49	-0.82↓
METHOXYCHLOR	5.34	5.31	-0.03

Table 6b. Ranking of selected chemicals based on the magnitude of the efficacy variation. The right column reported the quantification of the efficacy variations during the time course from 6 to 48 hours.
The colours represent the relationship between the ability to activate the estrogen receptor and the endocrine disrupting activity (ED) observed in vivo on male reproductive organs. Red = ED; Green = no - ED

COMPOUND	efficacy 6h	efficacy 48h	Efficacy decreasing/increasing
ZEARALENONE	3.42	5.30	1.88 ↑
17ß-ESTRADIOL	2.80	4.60	1.80↑
DIETHYLSTILBESTROL	5.40	5.70	0.30 ↑
GENISTEIN	6.60	6.20	-0.4
BISPHENOL A	3.66	1.94	-1.72↓
METHOXYCHLOR	3.00	2.12	-0.88↓

Table 7. Classification of ED compounds based on the combination of the variation of potency and efficacy in the time course with the absolute value of efficacy after 48h. The colours represent the relationship with the endocrine disrupting activity (ED) observed in vivo on male reproductive organs. Red = ED; Green = no-ED.

COMPOUND	RATIO POTENCY 48/6	RATIO EFFICACY 48/6	RATIO POTENCY*RATIO EFFICACY	RATIO POTENCY*RATIO EFFICACY*EFFICACY AT 48H
ZEARALENONE	0.99	1.55	1.54	8.17
17-ß ESTRADIOL	0.97	1.64	1.6	7.36
DIETHYLSTILBESTROL	1.02	1.06	1.07	6.12
GENISTEIN	0.82	0.94	0.77	4.76
METHOXICHLOR	0.99	0.71	0.7	1.49
BISPHENOL A	0.89	0.53	0.47	0.91

Thus, the combination of the variation of the potency and the efficacy "normalized" respect to the efficacy values at 48h was successfully used in discriminating positive and negative compounds for their endocrine disrupting activity as showed in table 7. It must be noted that compounds with no ED effect in vivo such as BPA or with a putative ED effect only at high level of exposure (not relevant for the humans), such as methoxychlor, have a very low score (right column) when compared to chemicals with known ED effect in vivo (estradiol, DES, zearalenone). Thus, these chemicals occupied the lowest position in table 7 (green lanes), and by this classification, resulted very interesting the score of genistein. It follows the score of DES, but it is higher than methoxychlor and BPA. Genistein has not any ED effect in vivo even if it is a good binder of ER. So, the position of this phytoestrogen could represent a putative threshold of non- concern for ED effect. Over this threshold there is ED effect. On the contrary below this threshold the chemical could not have any ED effect.

This in vitro classification fitted well with the *in silico* outcome where the strongest binders where ranked in the first position.

From these data, we may conclude that the novel dynamic ER transactivation assay is best marker of an ED activity than the classical transactivation or binding (computational or in silico) assays.

3.5 Results of in vivo bioluminescence imaging experiments

In transgenic reporter mice [76] already described in material and methods section, similar reporter-based biosensors like the one described in the transactivation assay were inserted in the genome of the mouse. In these reporter mice the luciferase expression is proportional to the activity of ER (ERE-Luc), NFkB (NFkB-Luc) rand Nrf2 (ARE-Luc), thus reporting the modulation of estrogen receptor, inflammation and oxidative stress pathway in any mouse tissue. To test the effects of Zearalenone, Bisphenol A, 17β-estradiol on these signals 4 reporter mice/experimental group were treated daily with two dosages of each substance administered in the drinking water for 21days. The compounds and their doses were chosen combining data obtained from the literature with in vitro results (see also Table 2). Since testing many doses was not possible for budget reason, we decided to choose a threshold dose, more realistic in term of human exposure, and a high dose, to force the system and see an effect, in case there was a low sensitivity. Zearalenone was both a strong binder (Ki was 4.8E-09 M) and activator of ER (EC_{50}s were 4.00E-10 M and 4.50E-10 M at 6 and 48 hours, respectively), similarly to endogenous hormone values. This mycotoxin is known to be an endocrine disruptor because of the adverse effect on female reproductive tract of immature pigs, thus it was interesting to investigate if it could be a putative ED on male reproductive organs. The doses of zearalenone were obtained considering to cover a low range of active concentrations retrieved in the literature (see table 2). Bisphenol A was a good binder in silico model (Ki was 7,6E-08 M) and moderate activator of ER in vitro assay (EC_{50}s were 4.80E-08 M and 3.20E-07 M at 6 and 48 hours, respectively). BPA was selected instead of Genistein, which showed good results from the previously approaches, because of this phytoestrogen is considered to have no ED effect or toxicity to human according to EFSA's opinion [73], even if it has an interesting action on ER such

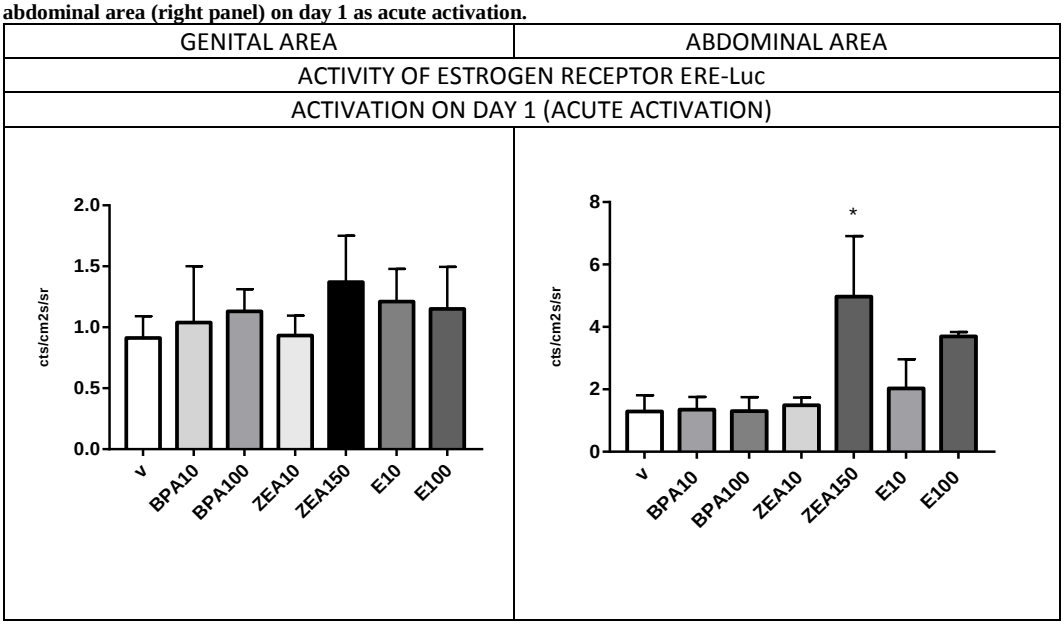
as demonstrated in our in silico and in vitro experiments. The BPA doses were selected to cover a low dose range of putative active concentrations retrieved in the literature (see also table 2).

The activation of these pathways was measured daily in each single mouse by bioluminescence in vivo imaging (see material and methods) for the 21 days of the experiment. After 21 days the animals were sacrificed, and organs were taken for ex vivo bioluminescence imaging analysis of the photon emission. Photon emission from the mouse body was quantified from the genital and abdominal areas in vivo and from prostate, testis, seminal vesicles and intestine ex vivo. Photon emission levels in time were normalized on the value measured before treatment (set to 1) in each animal. I have considered as parameters of activation: I) the acute effects observed after 24 hours, ii) the AUC over the 21 days, iii) the emission of the final day (day 21) and iv) the ex vivo photon emission measured from the explanted organs.

In the genital area the bioluminescence activity of estrogen receptor ERE-Luc at day 1 (acute activation), showed the presence of a trend of BPA10, BPA100; ZEA150, E10 and E100. Instead, in the abdominal area the ZEA150 showed a marked induction.

The ER signaling was clearly activated after 24 hours in the abdominal area by high doses of ZEA and E2 given per os, while no activation was visible by any treatment in the genital area of the mouse (Figure 11). Similar data were obtained analysing the AUC of the activation over the 21 days-acquisitions (Figure 12). The acquisition at day 21 (Figure 13) was demonstrating a persistent effect of ZEA and E2 on the abdominal area and a non-significant trend of increase the activity in the genital area for some treatment.

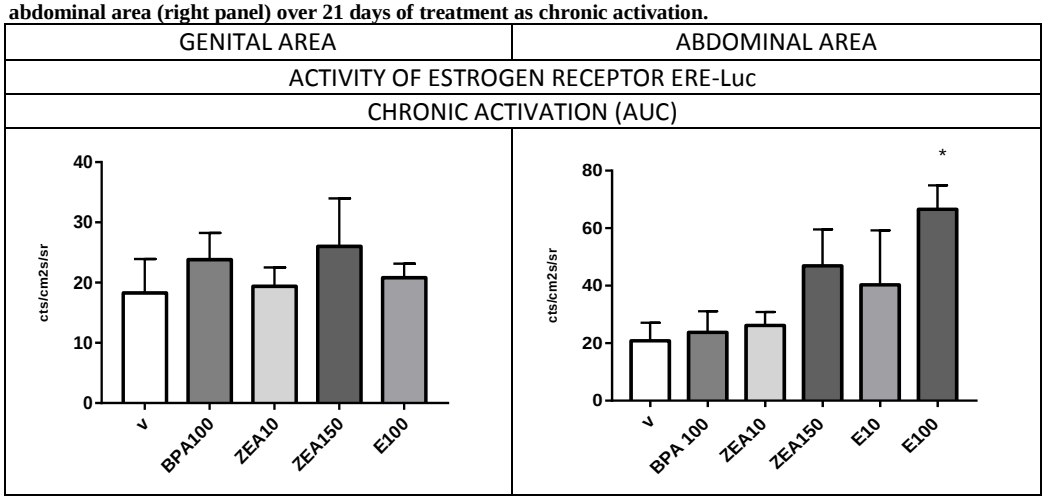
Figure 11. Quantification of bioluminescence of hormonal ERE-Luc pathway in genital area (left panel) and abdominal area (right panel) on day 1 as acute activation.



V = vehicle; BPA10 = bisphenol A 10 mg/Kg/day; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day. The values represent the means of photon emissions ± SEM calculated with GraphPad Prism v.7. *p<0.05 vs vehicle applying Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

In the chronic activation phase (Figure 12), the values of interactions with the reporter system are expressed as Area Under the Curve (AUC) calculated by the program GraphPad Prism v.7. In the genital area there was not a significant activity even if ZEA150 seemed to maintain its activation as on day 1. In the ERE-Luc of abdominal area E100 finally produced an important statistically significant induction of ER versus the vehicle ($p < 0.05$). On the contrary to day 1, ZEA150 showed a good activation. The other doses did not produce any induction.

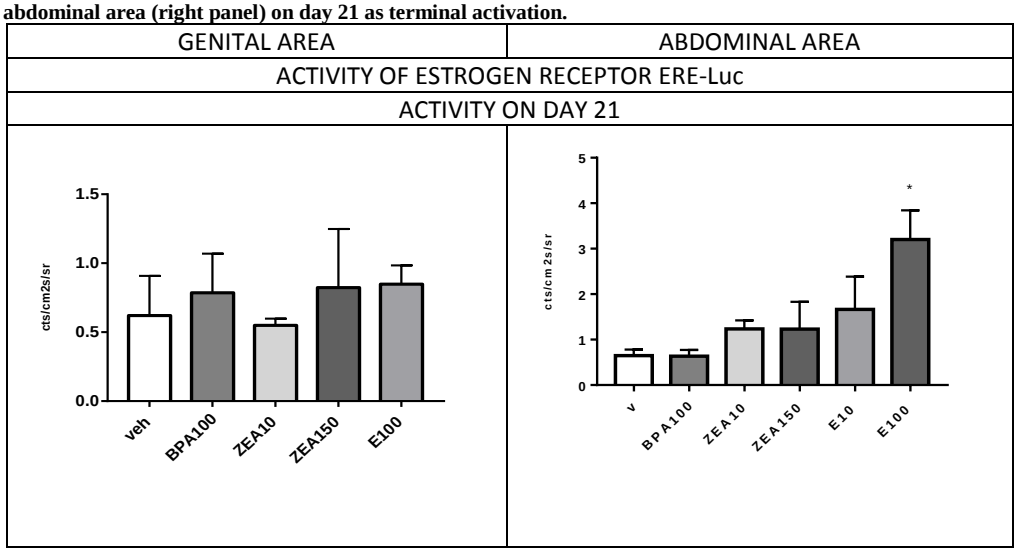
Figure 12. Quantification of bioluminescence of hormonal ERE-Luc pathway in genital area (left panel) and abdominal area (right panel) over 21 days of treatment as chronic activation.



V = vehicle; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day.
The values represent the means of photon emissions ± SEM calculated with GraphPad Prism v.7. *p< 0.05 vs vehicle calculated applying Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

In the analysis of terminal day 21 (Figure 13), only in the abdominal area the treatment E100 prolonged its activity, while the other treatments decreased the potency till control baseline in both observed area

Figure13. Quantification of bioluminescence of hormonal ERE-Luc pathway in genital area (left panel) and abdominal area (right panel) on day 21 as terminal activation.

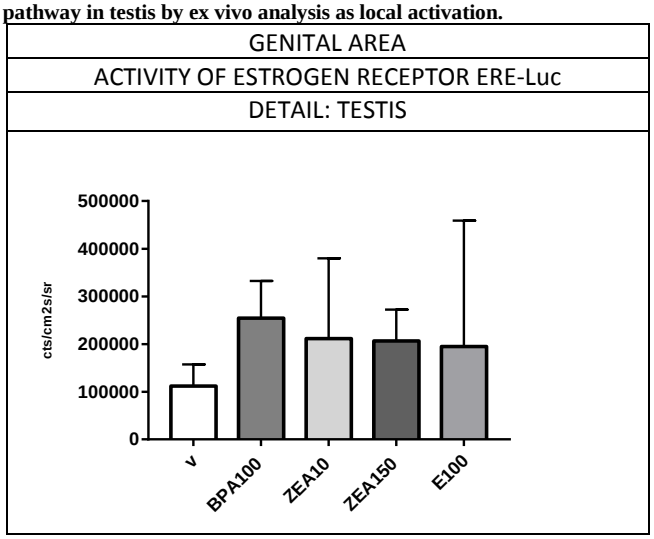


V = vehicle; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day. The values represent the means of photon emissions ± SEM calculated with GraphPad Prism v.7. *p< 0.05 vs. the vehicle calculated applying Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

The analysis of each single organ ex vivo demonstrated a weak trend of receptor activation in testis (Figure 14) and prostate (Figure 15). ZEA150 had a good but no

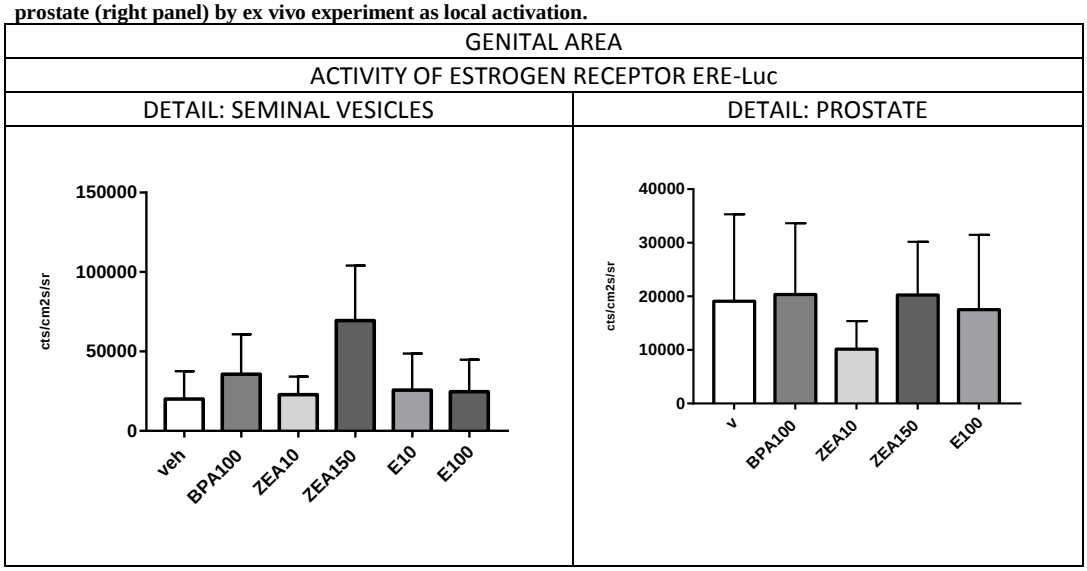
statistically significant activation in seminal vesicles (Figure 15). No interesting signaling were detected in the seminal vesicles by other treatments (Figure 15).

Figure 14. Quantification of bioluminescence of hormonal ERE-Luc pathway in testis by ex vivo analysis as local activation.



V = vehicle; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day. The values represent the means of photon emissions ± SEM calculated with GraphPad Prism v.7 and analysed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

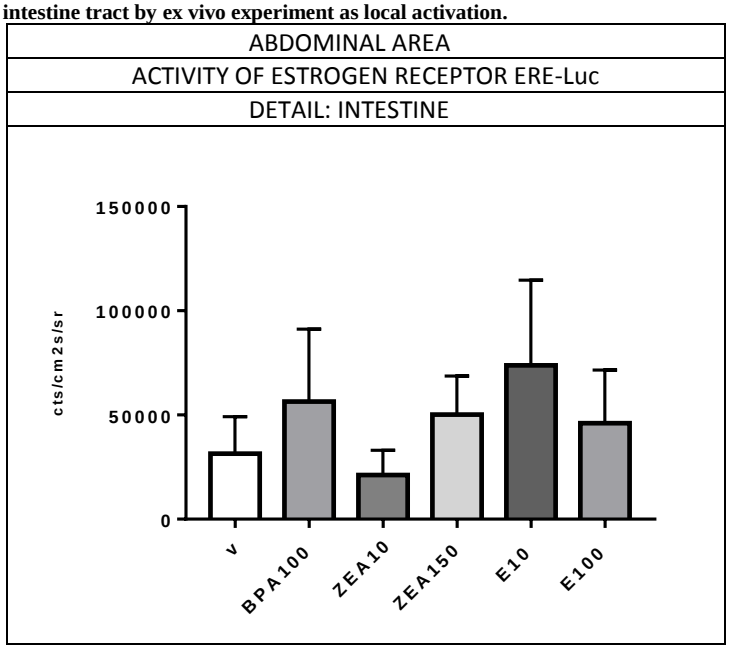
Figure 15. Quantification of bioluminescence of hormonal ERE-Luc pathway in seminal vesicles (left panel) and prostate (right panel) by ex vivo experiment as local activation.



V = vehicle; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day.
The values represent the means of photon emissions ± SEM and analysed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

In the intestine, BPA100 and E10 showed an receptorial ER activation but as the other doses, they did not produce a significant ER signaling (Figure 16). The activation observed by in vivo imaging analysis of E2 and ZEA150 in the abdominal area were not due to the intestine but possibly are mediated by other organs, e.g. kidney, live or spleen which express also the estrogen receptors.

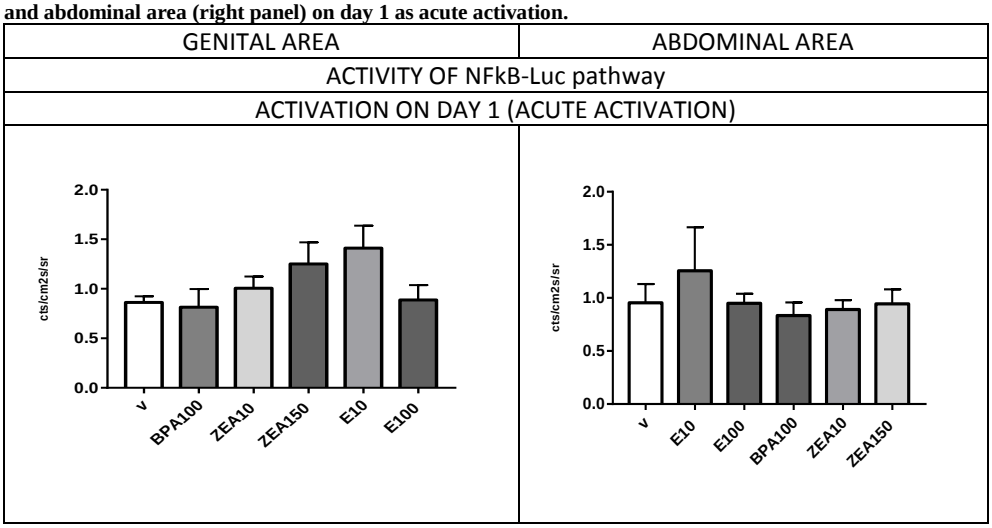
Figure 16. Quantification of bioluminescence of hormonal ERE-Luc pathway in intestine tract by ex vivo experiment as local activation.



V = vehicle; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day.
The values represent the means of photon emissions ± SEM and analyzed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

No significant activation of the inflammatory (NFkB) pathway was observed after acute administration of the compounds in both genital and abdominal areas even if E10 seemed to produce an interesting signaling (Figure 17).

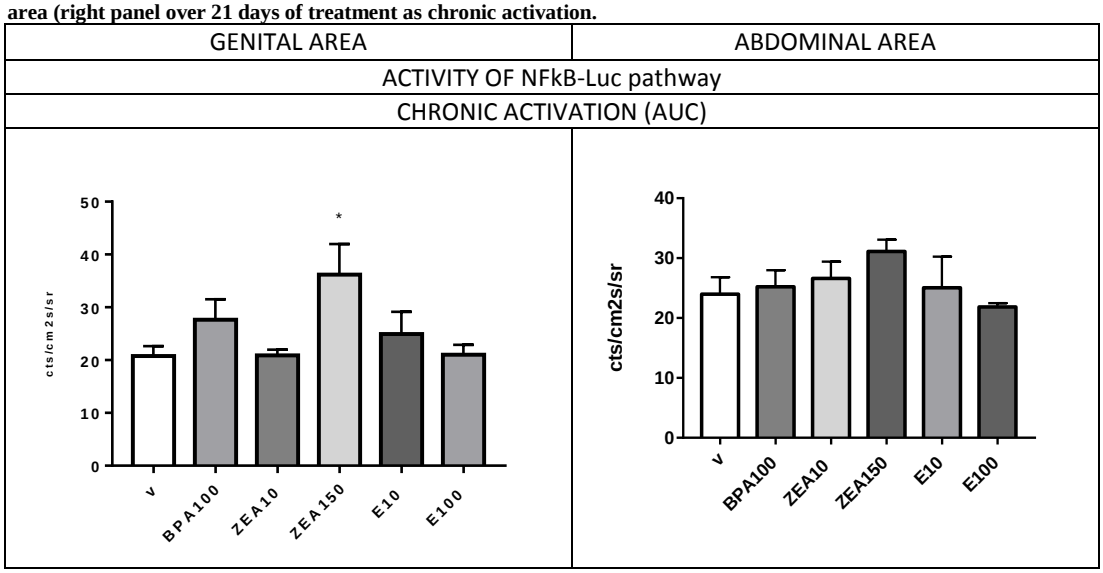
Figure 17. Quantification of bioluminescence of inflammatory (NFkB) pathway in genital area (left panel) and abdominal area (right panel) on day 1 as acute activation.



V = vehicle; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day.
The value represents the means of photon emissions ± SEM and analyzed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

The AUC analysis, which represents the whole activation of reporter system over the 21 days of repeated treatments, showed a clear activation of the inflammatory (NFkB) pathway upon administration of the highest dose of zearalenone, but only in the genital area (Figure 18).

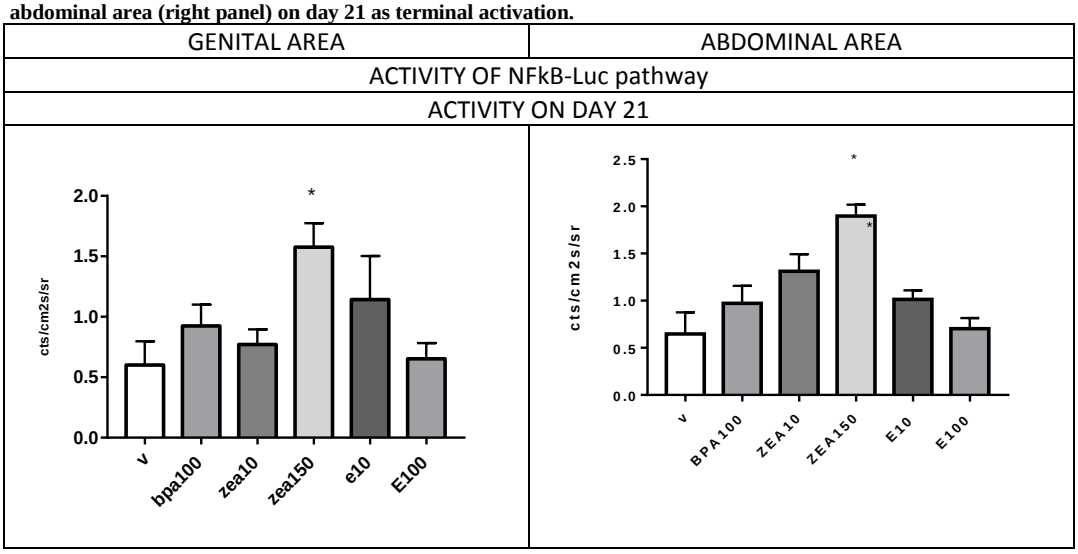
Figure 18. Quantification of bioluminescence of inflammatory (NFkB) pathway in genital area (left panel) and abdominal area (right panel over 21 days of treatment as chronic activation.



V = vehicle; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day.
The values represent the means of photon emissions ± SEM and analyzed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

Interestingly, at day 21 the NFkB pathway was found significantly active in the genital and abdominal area of the highest dose of the zearalenone group and on the low-dose group in the abdominal area. No other significant activation could be detected with the other treatment (Figure 19).

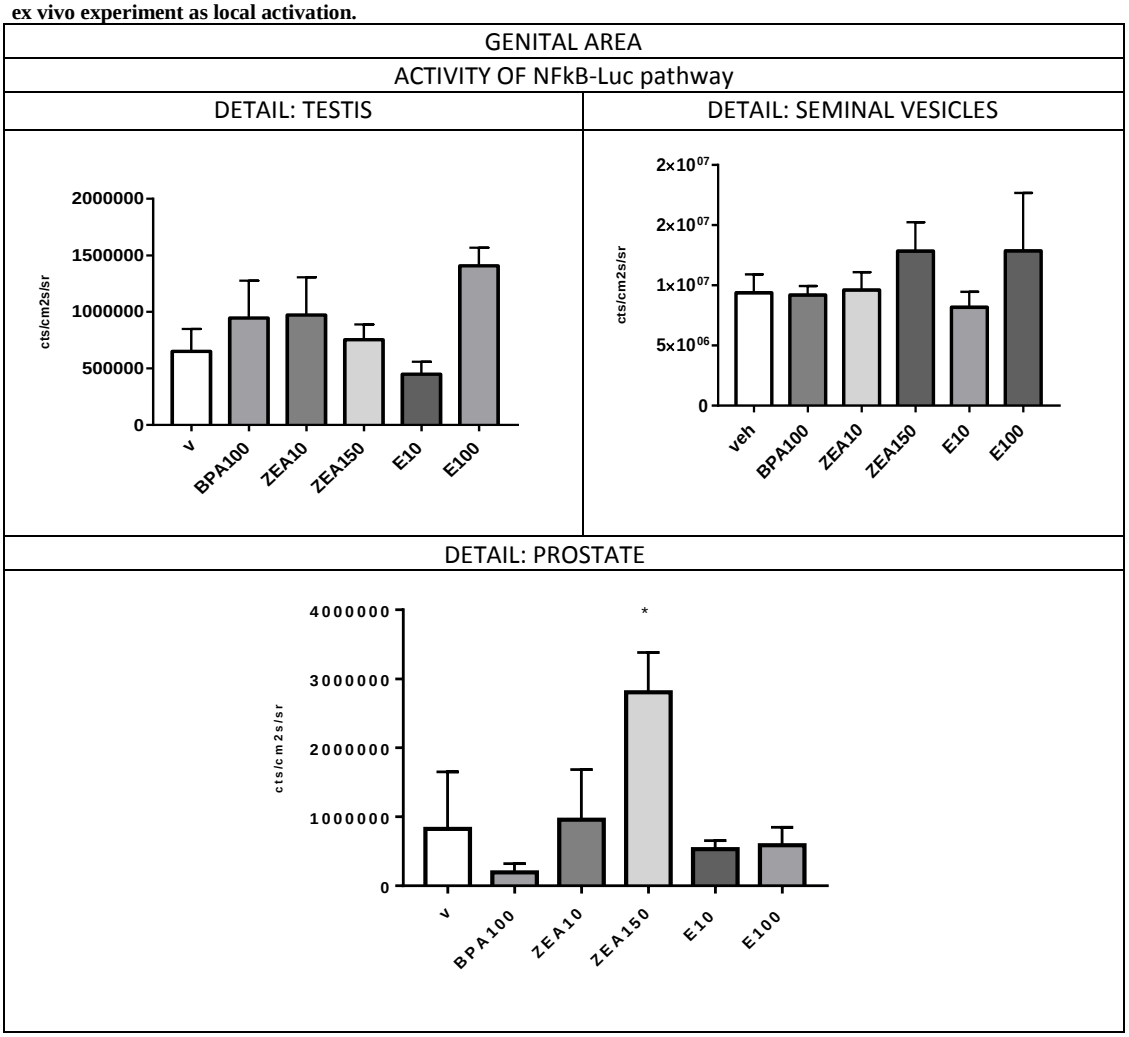
Figure 19. Quantification of bioluminescence of inflammatory (NFkB) pathway in genital area (left panel) and abdominal area (right panel) on day 21 as terminal activation.



V = vehicle; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day. The values represent the means of photon emissions ± SEM and analyzed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test (*p < 0.05 vs vehicle and abdominal area *p< 0.05 vs. vehicle).

The ex vivo bioluminescence imaging analysis of inflammatory pathway demonstrated that the significant activations produced by ZEA150 in the genital area were likely due to the NFkB signalling in the prostate (Figure 20). The other treatments did not produce any significant activation.

Figure 20. Quantification of bioluminescence of inflammatory (NFkB) pathway in testis, seminal vesicles and prostate by ex vivo experiment as local activation.

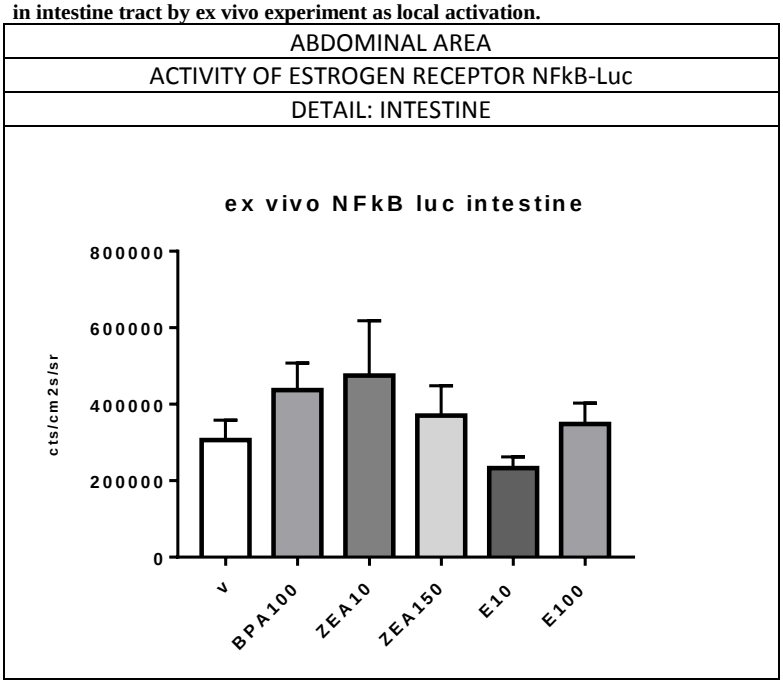


V = vehicle; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day.
The values represent the means of photon emissions ± SEM and analysed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test. Testis: *p < 0.051 vs. vehicle; prostate *p < 0.05 vs. vehicle.

The findings that ZEA150 was able to activate the inflammatory pathway in the genital area with a specific local effect in the prostate was very particular. Since no activity of the ER signaling was detected in the prostate by both doses of ZEA150, we may conclude that the strong NFkB activity elicited over the 21 days-treatment was due to the activation of other receptors (e.g. androgen receptor).

In the ex vivo NFkB analysis of intestine no significant signal was detected. This result explains that highest activation of ZEA150 at day 21 maybe was due to other organs in abdominal area (e.g. kidney, spleen, liver).

Figure 21. Quantification of bioluminescence of inflammatory (NFkB) pathway in intestine tract by ex vivo experiment as local activation.

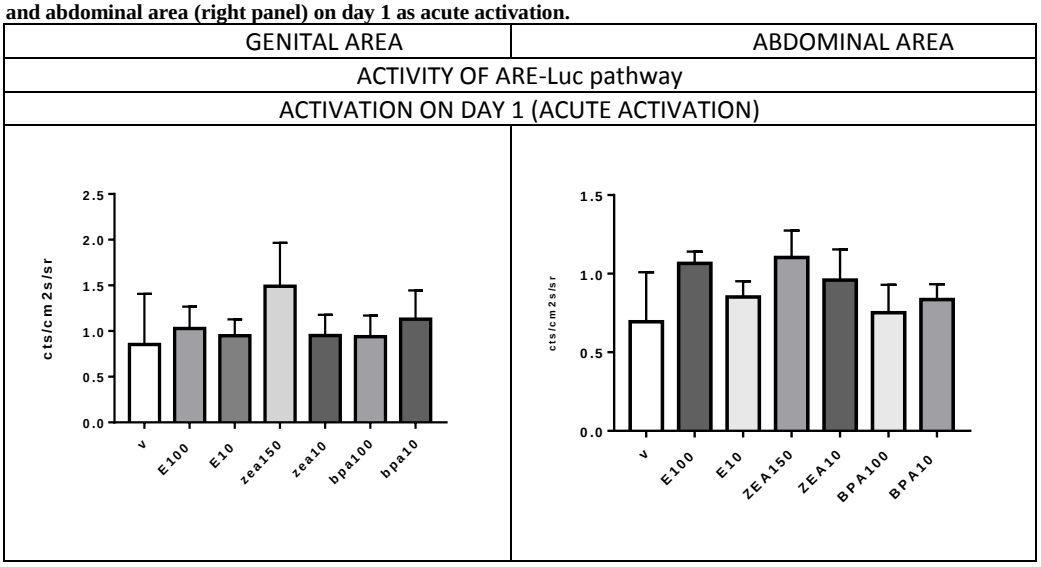


V = vehicle; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day. The values represent the means of photon emissions ± SEM and analyzed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

About the quantification of bioluminescence in the ARE-Luc reporter mice for the investigation of the activation of oxidative stress pathway, no significant signaling of the ARE-Luc pathway were observed after acute or chronic administration of the compounds (Figures 22-24, 26). Although an interesting activation in the ARE-Luc reporter mouse model was observed in the prostate upon treatment with zearalenone but it was not supported by statistical analysis (Figure 25).

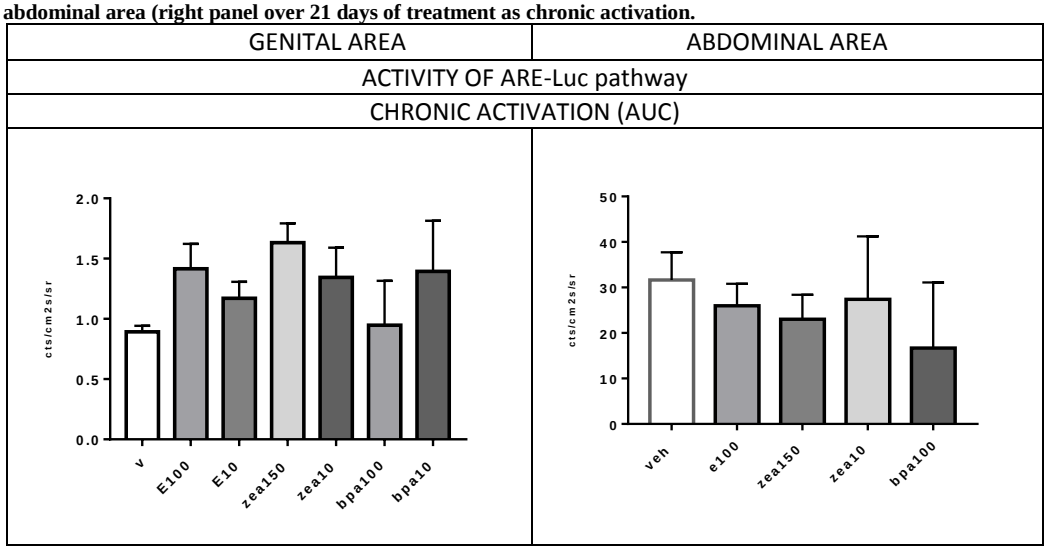
Those data are suggesting that this oxidative stress pathway is not involved in the toxicity as reported for these compounds in previous in vivo experiments.

Figure 22. Quantification of bioluminescence of oxidative stress (ARE-Luc) pathway in genital area (left panel) and abdominal area (right panel) on day 1 as acute activation.



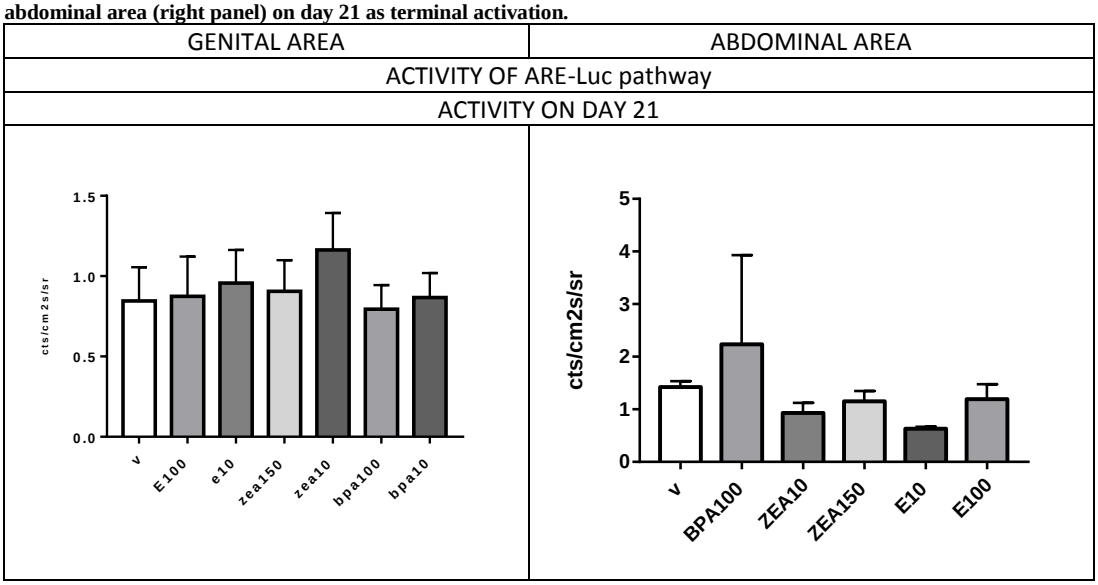
V = vehicle; BPA10 = 10 mg/Kg/day; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day. The values represent the means of photon emissions ± SEM and analyzed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

Figure 23. Quantification of bioluminescence of oxidative stress (ARE-Luc) pathway in genital area (left panel) and abdominal area (right panel over 21 days of treatment as chronic activation.



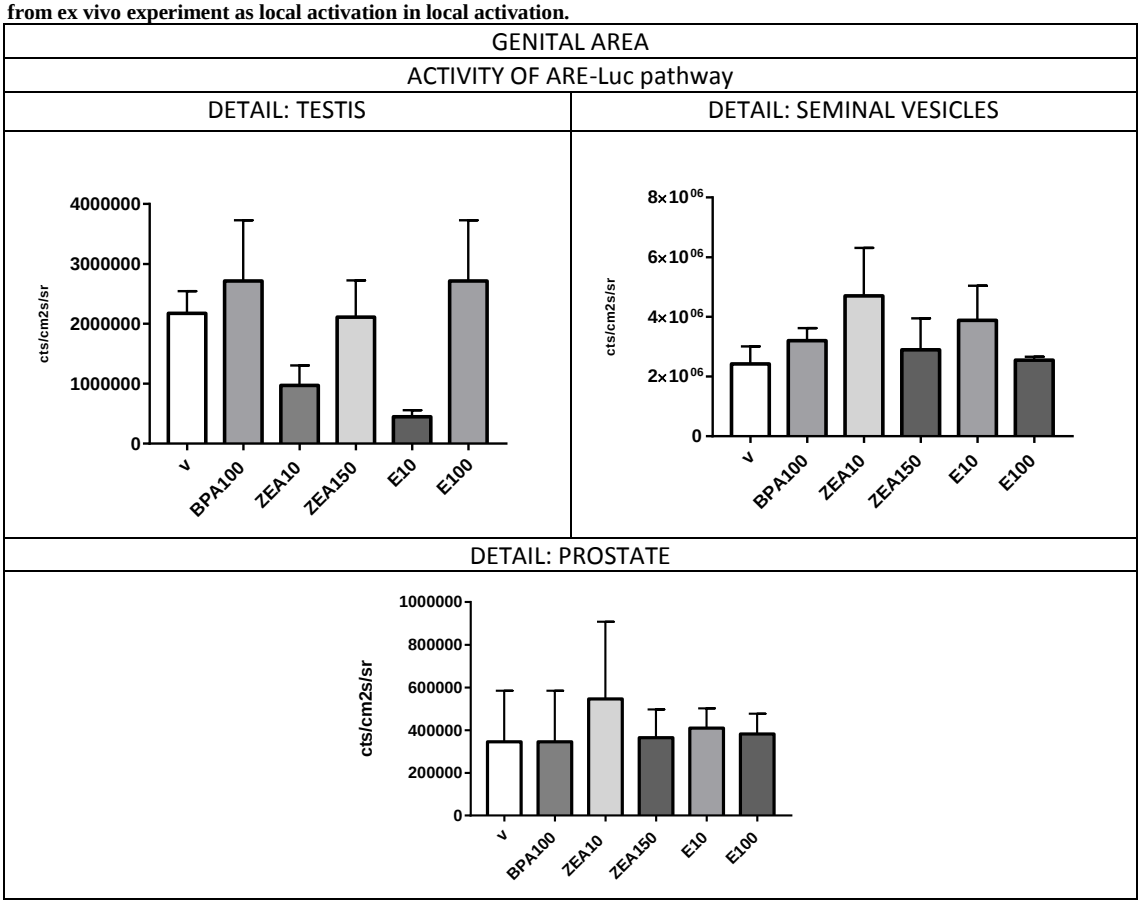
V = vehicle; BPA10 = 10 mg/Kg/day; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day. The values represent the means of photon emissions ± SEM and analyzed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

Figure 24. Quantification of bioluminescence of oxidative stress (ARE-Luc) pathway in genital area (left panel) and abdominal area (right panel) on day 21 as terminal activation.



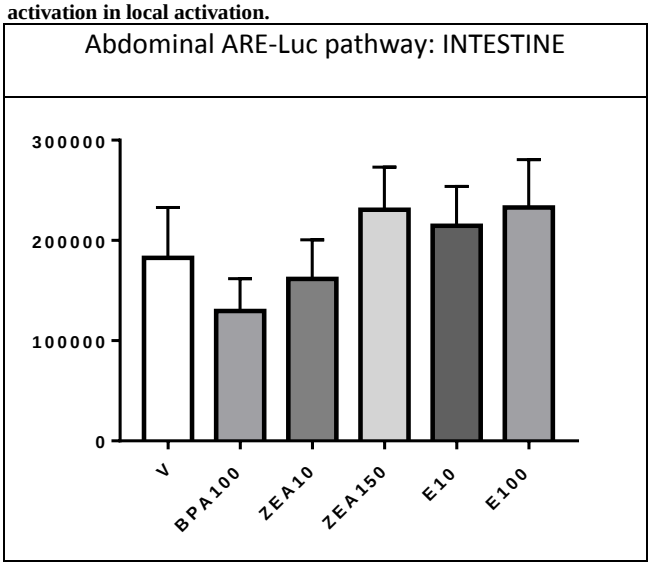
V = vehicle; BPA10 = 10 mg/Kg/day; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day. The values represent the means of photon emissions ± SEM and analyzed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

Figure 25. Quantification of bioluminescence of oxidative stress (ARE-Luc) pathway in testis, seminal vesicles, prostate from ex vivo experiment as local activation in local activation.



V = vehicle; BPA10 = 10 mg/Kg/day; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day. The Value are means ± SEM and analyzed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

Figure 26. Quantification of bioluminescence of oxidative stress (ARE-Luc) pathway in intestine tract from ex vivo experiment as local activation in local activation.



V = vehicle; BPA100 = bisphenol A 100 mg/Kg/day; ZEA10 = zearalenone 10 mg/Kg/day; ZEA150 = zearalenone 150 mg/Kg/day; E10 = 17β-estradiol 10 µg/Kg/day; E100 = 17β-estradiol 100 µg/Kg/day. The values represent the means of photon emissions ± SEM and analyzed by Ordinary one-way ANOVA plus Bonferroni's multiple comparisons test.

These findings indicate some remarkable correlation with the literature. Indeed, the weak evidences of ARE-Luc activation and significant activation of the NFkB-Luc pathways in the genital area with a specific focus on the prostate by the highest dose of zearalenone (150 mg/kg /day), match with the toxicological findings reported in the NTP study (NTP tr 235) (Table 2). In that study, mice and rats were fed for 90 day or 2 years with a wide range of concentrations (1 to 450 mg/kg bw day) of zearalenone. ZEA elicited adverse effects, such as an increase of incidence of squamous metaplasia in the prostate of B6C3F1 mice at 150 mg/kg bw day; inflammation in the prostate of F344/N rats at 1 mg/kg bw day and hyperplasia at 27-290 mg/kg bw day and an increase of atrophy in seminal vesicles and testis in both species, at low and at a higher dose (1-150 mg/kg bw day). Some evidence of cytoplasmic vacuolization increase (1.25; 2.5 mg/kg bw day) occurred in rats in the carcinogenicity study (Table 2).

The NFkB-Luc reporter mice seemed to be predictive of the indirect toxicological mechanisms of zearalenone, through the inflammatory pathway in the genital area.

In addition, zearalenone confirmed to be an ER activator only in the abdominal area of ERE-Luc mice (no activation in the genital area). This finding was not observed locally in the intestine, so other organs were involved in the estrogenic activation. These organs could be the liver or the kidney. Indeed, in the above mentioned NTP study [66], zearalenone was found to cause some toxicity to that organs.

Bisphenol A, for which there are controversial data in the literature, did not produce a significant activation in both the areas and in the ex vivo analysis, again in agreement with in silico/in vitro results.

The lack of ER activation of estradiol in the genital area was possibly due to oral administration of the compound in drinking water. This compound exerts a potent adverse effect in toxicological studies when administered by injection.

Discussion and Conclusive remarks

The extreme attention to the health of citizens and consumers to the involuntary exposure (through food, water, air) to endocrine disruptor, i.e. those substances capable of causing damage to any component of the endocrine system, has led to an increasingly stringent regulatory pressure in Europe.

Since the endocrine system responds physiologically with homeostatic mechanisms in response to external stimuli, it becomes important to identify and distinguish between substances able to cause a damage a harm and substances that interact with the body in a transitory manner causing an adaptive response without toxic consequences.

Being unlikely to solve the hormonal activity adaptation/ disruption issue with just experimental assay, we have tried to address the hazard characterization of substances with hormonal activity by the development of an integrated strategy of testing based on the combination of results of a hierarchy of different methodologies such as in silico models, in vitro and in vivo bioluminescence imaging assays.

The selected molecules ranged from known (DES) to suspected (BPA) endocrine disruptors and included both synthetic (DEHP) and natural (genistein) substances. As positive control 17β-estradiol has been used.

The first step used was in silico analysis with evaluation of the possible binding of selected substances to the estrogen alpha receptor to support the hypothesis that their hormonal activity occurred through a receptorial mechanism.

This approach is commonly used and is also part of the first level of investigation suggested by EFSA / ECHA in the recognition of EDCs.

Interest in computer-aided methods for investigations in the biological field has increased significantly in recent years. Despite their apparent simplicity, the results that derive from this approach must be carefully weighted and provide an indication of how to proceed further in the investigation rather than a definitive indication of a biological effect.

The computational methodology estimated different values of affinity of each ligand to hER Ligand Binding Domain (LBD). The value of using two different

approaches (XP Glide Score and MMGBSA) allowed to be taken into account solvation. That has meant that the MMGBSA docking protocol the solvation effect of the solvent (water molecules) towards the ligands, computing both the solvent/LBD and ligand/solvent interactions. The estradiol showed the best affinity values in both approaches as, being the endogenous hormone, is able to contrast the solvation effect.

The other ligands had greater values of MMGBSA scores because they, in the modelling conditions set, were not able to displace the water molecules into the LBD. Indeed, the binding free energy of DEHP (as XP GLIDE SCORE) was in the same range of BPA and Genistein while MMGBSA dG values of DEHP had the highest value (-2 kcal/mol). Thus, DEHP seems to remain in a soluble condition and it is not able to displace the water molecules into the LBD. There is a concordance between *in silico* results and literature data, where DEHP showed a high affinity to androgen receptor [see paragraph 1.7]. Zearalenone showed a binding free energy similar to that of the 17- β-estradiol but the MMGBSA score was less negative than the endogenous hormones because of the solvation effect.

The dissociation constant (K_i) values calculated from the XP GLIDE SCORE fits well with the K_i experimentally determined in vitro binding assay by other research groups. Thus, the computed K_i has been chosen as parameter for the prediction of putative endocrine disrupting activity (ED) taking into account the ED activity data in literature. However, the lack of correlation between the computed and experimental K_i and experimental ED activity retrieved in literature for all chemicals (only estradiol and zearalenone have similar affinity to ER and literature evidences of ED activity; genistein has a good K_i values but not ED activity such as BPA and methoxychlor), did not allow a prioritization of the investigated chemicals for ED activity through the results of their affinity.

In the next step, to check if the receptor binding was well correlated with the hormonal activity, the ER Reporter gene assay was performed, based on the ability of a compound to stimulate ER-dependent transcriptional activity in genetically engineered mammalian cells. The cell lines are MCF-7 cells which express human endogenous ERα. The cells are transformed (transfected) by introducing vectors containing DNA sequences for the receptor, along with EREs linked to a reporter gene, and the reporter gene itself. The reporter gene used in human cancer cells usually codes for luciferase (CALUX, chemically activated luciferase expression).

In the transactivation the EDCs show the estrogenic potency calculated as EC50, in respect to the positive control, 17β-estradiol. This system has enabled us to evaluate the kinetic and the biological consequences of cellular activation in the same cell monolayer, also taking into account the potential metabolism of the compounds or their possible degradation. In fact, a modification of the cellular response could also be attributed to the appearance of +/- active metabolites or to the degradation of the parent compounds. As mentioned in the results section, all compounds were stable and not significantly metabolized during the cell incubation period.

Three factors were taken into consideration, power, efficacy and trend over time. The in vitro dynamic ER activation showed that for some chemicals (genistein, BPA, methoxychlor), the potency (EC50) and the efficacy (fold induction) changed over time, but not for estradiol, zearalenone and DES.

Considering that estradiol, zearalenone and DES certainly have an activity in the animal and in man as endocrine disruptors, the duration of effect parameter combined with power and efficacy are likely to be associated in predicting the hormonal activity. Together with receptor affinity and the ability to induce a biological response, it also seems relevant how long the response is lasting. By consequence, the combination of the variation of the potency response and the efficacy, "normalized" for the efficacy at 48h, was successfully used in discriminating positive and negative compounds for their endocrine disrupting activity. 17β-estradiol, DES and zearalenone were put at the top of the list because of their ED effect observed in vivo, genistein resulted to represent a putative threshold of no-concern for ED activity, in supporting published data, while methoxychlor and BPA are definitely not considered a priority in terms of ED activity. This in vitro classification fitted well with the in silico outcome, since the strongest estrogen receptor binders where ranked in the first position. Besides, was no possible to calculate EC50s for 4-nonylphenol, DEHP and vinclozolin, not making possible to classify them as ED, totally in agreement with in silico results and in line with literature data (vinclozolin is mainly an androgen antagonist).

The third step of our stepwise approach was intended to verify in vivo the interaction of selected chemicals with the estrogen receptor and in addition the activation of other pathways triggering primary harmful effects.

We used three reporter mice designed in order to evaluate the response of some compounds to activate ER and causing oxidative stress and inflammation. The aim

of this phase was to investigate whether oxidative stress and inflammation could be correlated, and at which extent, with a ED response by analysing the activity of the compounds in a spatio-temporally manner (the bioluminescence image represents a picture of how far the receptor activation as photon emissions in the specific area (tissue), is extended at that precise time of observation.

We choose to test zearalenone (well-known endocrine disruptor and clearly identified as such by our in silico-in vitro approach), and BPA for which there are controversial data in the literature and that our approach has negatively classified as an ED.

Briefly, zearalenone disrupts oestrous cycle, ovulation, conception and implantation, cause embryonic death, reduce fetal weight and litter size and to impair neonatal survival on female pigs [24; 25]. BPA, at high doses, has raised concern for its high production volume and for the evidence of toxicity in animal studies mainly on reproductive and developmental systems, liver, kidney and some evidence in mammary gland [16,36-39].

Our data shows that zearalenone has been active on ER pathway in the abdominal area and significantly activated the inflammatory pathway in the genitals (in this specific case in the prostate, result of ex vivo bioluminescence analysis). These results are perfectly in line with the literature reports, in which prostate inflammation and metaplasia were detected in both mice and rats.

Bisphenol A did not produce a significant activation in both the areas and in the ex vivo analysis, again in agreement with in silico/in vitro results.

The lack of ER activation of estradiol in the genital area was possibly due to oral administration of the compound in drinking water. This compound exerts a potent adverse effect in toxicological studies when administered by injection.

Several works in the literature made a correlation between in vitro and in vivo experiments. Sonneveld et al., established a good correlation between in vitro ERα-CALUX and in ovariectomized test in young rats by scoring the vaginal cornification (Allen-Doisy test) [31]. Nevertheless, the plausibility of the in vivo model, has some limitations including a lack of certainty regarding the adequacy of animal species to the treatments (its sensitivity to the treatments), the life stages of animals; a lack of correlation between the mechanistic of action, that are diagnostic or predictive of endocrine disrupting activity, and apical response observed in vivo, that is the adverse outcomes [32]. In the last period new in vivo approach based on

transgenic mouse model have been generated to measure estrogen receptor (ER) transcriptional activity in living organisms [33].

From our results, in silico approach emerges as a computational methodology for the evaluation of the ER affinity (prioritization of assessment) of a large number of molecules. During a safety evaluation process, the molecules that show the lowest affinities should in principle be disregarded and the attention should be focused on the molecules that show the highest affinities.

While the in silico screening based on the calculation of K_i cannot be used as a stand-alone procedure due to its intrinsic lack of biological meaning, it can be successfully used as a first prioritizing step in a tier approach. The second mandatory check for the in silico positive hits should be an in vitro evaluation procedure, in which the affinity of the positive hits is measured through a reference cellular assay. Then integrating the time variable in the evaluation of the potency and efficacy the tested compounds could be classified as ED or no-ED as obtained in our experiment. While the in vivo experiment highlighted that potent estrogenic compound, as zearalenone, could also raise concern for the activation of other toxicological pathway such as the inflammatory ones.

We are aware that this indication of procedure must be evaluated on dozens of molecules whose in vivo activity is already known before arriving at its use to predict the possible activity of ED of unknown molecules, but we think that this approach deserves to be implemented.

Table 8. Summary of results relevant for estrogen receptor. The table shows the result of each chemical along the three different methodologies, where the chemical was able to interact within each system. Estradiol, as expected, ZEA were the strongest estrogen like compound in all assays (symbol X) except the boxes containing the symbol "- ". BPA showed positive results in silico and in vitro, while not statistical significant or controversial results were in the animal assays ("- "or +/-).

Summary of results relevant for estrogen receptor								
Compound	In silico model	in vitro bioluminescence imaging	In vivo bioluminescence imaging					
			Genital area			Abdominal area		
	hERα	ERα	ERE-Luc	NFKB-Luc	ARE-Luc	ERE-Luc	NFKB-Luc	ARE-Luc
E2	X	X	+/-	-	+/-	X	-	-
ZEA	X	X	+/-	X (prostate)	+/-	X	X	-
BPA	X	X	+/-	+/- (testis)	+/-	-	-	-
DES	X	X						
GEN	X	X						
4-NP	X	-						
MXC	X	X						
VCZ	X	-						
DEPH	X	-						

E2 = 17β-estradiol; ZEA = zearalenone; BPA = bisphenol A; DES= diethylstilbestrol; GEN = genistein; 4-NP = 4-Nonylphenol; VCZ = vinclozolin; MXC = Methoxychlor; DEHP = Bis(2-ethylhexyl) phthalate; X = interaction with receptor ($p < 0.05$); - =, no interaction or $p > 0.05$; Trend = +/- not statistically significant

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APPENDIX A

Figures and table of LC-MS analysis of samples from in vitro experiment. Figure 1A, 1B, 1C represent the chromatograms of determination of analytes (bisphenol A, zearalenone, genistein) from the medium.

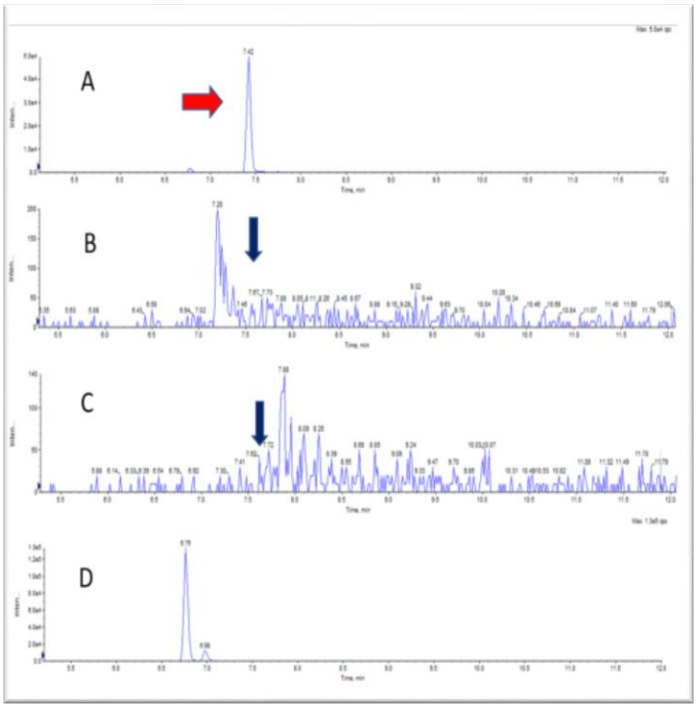

Figure 1A. example of LC-MS / MS trace of BPA extracted from the ID1sample. A: presence of the analyte (405 pg./µl, or 10^{-6}M) (indicated with red arrow); B and C: absence of analyte (indicated with blue arrow); D: internal standard

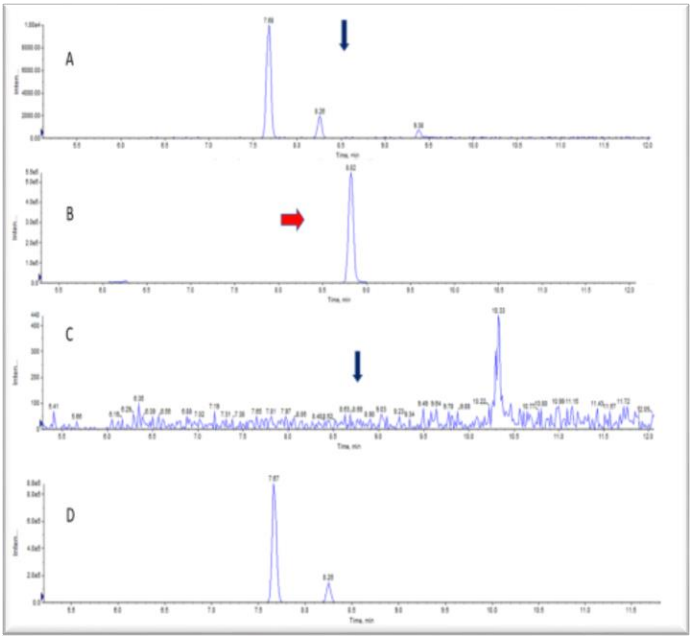

Figure 1B example of LC-MS / MS trace of Genistein extracted from the ID2 sample. B: presence of the analyte (130 pg./µl or 10^{-6}M) (indicated with red arrow); A and C: absence of analyte (indicated with blue arrow); D: internal standard

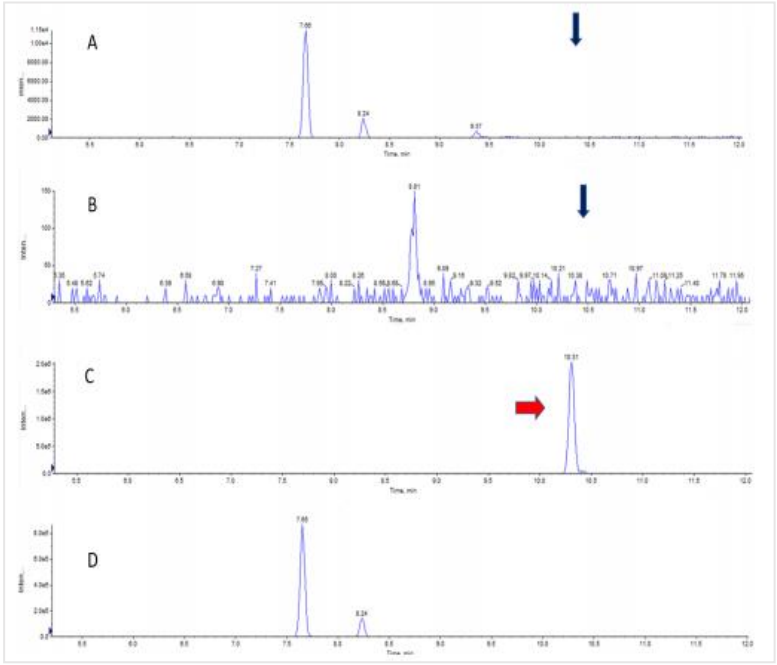

Figure 1C. example of LC-MS / MS trace of Zearalenone extracted from the ID3 sample. C: presence of the analyte (488 pg./µl or 10-6M) (indicated with red arrow); A and B: absence of analyte (indicated with blue arrow); D: internal standard

Summary of quantification of Bisphenol A, Zearalenone and Genistein n characterized in the cleaned medium(control) and in medium cell conditioning

Table A) characterization of BPA in sample extracted from the medium and medium cell conditioning at 6 hours

	samples 6 hours						
	sample	treatment- 20.6.2018	pg/µl	mean	DV STD	t-test 6hours	t-test 6hours-48hours only MEDIUM
BPA	31	only MEDIUM 1a	103	**93**	6	**0.000019**	**0.000**
	33	only MEDIUM 2a	90				
	35	only MEDIUM 3a	92				
	37	only MEDIUM 4a	88				
	39	only MEDIUM 5a	89				
	71	MEDIUM COND 1a	127	**124**	5		t-test 6hours-48hours MEDIUM cond **0.164**
	73	MEDIUM COND 2a	127				
	75	MEDIUM COND 3a	125				
	77	MEDIUM COND 4a	126				
	79	MEDIUM COND 5a	116				

Table B) characterization of BPA in sample extracted from the medium and medium cell conditioning at 48 hours

	samples 48 hours					
	sample	treatment- 22.6.2018	pg/µl	mean	DV STD	t test 48 hours
BPA	131	only MEDIUM 1a	155	**134**	13	**0.313**
	133	only MEDIUM 2a	123			
	135	only MEDIUM 3a	123			
	137	only MEDIUM 4a	131			
	139	only MEDIUM 5a	138			
	171	MEDIUM COND 1a	108	**159**	51	
	173	MEDIUM COND 2a	192			
	175	MEDIUM COND 3a	218			
	177	MEDIUM COND 4a	104			
	179	MEDIUM COND 5a	174			

Table C) characterization of genistein in sample extracted from the medium and medium cell conditioning at 6 hours

	samples 6 hours						
	sample	treatment- 20.6.2018	pg/µl	mean	DV STD	t-test 6 hours	t-test 6hours-48hours only MEDIUM
GENI	21	only MEDIUM 1a	210				
	23	only MEDIUM 2a	198				
	25	only MEDIUM 3a	177	**205**	18		**0.413**
	27	only MEDIUM 4a	219				
	29	only MEDIUM 5a	221				
	61	MEDIUM COND 1a	220				T test 6hours-48hours MEDIUM cond
	63	MEDIUM COND 2a	219				
	65	MEDIUM COND 3a	233	**216**	12		**0.286**
	67	MEDIUM COND 4a	206				
	69	MEDIUM COND 5a	202			**0.293318**	

Table D) characterization of genistein in sample extracted from the medium and medium cell conditioning at 48 hours

	samples 48 hours					
	sample	treatment- 22.6.2018	pg/µl	mean	DV STD	t test 48 hours
GENI	121	only MEDIUM 1a	196			
	123	only MEDIUM 2a	213			
	125	only MEDIUM 3a	153	**193**	26	
	127	only MEDIUM 4a	217			
	129	only MEDIUM 5a	186			
	161	MEDIUM COND 1a	220			
	163	MEDIUM COND 2a	281			
	165	MEDIUM COND 3a	309	**243**	51	
	167	MEDIUM COND 4a	228			
	169	MEDIUM COND 5a	179			**0.086**

Table E) characterization of zearalenone in sample extracted from the medium and medium cell conditioning at 6 hours

	samples 6 hours						
	sample	treatment- 20.6.2018	pg/µl	mean	DV STD	t-test 6hours	t-test 6hours-48hours only MEDIUM
ZEA	11	only MEDIUM 1a	212				
	13	only MEDIUM 2a	247				
	15	only MEDIUM 3a	159	**226**	89		**0.095**
	17	only MEDIUM 4a	367				
	19	only MEDIUM 5a	147				
	51	MEDIUM COND 1a	133				t-test 6hours-48hours MEDIUM cond
	53	MEDIUM COND 2a	135				
	55	MEDIUM COND 3a	109	**126**	13		**0.036**
	57	MEDIUM COND 4a	117				
	59	MEDIUM COND 5a	137			**0.037**	

Table F) characterization of zearalenone in sample extracted from the medium and medium cell conditioning at 48 hours

	samples 48 hours					
	sample	treatment- 22.6.2018	pg/µl	mean	DV STD	t-test 48 hours
ZEA	111	only MEDIUM 1a	187			
	113	only MEDIUM 2a	133			
	115	only MEDIUM 3a	133	**149**	22	
	117	only MEDIUM 4a	141			
	119	only MEDIUM 5a	153			
	151	MEDIUM COND 1a	93			
	153	MEDIUM COND 2a	128			
	155	MEDIUM COND 3a	104	**99**	21	
	157	MEDIUM COND 4a	98			
	159	MEDIUM COND 5a	69			**0.006**

Summary of quantification of Bisphenol A, Zearalenone and Genistein in cells extracts of MCF7 subclone B17

Table F) characterization of BPA in sample extracted from MCF7 pellet

	samples 6 hours	treatment 20.6.18	ng/camp	mean	DV STD	t-test 6hours-48hours
BPA	96	MCF7 pellet 1 BPA 6h	0.51	**0.46**	0.09	
	97	MCF7 pellet 2 BPA 6h	0.36			
	98	MCF7 pellet 3 BPA 6h	0.57			
	99	MCF7 pellet 4 BPA 6h	0.39			
	100	MCF7 pellet 5 BPA 6h	0.45			
	samples 48 hours	treatment 22.6.18	ng/camp	mean	DV STD	
BPA	196	MCF7 pellet 1 BPA 48h	0.21	**0.47**	0.16	
	197	MCF7 pellet 2 BPA 48h	0.50			
	198	MCF7 pellet 3 BPA 48h	0.55			
	199	MCF7 pellet 4 BPA 48h	0.65			
	200	MCF7 pellet 5 BPA 48h	0.47			**0.864**

Table G) characterization of genistein in sample extracted from MCF7 pellet

	samples 6 hours	treatment 20.6.18	ng/camp	mean	DV STD	t-test 6hours-48hours
GENI	91	MCF7 pellet 1 GENI 6h	2.50	**3.04**	0.55	
	92	MCF7 pellet 2 GENI 6h	3.44			
	93	MCF7 pellet 3 GENI 6h	2.72			
	94	MCF7 pellet 4 GENI 6h	3.79			
	95	MCF7 pellet 5 GENI 6h	2.76			
	samples 48 hours	treatment 22.6.18	ng/camp	mean	DV STD	
GENI	191	MCF7 pellet 1 GENI 48h	4.91	**4.76**	0.59	
	192	MCF7 pellet 2 GENI 48h	4.19			
	193	MCF7 pellet 3 GENI 48h	4.43			
	194	MCF7 pellet 4 GENI 48h	5.71			
	195	MCF7 pellet 5 GENI 48h	4.55			**0.001**

Table H) characterization of zearalenone in sample extracted from MCF7 pellet

	samples 6 hours	treatment 20.6.18	ng/camp	mean	DV STD	t-test 6hours-48hours
	86	MCF7 pellet 1 ZEA 6h	1.78			
	87	MCF7 pellet 2 ZEA 6h	1.18			
ZEA	88	MCF7 pellet 3 ZEA 6h	2.52	**1.74**	0.49	
	89	MCF7 pellet 4 ZEA 6h	1.68			
	90	MCF7 pellet 5 ZEA 6h	1.54			
	samples 48 hours	treatment 22.6.18	ng/camp	mean	DV STD	
	186	MCF7 pellet 1 ZEA 48h	2.20			
	187	MCF7 pellet 2 ZEA 48h	2.17			
ZEA	188	MCF7 pellet 3 ZEA 48h	3.43	**2.45**	0.59	
	189	MCF7 pellet 4 ZEA 48h	1.92			
	190	MCF7 pellet 5 ZEA 48h	2.51			**0.073**