Theodore Eliades
George Eliades *Editors*

Plastics in Dentistry and Estrogenicity

A Guide to Safe Practice

Springer

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Theodore Eliades • George Eliades
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Editors
Theodore Eliades
Department of Orthodontics
and Paediatric Dentistry
Center of Dental Medicine
University of Zurich
Zurich
Switzerland

George Eliades
Department of Biomaterials
School of Dentistry
University of Athens
Athens
Greece

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Foreword

It is estimated that 370 million direct dental restorations are placed in Europe annually [1]. Of these an increasing proportion are resin-bonded composite (RBC) restorations, because of the declining use of dental amalgam. Whereas there have been healthcare concerns over the use of amalgam, along with environmental issues, it is understandable that attention is now focussed upon RBC biomaterials as their deployment expands considerably. In this connection, it is the organic 'resin' phase of dental composites that attracts the main notice. This is because the 'resin' is supplied as a mixture of monomers that undergo polymerisation to create the composite matrix in situ. Adverse outcomes can ensue if either (a) inadequate polymerisation leaves substantial elutable monomer concentrations or (b) the monomers or polymer network contains or biodegrades to release undesirable substances such as BPA.

Although disputed by some experts, it is evident that BPA is released in non-minimal quantities from various polymers (dental composites, polycarbonates) that are used intra-orally. Many research groups have identified effects in vitro and in animals with concentrations far below the ones measured to be released from materials. Nevertheless, there is absence of proof that dental RBCs and related materials constitute a 'clear and present danger' to patient health. That is, a potential risk does not necessarily translate into an actual risk. The reality is that there are considerable variations in the mode of application of dental resins, in the patient's ages and in the amounts of BPA released from different classes of material that all modify the exposure to hazard. It is likely that as the situation clarifies, different subcategories may have different risk/benefit ratios attached to them.

This book introduces and considers these issues in a careful and responsible manner showing that the evidence is not yet complete. So we need to read and build upon this evidence, meanwhile adopting a cautious attitude to the possible risk.

Manchester, UK — David Watts

Reference

1. European Commission (2012) DG ENV – final report

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Part I
Introduction and Overview

Chapter 1
Endocrine Disruptors (Xenoestrogens): An Overview

George Dimogerontas and Charis Liapi

1.1 Introduction

In the last decades, a large number of natural and synthetic chemicals have been identified as interfering with the endocrine system; they are collectively termed endocrine-disrupting chemicals (EDCs) or endocrine disruptors. According to the working definition of the Environmental Protection Agency (EPA), an endocrine disruptor is "*an exogenous agent that interferes with the synthesis*, *release*, *transport*, *metabolism*, *binding*, *action*, *or elimination of natural hormones in the body responsible for the maintenance of homeostasis*, *reproduction*, *regulation of developmental processes and/or behavior*"[1]. Endocrine disruptors comprise more than 100.000 synthetic chemical compounds that belong to different classes. A subset of the endocrine disruptors, including synthetic estrogens, natural products, commercial chemicals, industrial compounds, or by-products among which plastics, are known as environmental estrogens or xenoestrogens; they confer estrogenic potential ("estrogenicity") translated as affinity to the estrogen receptors (ER) (α or β), thus ability to activate expression of estrogen-dependent genes or stimulation of cell proliferation of ER-competent cells [2].

Estrogens consist of an important group of steroid hormones found not only in humans but in all vertebrates, insects, and plants. In humans, estrogens (estradiol, estrone, and estriol) are primarily produced by developing follicles in the ovaries as well as by the corpus luteum of the placenta; adrenal cortex, brain, testicles, liver, and adipose tissue are smaller sources of estrogens but the only source during menopause. Estrogens are formed from the aromatization of either androstenedione or testosterone (immediate precursors) by the enzyme aromatase which is located in many tissues including adipose tissue and brain. Thus, in men, the primary source

G. Dimogerontas • C. Liapi (✉)
Department of Pharmacology, School of Medicine,
University of Athens, Athens, Greece
e-mail: charis.liapi@gmail.com

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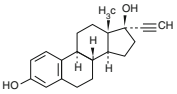
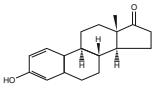
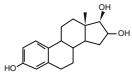
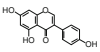
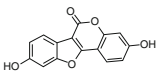
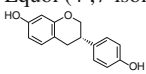
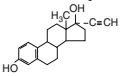
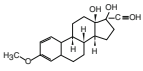
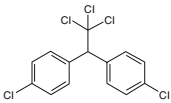
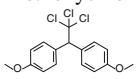
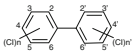
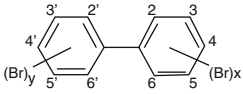
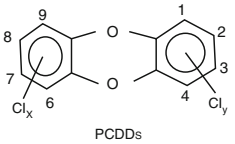
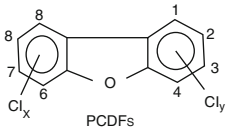
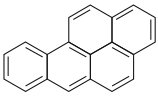
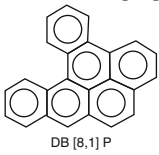
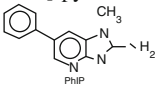
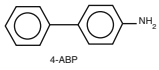
of estradiol is from conversion of testosterone by aromatase. In the plasma, estrogens are bound to the glycoprotein SHBG (sex hormone-binding globulin), which regulates access to the receptor [3]; SHBG serum levels are relatively high until puberty with many sites unoccupied; the large numbers of SHBG binding sites that remain unoccupied in men and women, 44 and 80 %, respectively, and especially in women taking oral contraceptives (they cause a three to five times increase in SHBG levels) are available to bind nonsteroidal ligands [4–6].

Estrogens are regulating the development, maintenance, and function of the reproductive system in both sexes, but they also exert important biologic effects in many tissues and organs, influencing many physiological processes. Given the widespread role for estrogens in many body functions, xenoestrogens, binding to estrogen receptors and acting as inappropriate estrogens, can disturb the physiology not only of the genital system, but they can also influence the integrity of many systems; they cause among others cancer, immunological, and neurological problems [7–9] in a wide range of organisms including, except for mammals, fish, birds, and reptiles [10]. Taking into consideration that the compounds with estrogen-like biological effects are ubiquitous in nature and that the endocrine systems are interlinked with each other and with other systems, EDCs have been considered as a threat for human health and for wildlife species, raising scientific, public, and political concern [11] not only at the level of health issues but also from the consequences to the economy of a country; the Belgian dioxin crisis caused an estimated damage to the Belgian economy of many million euros in addition to the number of cancers that according to estimations could reach the 8,000 as a result of the ingestion of PCBs and dioxin [12, 13]. In view of the importance of the issue, international agencies like the European Commission, the European Parliament, the US Environmental Protection Agency, the Organization for Economical Cooperation and Development, the WHO International Program on Chemical Safety, nongovernmental organizations, and the chemical industry have addressed the issue in an attempt to identify the potential risks and to develop an international research strategy [14].

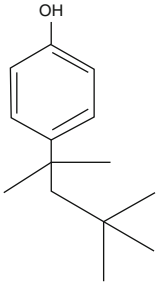
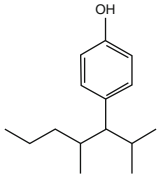
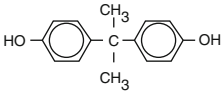
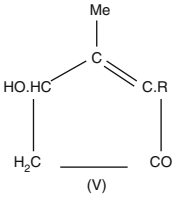
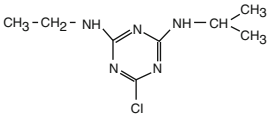
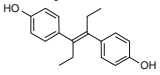
More than 80,000 chemicals with estrogenic activity are used in a vast array of industry and household products, including compounds used as pesticides such as DBCP, vinclozolin, endosulfan, dieldrin, kepone, methoxychlor o,p-DDT, toxaphene, phenolic derivatives, and polychlorinated biphenyls (PCBs); compounds used in the food industry; antioxidants such as t-butylhydroxyanisole; plasticizers such as benzylbutylphthalate and 4-OH-alkylphenols; products associated with plastics such as bisphenol A and phthalates; industrial chemicals and by-products such as polychlorinated biphenyls (PCBs), dioxins, and benzo(a)pyrene; and heavy metals [9, 10]**.** The chemistry of these compounds is significantly different from the hormones they mimic, and their chemical structure does not predict the estrogenic activity they dispose (Table 1.1). Some of the most common products, except for pesticides, are flame retardants, electronic enclosures, wood preservatives, glues, cleansing and degreasing agents, polyesters, textiles, paints, lubricants, toys, personal care products, cosmetics, food and beverage containers, and dental material [15–17]. Thus, the exposure to EDCs can be through different routes such as diet, drinking water, air, and skin; dermal and inhalation exposure in industry

Table 1.1 The chemical formulas of the main estrogenic substances

Natural estrogens		
Estradiol	Estrone	Estriol
		
Phytoestrogens		
Genistein	Coumestrol	Equol (4′,7-isoflavandiol)
		
Synthetic steroid estrogens		
17a-ethinylestradiol	Mestranol	
		
Synthetic nonsteroid estrogenic compounds		
Chlorinated hydrocarbons	DDT	Methoxychlor
		
Polychlorinated Biphenyls (PCBs)		
Polychlorinated Biphenyls (PBBs)		
Aromatic heterocyclic compounds	PCDDs (Dioxins)	PCDFs
	 PCDDs	 PCDFs
Polycyclic Aromatic Hydrocarbons (PAHs)	B[a]P: Benzo-a-pyrene	DB[a,l]P: Dibenzo-[α.l]-pyrene
		 DB [8,1] P
Aromatic Amines (AA) and Heterocyclic Aromatic Amines (HAA)	PhIP: 2amino-1-methyl-6phenylimidazolo[4,5-b]-pyridine	4-ABP: 4aminobiphenyl
	 PhIP	 4-ABP

(continued)

Table 1.1 (continued)

Alkylated phenols	Octy-phenols 	Nonyl-phenols 
Monomers of polymeric plastics	Bisphenol-A 	
Synthetic pyrethrines	Allethrin 	
Triazines	Atrazine 	
Pharmacological substances	Diethylstilbestrol (DES) 	

workers and exposure to agriculture workers are common ways of occupational exposition [18].

Surface water, municipal effluents from sewage treatment plants, and sediments are among the important contamination sources in many European and other countries [19–21] with consequent adverse effects in wildlife (fish, roach, etc.) [22, 23]; the major intake of estrogenic chemicals is considered to be through food [24, 25]. Fish products may represent an important dietary source of EDC contamination in food, but edible plants may also take up estrogenic compounds from terrestrial or aquatic environments [26, 27]. Note that weak estrogenicity has also been detected in mineral water and milk as a result of the leach from the polyethylene terephthalate (PTE) in baby bottles [28–30]. EDC chemicals are present in higher amounts in humans because humans are at the top of the food chain, having ingested plants and animals that contain low levels of these persisting compounds.

EDCs share physical and chemical properties such as chemical stability, lipid solubility, accumulation in fat, slow rate of biotransformation, and biodegradation. They are weak estrogens (most of them about 1/1,000 to 1/1,000,000 of the activity of estradiol), but small changes in more innocent compounds can give rise to persistent and bioaccumulative compounds (replacement of chloride by bromide leads to lipophilic brominated organic compounds that, although they show a weak estrogenic activity, tend to accumulate much more in fat compared to chlorinated ones) [31]. The major difference between naturally occurring biochemical molecules and man-made compounds is that the former are assembled and disassembled very rapidly in the human body, while the latter resist biodegradation in the environment and consequent bioaccumulation and biomagnification within various food chains. Thirteen years after Yu-cheng accident (literally oil symptoms), in which people in Taiwan had consumed PCB- and PCDF-contaminated cooking oil for 9 months (estimated consumption 1 g of PCBs and 3.8 mg of PCDFs), the concentrations in women that had born a child were 7- up to 130-fold higher (depending on the compound) compared to nonexposed population [32].

In contrast to endogenous hormones that bind to carrier proteins and thus become biologically inactive, EDCs remain unbound and active. The half-life of these compounds is ranging from weeks to years (i.e., half-life of methoxychlor is 2 weeks; of DDT, 6 months; of PCBs, PCDDs, and PCDFs, 7–10 years) [33].

Many estrogen-like compounds with high biologic activity are present in trace amounts, but since man is exposed to a plethora of these chemicals, the overall estrogenicity might be important and may contribute to overall risk and health implications [34]. Because of the long half-life and bioaccumulation of many EDCs, the "safe" concentrations today may become responsible for adverse effects in the following years [35].

1.2 Mechanism of Action of Estrogens and Xenoestrogens

1.2.1 Estrogen Receptor Signalling Pathway

The pleiotropic effects of estrogens in the body are mainly effectuated by binding to the estrogen receptors, ERα and ERβ [36], representing products of two different genes localized on human chromosomes 6 and 14, respectively [37]. Although both isotypes exist in the various systems, ERα is the main isotype in the genital system and mammary gland, while Erβ is the main isotype in the central nervous, the cardiovascular, and the immune systems; the urogenital and gastrointestinal tracts; the kidneys; and the lungs [38–42]. Various ERα and ERβ isoforms and splicing variants (hERβ1 long, hERβ1 short, hERβ2, hERβ4, hERβ5, hERα-46) have been described [43, 44].

The ERs (α, β) are composed of three independent but interacting functional domains: the NH_2-terminal transcriptional AF1 (activation function-1)

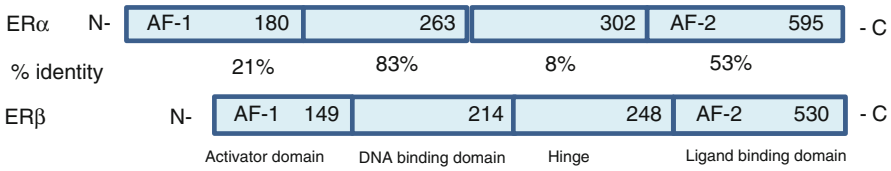


Fig. 1.1 The functional domains of ERα (*top*) and ERβ (*bottom*) with the amino acids counting and the identity percent's (%) are shown. The DNA-binding domain and the hinge region are highly conserved between the two receptors. *AF-1* Activation function 1; DBD - DNA-binding domain; *H* Hinge; *LBD* Ligand binding domain; *AF-2* Activation function 2

domain, the DNA-binding domain, and the ligand-binding domain that contains a ligand-dependent transcriptional AF2 (activation function-2) domain [45]. Although the DNA-binding domains of ERα and ERβ show a high degree of homology (only three amino acids difference), the ligand-binding domain shows only 53 % homology (Fig. 1.1).

The classical mechanism of activation of ERs, through which genomic effects take place, depends on ligand binding to the receptors, after which the receptors dimerize and bind to estrogen response elements (EREs) located in the promoters of estrogen-responsive genes to activate gene transcription [46, 47]. ERα (but not ERβ) has also the ability to bind to the orphan nuclear hormone receptor SF-response elements (SFREs) that serve as its EREs [48].

Maximum transcriptional activity requires the concerted actions of the ligand-independent AF1 domain and the ligand-dependent AF2 domain. Regulatory cofactors of the transcriptional activity include coactivators, corepressors, and chromatin-remodeling complexes (chromatin is regulating the basal activity of many promoters) [46, 49–52] (Fig. 1.2).

Most of the coregulators of the activator protein-2 (AP-2) (i.e., RIP 140, TIF-2, SRC-1, and SHP) interact equally well with ERα and ERβ [53–55], while others, such as the TRAP 220 coregulator, show significant differences in the interactions with ERα and ERβ [56, 57]; corepressors preferentially associate with ER antagonist [58–60]. Since distinct ERα and ERβ ligands are known to effect preferential recruitment of different coactivators [61, 62], the selective receptor/coactivator interactions represent an efficient system through which the pleiotropic effects of ER ligands might be mediated and are likely further determined by tissue-specific patterns of posttranslational modification of coactivators [63].

In summary, transcriptional activity of ERs is strongly influenced by ligands and the conformational changes induced upon ligand binding of ERα or ERβ, the formation of dimers (i.e., ERα/α and ERβ/β homodimers or ERα/β heterodimers), and the cofactor recruitment including interaction with chromatin. The co-expression of ERα and ERβ in different tissues results in a heterogeneous pool of pro-proliferative ERα/α and antiproliferative ERβ/β homodimers and in ERα–ERβ heterodimers that have different biologic effects than the homodimers [64–69]. Thus, according to receptor subtype and the cell type [70, 71], gene activation or repression can happen [72–75].

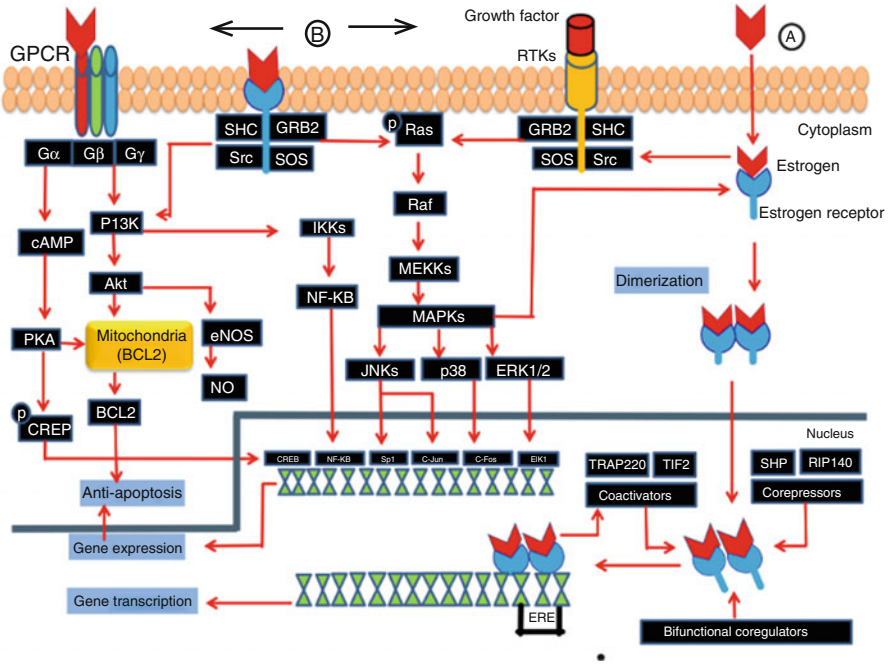


Fig. 1.2 Schematic Illustration of Classical (genomic) and Non-Classical (non-genomic) estrogen signaling pathways: (**A**) Classical pathway: ER complex, homodimerization and translocation from cytoplasm to to the nucleus. In the nucleus it induces two pathways: 1. Direct binding to responsive elements in the target gene promoters, subsequently the receptor-ligand complex binds to the palindromic ERE, and stimulates gene transcription with the recruitment of corregulators (coactivators, coreprossors and bifunctional coregulators). 2. ER complex interacts with transcription factors such as NF-κbB, activator protein-1 and SP1 to influence gene transcription. (**B**) Non-Classical pathways (non-genomic): Estrogen interaction with Nonsteroidal hormone receptors or Steroid hormone receptors in the membrane. Both non-classical pathways activate kinases that ultimately regulate transcription of specific genes. These signaling cascades recruit second messengers including NO, RTKs, GPCRs, and protein kinases including PI3K, serine-threonine kinase Akt, MAPK family members, and PKA and PKC. A typical example is the induction of antiapoptosis: ER associated MAPK pathway induce rapid phosphorylation of the adaptor proteins, Src and SHC, resulting in a SHC-GRB2-SOS complex formation; this leads to the subsequent activation of Ras, Raf, and MAPKs, including ERK1/2, JNK, and p38. They are then translocated to the nucleus and participate in gene transcription. (Courtesy of Hussam Al-Humadi, MD)

Peptide growth factors are also capable of eliciting estrogen receptor-dependent activation of an ERE of DNA; ER-dependent transcriptional activation can also be elicited by both protein kinase A and protein kinase C pathways [76–79]. In some cases, genomic effects are effectuated through protein–protein interactions [80]. In the absence of an ERE (around one third of the genes in humans that are regulated by ERs do not contain ERE-like sequences [81]), the ER–ligand complexes can bind to activator protein-1 (AP-1, a transcription factor which is a heterodimeric protein composed of proteins belonging to thec-Fos, c-Jun, ATF, and JDP families)

or interact with transcription factors NF-κB (nuclear factor-κβ), and the SP (specific protein-1) to influence gene transcription [70, 72, 82, 83].

All abovementioned genomic mechanisms of action of estrogens mediated through the ERs activation upon ligand binding take time to be effectuated, but estrogens exert rapid and transient membrane-initiated effects as well; these effects occur within seconds or minutes, are known to involve several signalling cascades, and may also influence gene transcription in the nucleus (Fig. 1.2) The second messenger signalling events include stimulation of adenylate cyclase and production of cAMP [76, 84], mobilization of intracellular calcium[85], stimulation of PI3K and PKB [86, 87], and activation of MAPK pathway of Src with consequent activation of the extracellular-regulated kinases Erk1 and Erk2 [88–92].

Although most of the rapid effects of estrogens are believed to be mediated through activation of nuclear ERS (ERα and ERβ) localized near the cell surface (a small amount, approximately 2 %, of either ERα or ERβ can associate with the cell membrane [93]), novel membrane ERs (mERs) have been identified in a number of tissues. Membrane receptors are located in caveolae (specialized membrane invaginations enriched in the scaffold protein caveolin-1) at the membrane [94] and can bind to caveolin-1, G proteins, PI3 kinases, Src kinase, Ras, etc. (Fig. 1.2) [95–101].

An indirect induction of nongenomic effects can indirectly activate the gene transcription (i.e., the activation of a nuclear ER through phosphorylation by both Src/Erk and PI3K signalling in the absence of a ligand), and thus, the modulation of the functions of ERs by nongenomic actions of estrogens may augment the classical mechanism of ER action.

The possible convergence of genomic and nongenomic actions on target genes is an attractive mechanism by which ERs can finely regulate gene expression [78, 102]. It has been suggested that some of the responses to selective estrogen receptor modulators (SERMs) are mediated through nongenomic actions, which subsequently lead to genomic responses [103].

Similarly to estradiol, EDCs with estrogenic activity interfere with the functioning of the complex endocrine system acting through the ERα and ERβ receptor-mediated mechanism. The EDC receptor–ligand complex results in conformational changes and may activate EREs in a different way than the natural estrogen and thus influence the response in a qualitative and quantitative way, i.e., by mimicking the action of naturally produced hormones, they set off similar chemical reactions in the body, and by blocking the receptors in cells, they prevent the action of normal hormones, sometimes in a nonreversible manner [104]. Xenoestrogens seem to have equal binding affinity either to ERα or to ERβ [105] [the final effect in a specific tissue seems to be regulated by the ratio of the two ER isoforms (ERα, ERβ)], and they can selectively activate or repress estrogen-responsive genes in a different mode than the natural estrogens [104].

EDCs can bind either to estrogen receptors acting as estrogens or antiestrogens [106] or to androgen receptors acting as androgens or antiandrogens [106], but some EDCs can activate both receptors (bisphenol A binds to ER and acts through the genomic pathway [107], the pesticide o,p′-DDT also binds and activates the ER

[108, 109], while the p,p′-DDE (the DDT metabolite) acts as an androgen antagonist but also as a weak estrogen receptor agonist compared to o,p′-DDT [110, 111]). The chemical structure of these compounds does not predict their activities, and small changes can alter affinity for the receptor; a typical example is the 5-carbon DPP that has 3-fold increased antiandrogenic potency compared to 4-carbon DBP. It seems that the structure function relationship is very complex.

Except for the genomic effects, some xenoestrogens, such as endosulfan, nonylphenol, and o,p′-DDE, induce rapid nongenomic effects by binding to membrane estrogen receptors (mERα, mERβ, and GPR30); the consequent activation or inhibition of several kinases including Erk1/Erk2, PI3K, MAPK, PKC, and PKA kinases triggers signal cascades. Activation of Ca^{2+} and K^{+} channels, intracellular Ca^{2+} concentration signals, cell proliferation, and apoptosis are effectuated through these pathways in several cell types [112–115].

Nongenomic effects of xenoestrogens have been observed in many cell types including pituitary cancer cells, breast cancer cells, cells of the immune system, neuronal cells, and bone tissues [109, 115–119].

The different classes of EDCs show a diversity of effect patterns and a distinct effect profile: they can induce genomic (nuclear) and nongenomic (extranuclear) effects or both of these effects, independently of each other and thus in conjunction with the activation or inhibition of other signalling pathways (e.g., PI3K); this might lead to an indirect promotion of the transcriptional activity of the ER [107, 120]. The substantial differences in the way they exert their effects through steroid receptors and the ability of compounds to activate either or both pathways are mostly influenced by their chemical structure: as an example, the long-carbon-side-chain alkylphenols show weak estrogenic activity in genomic assays and the shorter-side-chain versions even weaker, while the short- or long-carbon-chain variants show quite robust nongenomic activities [121–123].

Some EDCs, such as the chlorinated hydrocarbon β-HCH, although not binding to ER, are capable of activating ER target genes in a pattern very similar to the profile observed with estrogens. In order to explain these type of estrogenic effects of some compounds, Norman et al. [124] have proposed for the ERS the "two ligand-binding domains," the "*classical*" and the "*alternative*" ligand-binding domain, responsible for the prolonged genomic events and the rapid nongenomic signalling, respectively. According to this theory, a ligand binds to the binding site it better fits; the conformation of a membrane-bound receptor favors binding to alternative site. Thus, compounds such as β-HCH and p,p′-DDE might have different affinities for the two proposed binding domains of the ER: p,p′-DDE fails to interact with the "alternative" domain in the membrane ER and consequently nongenomic effects cannot happen; on the contrary, β-HCH shows affinity to the "alternative" domain and thereby a sustained activation of Src/Ras/Erk pathway that may also lead to the strong activation of a number of other signalling cascades (such as PI3K and PKC), in addition to Src/Ras/Erk pathway [125–127].

As many estrogen agonists and antagonists, xenoestrogens have the ability to selectively bind membrane estrogen receptor [selective membrane receptor modulators (SmERMs)].

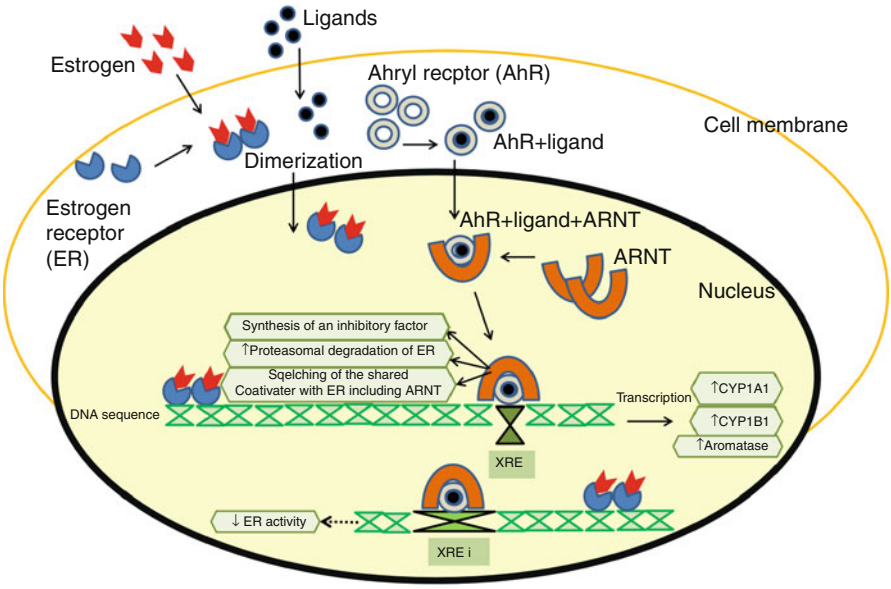


Fig. 1.3 Activation of ER and Ahryl receptor including relevant proposed mechanisms of crosstalk between their signaling pathways from the step of heterodimerization for Ahryl with ARNT and homodimerization for ER. AhR has been reported to inhibit ER activity through a combination of several different mechanisms: direct inhibition by the activated AhR/ARNT heterodimer through binding to inhibitory xenoestrogen receptor (iXRE) present in ER target genes; squelching of shared coactivators, including ARNT; synthesis of an unknown inhibitory protein; increased proteasomal degradation of ER; and altered estrogen synthesis/metabolism through increase in aromatase, cytochromeP450 1A1 and 1B1 expression. ((Courtesy of Hussam Al-Humadi, MD)

Although many of the EDCs' effects are through binding to estrogen receptors, acting as agonists or antagonists, some of them bind to androgen or aryl hydrocarbon receptor (AhR) [128] (Fig. 1.3). The aryl hydrocarbon receptor (AhR) is a member of the basic helix–loop–helix Per (Period)–ARNT (aryl hydrocarbon nuclear translocator)–SIM (single-minded) (bHLH-PAS) family [129]. Upon ligand binding, the AhR translocates from the cytoplasm to the nucleus where it binds its dimerization partner ARNT. The activated AhR/ARNT heterodimer complex binds to xenobiotic response elements (XREs) and activates the expression of AhR target genes, such as cytochrome P4501A1 (CYP1A1) and CYP1B1 [130]. As shown in AhR-null animals, the AhR mediates most, if not all, of the toxic effects of 2,3,7,8-tetrachlorodibenzo-[p]-dioxin (TCDD) [131]. Although its physiological role is unknown, the AhR has been shown to be important in liver development and female reproduction [132]. Since AhR is a receptor for many ligands, striking synergistic effects can be anticipated.

Inhibitory crosstalk between the AhR and ER signalling was suggested by early experiments examining the long-term effects of TCDD treatment in Sprague Dawley rats [133]. The first observations, that the incidences of both mammary and uterine

tumors decreased in female rats [133] after exposure to dioxins, were supported by other reports demonstrating that TCDD inhibits the formation of 7,2-dimethylbenz[a] anthracene (DMBA)-induced mammary tumors [134].

The precise molecular mechanisms for this crosstalk are unclear and may be a combination of several different mechanisms. Several studies have reported that the activated AhR inhibits the expression of E_2-induced genes [135, 136], causes decrease of the levels of nuclear ER, and is involved in mediating the antiestrogenic responses in target cells/organs [137–140]. Thus, it is possible that the primary antiestrogenic action of TCDD is to downregulate expression of the ER gene, thereby reducing cellular ER levels.

EDCs can disrupt the homeostasis of a multicellular tissue by inhibiting the gap junctional communication (the intact communication between adjacent p cells through the connexin-lined gap junctions (Gjs) is a requisite for maintaining homeostasis) [141]. Furthermore, they can dysregulate the effects of effects in an indirect way by disrupting hormone levels; they can inhibit or activate the expression of the P450 enzymes, with consequent alterations in the synthesis, transport, metabolism, and excretion of endogenous hormones, i.e., inhibition of enzymatic activity of the P450 family members CYP19 and CYP3A1, which convert testosterone to estradiol, decreases the hormone synthesis.

EDCs may act at the cellular and molecular levels, binding to both steroid and aryl hydrocarbon receptors exhibiting both dependent and independent receptor modulations of specific gene transcriptional elements [142–144]. As a result, xenoestrogens have the potential to variably modulate cell proliferation, cell cycle progression, apoptosis, and cytokine production in much the same way as 17-β-estradiol does [145, 146].

1.2.2 Cytochrome P (CYP) Induction

Exposure to EDCs can interfere with the induction of the phase I enzymes of the cytochrome P450 family. An example is CYP1A that is inducible by several classes of EDCs including the halogenated aromatic hydrocarbons (HAHs), the polycyclic aromatic hydrocarbons (PAHs), and the dioxins [2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)] [147]. In the presence of a ligand, the AhR/ARNT heterodimer binds to the xenoestrogen responsive elements in the promoter of the CYP1A gene [147, 148] while at the same time the induction of many other genes happens [149, 150] (Fig. 1.3). CYP induction that occurs by a process involving de novo RNA and protein synthesis [151] has been shown to be important in the metabolism of xenoestrogens and the generation of reactive genotoxic metabolites [152, 153]. By this procedure, weakly active procarcinogens can be transformed into electrophilic intermediate metabolites capable of reacting with DNA, raising the risk of developing cancer; a typical example is the case of breast cancer in which the induction of the ile/val and val/val alleles of the cytochrome P450 1A1 gene, under certain circumstances, may result in increased risk [154].

1.3 Effects

The endocrine system regulates complex functions and thus hormone dysregulation results in a wide array of effects. Endogenous estrogens (17-β-estradiol, estrone, and estriol) are not only regulating the development, maintenance, and function of the reproductive system in both sexes [155–157] but they also exert important biologic effects in many tissues and organs: they affect cognition and behavior in the central nervous system; they are involved in the cardiovascular health, have a significant impact on cell-mediated and humoral immune and autoimmune responses, and play a role in adipocyte development and function as well as in bone growth and epiphyseal plate closure in both sexes [158–163]. They are implicated in the development or progression of numerous diseases including breast and colon cancer, osteoporosis, cardiovascular and neurodegenerative diseases, endometriosis, and obesity [164–167].

In relation to estrogens, EDCs can have direct toxic effects on an endocrine gland and indirect endocrine toxicity to non-endocrine organs [168]. The effects of EDCs in wildlife have been documented by many studies; the most prominent include masculinization in snails, hermaphroditism in fish, distorted sex organ development and function in reptiles (alligators and turtles), abnormal nesting behavior and induced eggshell thinning in birds, and disturbed reproduction and immune functions in grey seals [169, 170].

In humans, xenoestrogens have been mainly accused for cancer, neurological and immunological effects, reproduction failure, and osteoporosis, but data are still contradictory. The link between man-made chemicals and adverse effects that usually appear as domino effect is not quite clear. The causative role of chemical substances in diseases and abnormalities related to endocrine substances has not been well documented in human health, even though various articles have appeared describing the growing evidence that man-made chemicals are causing adverse effects in both humans and wildlife by poisoning the hormone system. The fact that adverse effects in animals do not predict the same results in humans and many effects appear to be species specific makes the issue even more difficult; i.e., exposure to phthalates causes suppression of testosterone in rat and stimulation of testosterone in the mouse, while no clear effects have been demonstrated in humans [171, 172]. The accidents in Seveso and in Taiwan (Yu-cheng disease) gave a lot of information about the connection of EDCs and human health [171, 173], but in order to establish a clear cause–effect relationship, geographical, social, diet, lifestyle and inter-population variations should be taken into consideration.

The diversity of mechanisms, the complexities and interactions of endocrine signalling mechanisms, the variety of possible end points, and the broad range of chemicals possibly involved in the adverse effects in humans and wildlife make the issue difficult to understand in its various aspects; thus, before the hypothesis becomes a certainty, it might take a lot of time.

Some of the effects associated to the exposure to xenoestrogens are presented in the following sections.

1.3.1 Effects on Female Genital System

The fundamental role of estrogens in females during puberty and reproductive cycling is well known [174, 175]. Estradiol exerts complex effects on gonadotropin-releasing hormone (GnRH) neuronal function including long-term genomic effects through binding to ERα and/or ERβ subtypes and rapid nongenomic effects such as glutamate-induced currents in hippocampal neurons and second messenger cascades in hippocampal or hypothalamic neurons [85, 176–179].

Xenoestrogens seem also to affect the physiology of the genital system in women, since in epidemiological studies, they have been associated with menstrual disorders, abnormal ovulations, endometriosis, and spontaneous abortions.

Similarly to estrogens, EDCs, in particular o,op′-DDT, can modulate the GnRH secretion in vitro in the immature female hypothalamus through both slow and rapid effects; in these effects, glutamate plays an important role with the participation of genomic and nongenomic pathways involving several receptors (ERs, AHR, and AMPA) and intracellular kinases (A, C, and MAPK) [180]. The early onset of puberty observed after exposure to EDCs is probably connected to GnRH stimulation [181–183].

Epidemiological studies have shown that women who consumed fish from Lake Ontario, polluted with organic pollutants, had reduced cell cycle length, while endocrine dysfunction was found in women exposed to pentachlorophenol [184–186]. Endometriosis represents a common gynecological condition reaching 5–15 % of childbearing-age women and up to 3–5 % of postmenopausal women. Although endometriosis has developed in rats [187, 188] and in monkeys [189] after exposure to dioxins, the data in women are still conflicting; thus, in some studies, EDCs acting through an Ah receptor mechanism [190] have been associated with endometriosis, while in others, there is no evidence of such association [191, 192]. Early menopause has also been referred in women exposed to perfluorocarbons [193].

The concentrations of estrogens in the plasma seem an important factor for the manifestation of the effects after exposure to chemicals with estrogenic activity; thus, menopaused women under hormone replacement therapies are less vulnerable than those who do not take estrogens, while prepubertal girls are more vulnerable compared to older ones [194]. Several of the effects of the estrogenic compounds are also due to the alterations in the aromatase activity and thus changes in the estrogen concentrations [195, 196].

Reproduction problems have also been connected to the extensive use of EDCs. A decrease in fertilization rates after IVF has been found in couples in which the husband was exposed to EDCs (pesticides) [197]. Furthermore, exposure to organochlorides has been associated in some studies with spontaneous abortions [198–200], but according to other reports, pregnancy outcome was not affected [201].

A causal relationship between malformations in the urogenital system and exposure to EDCs had been strongly suggested by the “feminization” of the population in some areas with high discharge of these compounds [202]. The prevalence of birth of more girls than boys from young fathers in the Seveso accident in 1976, a fact observed

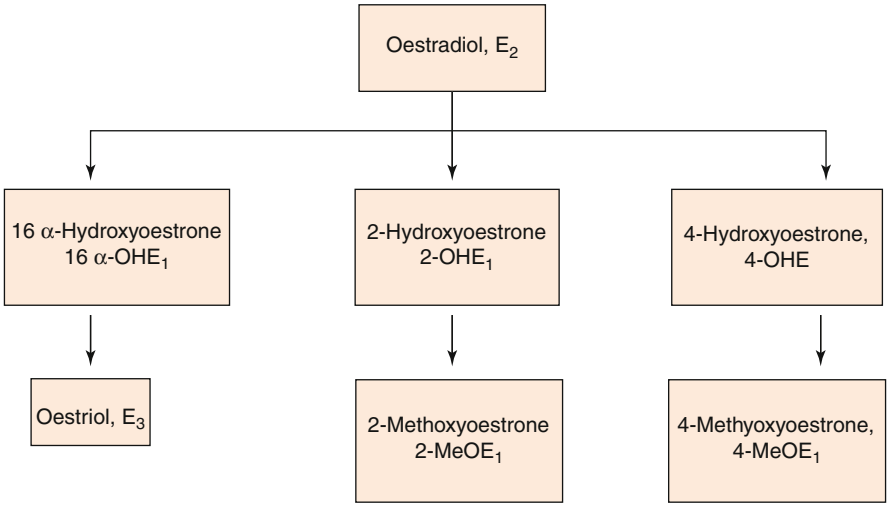


Fig. 1.4 Metabolic products of Oestradiol (E2)

and in many industrial countries, has been connected with exposure to dioxins [203, 204]. Structural and functional defects in the female reproductive tract have been observed after exposure to diethylstilbestrol and other xenoestrogens such as the pesticide methoxychlor; these compounds have been shown to disrupt the development of the female reproductive tract by altering HOX gene expression (HOX gene determines the differential developmental identity of the Müllerian duct) [205, 206].

Estrogens have also been implicated in endometrial cancer through involvement of G protein-coupled estrogen receptor GPR30 and consequent activation of the PKC pathway [207]. Another possible mechanism is that the 2-OH and 4-OH estrogen metabolites can be further oxidized to semiquinones and quinones, which can form bulky DNA adducts and initiate carcinogenesis (Fig. 1.4).

1.3.2 Effects on Male Genital System

Estrogens have a fundamental role in male genital system and consequently in male fertility. Estrogen receptors have been found in mature and fetal testis and in epididymis as well, indicating their importance in regulation of spermatogenesis: ERα is mainly localized in Leydig cells, and ERβ is mainly localized in Leydig and most germ cells, while aromatase, the enzyme that converts testosterone or androstenedione to estradiol, is found in Leydig cells, Sertoli cells, and germ cells [208–210]. Estrogens induce also both proliferative and antiproliferative effects; some of these effects are mediated through binding to ERβ, and consequent downregulation of the androgen receptor ending in induction of apoptotic mechanisms [211].

In view of the important role of estrogens in the male genital system, and since men are routinely exposed to estrogen-like compounds, various changes in male physiology and fertility have been attributed to these chemicals [212, 213].

Studies in wildlife have shown that some of the EDCs, as the metabolite of p,p′ DDE, exhibit antiandrogenic activity; exposure of the alligators in Lake Apopka to DDT showed a progressive decline in the population and abnormal genital structure [214]. Impaired fertility was also shown in experimental animals after exposure to lindane or PCBs [215, 216].

Prenatal exposure of experimental animals to DES resulted in increased incidence of cryptorchidism, urethral abnormalities, testicular hypoplasia, poor semen quality, rete testes adenocarcinoma, and cell hyperplasia [217–219]. Similarly, DES-exposed males have shown pseudohermaphroditism, genital malformations (small testes, testicular abnormalities, microphallus), and reduced semen quality [220]. The genital system seems to be vulnerable when the exposure to estrogenic chemical compounds happens only at a critical period of neonatal life [221]. The effects of some xenoestrogens on sperm quality seem to be effectuated through a nongenomic pathway [222].

Epidemiological studies in various European countries including France, Sweden, Scotland, and Greece have shown a progressive decline in sperm analysis attributed to exposure to compounds with estrogenic activity (i.e., the pesticide dibrochloropropane, DBCP) [223–227]. Reduced sperm concentration and motility was found in higher prevalence in semirural and agricultural areas compared to more urban areas. Workers exposed to dioxin had decreased serum testosterone and increased LH [228]. The increased incidence of hypospadias and cryptorchidism in some countries has also been associated with prenatal or paternal exposure to EDCs [229, 230].

Increased incidence of testicular cancer, a malignancy more common in young men, has been observed in many countries [231–233]. Etiologic agents or conditions for testicular cancer include, among others, exposure to pesticides and field exposure to hydrocarbons and polyvinyl chloride, but many authors have linked the increased incidence with embryonal exposure to EDCs [234–237]. The mothers of men with testicular cancer showed higher concentrations estrogenic compounds [238].

Although the data are not conclusive and sometimes are contradictory, the most possible etiology for the testicular dysgenesis syndrome (TDS) (a disorder of the male reproductive function including decrease of sperm count and increased incidence of testicular cancer and hypospadias and cryptorchidism), which has shown an increase in a small time period, seems to be rather an environmental and not a genetic factor [234, 238, 239].

According to recent studies, xenoestrogens can affect male fertility through a transgenerational epigenetic action on male reproduction system; thus, a transient in utero exposure to a xenoestrogen influences the embryonic testis transcriptome and through epigenetic effects results in abnormal germ cell differentiation that subsequently influences male fertility [240].

1.3.3 Breast Cancer

Estrogens are hormones with genotoxic potential and may act as carcinogens at non-physiological doses. Their carcinogenic effects seem to be independent of ERs,

although ERs could play a role in the early stages of cell transformation, invasion, and tumorigenesis [241]. Increased expression of specific proteins and induction of oxidants and aldehydes ending to and cause lipid peroxidation are among the mechanisms estrogens cause cancer. Furthermore, several oncogenes have been shown to encode the growth factors and their receptors that are activated by estrogens (close relationship), i.e., c-erb-1 oncogene encodes the EGF-r (transmembrane receptor protein, whose extracellular domain is overexpressed in many cancers) [242, 243]. Estrogenic compounds, given their capacity to perturb normal hormonal actions, have been associated to the development of hormone-dependent cancers, such as breast and endometrial cancers and testicular cancer [244].

Similarly to estrogens, many EDCs induce an increased activity in a series of genes in which transcription products are growth factors involved in the carcinogenic process, i.e., EGF, TGFa, IGF1, and their receptors; this fact makes proliferation uncontrollable [245]. The first well-studied case of the association between cancer and estrogenic compounds is the example of diethylstilboestrol (DES), in which the daughters of the pregnant women that had been exposed to DES developed a clear cell adenocarcinoma of the vagina and the cervix [246].

Cancers are traditionally presumed to occur without a threshold; as a consequence, any dose of a carcinogen is associated with an increased risk.

Estrogens have been implicated in the development of breast cancer. In the USA, each year 44,000 women die of breast cancer, making it the leading cause of cancer deaths among American women that do not smoke and among those aged 40–55 years. Increased incidence of breast cancer in all age groups has been shown in various countries. The elevated incidence of breast cancer has been associated with prolonged and cumulative exposure to high levels of estrogens, i.e., early onset of menarche and late menopause, obesity, and hormonal replacement therapy (HRT) [247–251]. Decreased levels of SHBG (sex hormone-binding globulin) have also been associated with increased incidence of breast cancer due to increased levels of free estrogens. Estrogen metabolites have also been implicated in the increased incidence of breast cancer. A shift of the normal metabolic pathways of estrogens to alternative routes may involve carcinogenic metabolites; thus, if instead of the activation towards the 2-hydroxyestrone metabolite (2OHE1) production that acts as weak antiestrogen and is not carcinogenic, a shift to 16a OHE1 pathway occurs, it gives rise to fully potent active estrogens (Fig. 1.4). The 16a OHE1 pathway metabolites are genotoxic and carcinogenic; they circulate in very small amounts but they remain unbound due to their low affinity for SHBG and thus are free to covalently bind to the nuclear ER and to form stable adduct interacting with nuclear histone proteins [252].

ERα, the important receptor for breast development [253], is the mammary mediator of estrogenic effects in breast cancer (both in cell cultures and in breast tissue) [254]. Several sequence variations or single-nucleotide polymorphisms (SNPs) in the ERα gene (*ER1*) have been associated with increased risk of cancer [255].

Reduced binding of estradiol to SHBG may increase risk for breast cancer development [256].

Increased levels of androgens have also been implicated in breast tumor development mainly serving as substrates for estrogen [257].

In a number of epidemiological and cross-sectional studies, EDCs, such as PCBs, DDE, and dieldrin, are included among the risk factors for breast cancer [258, 259]. Increased risk for breast cancer has been shown in countries with medium or high levels of exposure to various EDCs (i.e., triazine pesticides) [260]; a positive correlation between organochloride concentration in adipose tissue and the development of breast cancer [261, 262] has been shown as well. A possible connection between the levels of some pesticides acting as xenoestrogens in breast milk and adipose tissue cannot be excluded [261, 263]. The fact that in Seveso a decrease incidence in breast cancer was reported shows the complexity of the whole issue [264].

Epidemiological data have linked early-life TCDD exposure and diets high in fat to increased risk for breast cancer in humans; high-fat diet has been shown to increase sensitivity to maternal TCDD exposure, resulting in increased breast cancer incidence, by changing metabolism capability [265]. Even a single exposure to a xenoestrogen, if it happens during a critical period of life, may alter epithelial differentiation and lead to increased multiplicity of tumors or decreased latency of tumor formation [266]. P 53 mutations may also be implicated in the susceptibility to EDCs for breast cancer development [267].

The carcinogenic or noncarcinogenic effects of estrogens have been associated to the initiation of estrogen metabolism by cytochrome P450 enzymes CYP1B1, CYP1A1, and CYP1A2 [147, 268–270]. Estrogenic compounds like dioxins, PCDFs, and some PCBs, acting in a similar way, induce CYP1A1, CYP1A2, and CYP1B1 gene expression by serving as aryl hydrocarbon receptor (AhR) agonists; CYP1A1 and CYP1B1 catalyze hydroxylation of the A-ring of estradiol (E2) to form the catechol estrogen 2- or 4-hydroxylestradiol (2-OH-E2 or 4-OH-E2, respectively) (Fig. 1.4) [271].

The discrepancies between EDCs as risk factors and breast cancer found in various studies are probably due to the fact that in the breast cancer development a complex mixture of estrogenic chemicals is involved and not only one factor [272].

1.3.4 Obesity

Nowadays, obesity has risen dramatically not only in industrialized countries but also in poorer countries reaching epidemic proportions [273].

Estrogens through ERα and ERβ are also involved in the regulation of body fat distribution and metabolism [274–277]; ERβ has been shown to have anorectic effects mediated via the central nervous system [278], while disruption of ERα in the ventromedial nucleus of the hypothalamus leads to weight gain, increased visceral adiposity, hyperphagia, hyperglycemia, and impaired energy expenditure in female mice [279].

Studies in DES-exposed mice have indicated that the increase in body weight was associated with an increase in the percent of body fat [280, 281], and significant alterations in genes involved in fat distribution were altered [282]. Similarly to endogenous estrogens, a link between exposure to environmental chemicals (such

as estrogenic chemicals, BPA, PCBs, DDE, and persistent organic pollutants and heavy metals) and the development of obesity has been shown in epidemiologic studies, in support of the findings in experimental animals and show [283–287].

Polychlorinated biphenyls (PCBs) and organochlorine pesticides have been associated with high levels of total serum lipids, fat mass, and BMI, while non-dioxin PCBs were shown to be inversely associated with BMI [288–291]. Moreover, prenatal and early-life PCB exposures have been associated with increased weight in boys and girls at puberty [292]. Other studies report a link between some persistent organic pollutants and increased body weight and diabetes [293].

1.3.5 Diabetes

Diabetes mellitus represents one of the most serious health problems worldwide with more than 177 million people suffer from it, and it is among the leading causes of death [294]. Estradiol seems to play an important role in energy balance, lipid metabolism, and glucose homeostasis [294–299]. Estradiol increases insulin biosynthesis and release in an ERα-dependent manner [300, 301], rapid nongenomic insulinotropic action on b cells is effectuated through ERβ [302], and both receptors (ERα and ERβ) can modulate GLUT4 expression in skeletal muscles of mice [303]. Similarly to E_2, exposure to BPA and other persistent organic pollutants (POPs) like dioxins, furans, polychlorinated biphenyls (PCBs), or organochlorine pesticides, stored in white adipose tissue, have been strongly associated with type 2 diabetes and with most of the components of the metabolic syndrome; cardiovascular disease and liver enzyme abnormalities are established in several cross-sectional studies [290, 304–307]. BPA exposure disrupts pancreatic β-cell function and causes hyperinsulinemia [300] and mild insulin resistance, increases basal and insulin-stimulated glucose transport (due to an increased amount of GLUT4 glucose transporter) [308], stimulates adipogenesis [309, 310], and inhibits adiponectin release leading to increased risk for metabolic syndrome [311]. A significant relationship between BPA concentration in urine and type 2 diabetes has been found [304].

1.3.6 Neurologic Defects

The development of the central nervous system both in utero and during childhood is a continuous process in which many morphologic changes take place. Estrogens play an important role in neural development. Both ER receptors are highly expressed in the brain; ERα receptors are present in higher concentrations in the hippocampus, and ERβ receptors are present in higher concentrations in the basal forebrain and cerebral cortex. The neuroprotective effects of estrogens against neuronal cell death [312, 313] have been documented both in vitro and experimental animals: estrogens regulate the dopaminergic neurotransmission [314–316], promote the growth and survival of cholinergic neurons, and increase cholinergic

activity, but they also have antioxidant and antiapoptotic effects [317–320]. The data from clinical studies in neurodegenerative diseases (Alzheimer's disease and Parkinson disease) are inconsistent and even controversial; estrogens have been positively, negatively, or with no effect correlated with the onset and the severity of the diseases [319, 321–324]. Increased risk of dementia has been associated with lower lifetime endogenous estrogens [325, 326]. Male–female differences in the clinical and cognitive characteristics of several diseases have also extensively been discussed [327–332]. The protective effects have been connected to ERα rather than ERβ activation, through ER-dependent and ER-independent mechanisms or both are involved [313, 333].

Similarly to estrogens, EDCs act directly on CNS, and since the brain is a very sensitive target of steroid action, especially during development, EDC exposure might cause severe problems. Reproductive behavior, learning and memory, and other functions are permanently impaired after perinatal exposure of experimental animals [334]; male rats exposed to TCDD during the perinatal period have shown altered sexual differentiation in the brain, involving sexually dimorphic reproductive and nonreproductive neural end points. EDCs also affect neuronal synapse formation [335]. Many of the effects of the EDCs in CNS are mainly effectuated through the ERβ receptor, but the effects vary depending on the chemical compound, i.e., bisphenol A and methoxychlor affect the dopaminergic and noradrenergic systems in rodents [334, 336], but they are associated to sensory or cognitive deficits after exposure during the neurodevelopment period [337]. The effects of TCDDs though seem to be effectuated through the Ahr receptors found on GABAergic neurons (GABA and glutamate regulate learning and memory functions, stress responses, social behaviors, and mood [338, 339]).

Exposure of humans to EDCs has resulted in effects on behavior changes, learning problems, memory, attention deficit, and impairment of sensory and psychomotor development [340, 341]. Neurologic disorders and cognitive (impairment of memory and attention) and behavioral problems had been reported in young children whose mothers had consumed food contaminated with PCBs in addition to growth retardation [342, 343].

Exposure to PCBs has been associated with a memory deficit at 7 months and at 4 years of age, while up to 11 years of age, a negative association was reported between deficit of IQ (intelligence quotient) and PCBs' concentrations index (the index was comprising maternal and cord serum and maternal milk concentrations). Other studies have reported a significant decrease in mental developmental index score as a function of maternal breast milk levels of PCBs at 2 weeks postpartum, which probably reflects maternal body burdens during pregnancy. Most of the studies showed a decreased IQ and poorer cognitive functioning in preschool children [344–346]. Verbal functions are longlasting, while visual–spatial functions, episodic memory, and sustained attention may be less sensitive to prenatal PCB exposure [347].

Some EDCs cross the placenta readily and the blood–brain barrier in the fetus; thus, exposure to these agents can impair mental and physical ability due to altered bioavailability. It is becoming clear that developmental exposure to EDCs and dioxin-like compounds can permanently impair neuroendocrine functions. Several studies, in various countries, have been conducted in order to establish the

association between prenatal exposure to xenoestrogens and several aspects of psychomotor development [348].

The data are not conclusive and the discrepancies found between the clinical studies are probably due to the different methodologies used for the assessment of the neuropsychological problem and the parameter examined [349, 350]. Another possible reason is that isolated xenoestrogens do not reflect the effect of exposure to a mixture [351].

1.3.7 Immunologic Effects

The relationship between autoimmune system and endogenous estrogen levels is well established [352]. Estrogens mediate their effects via estrogen receptors (nuclear isoforms and/or membrane receptor) in different cell types of the immune system (B cell, T cells, dendritic/macrophages, monocytes) [353]. They regulate T cytokine gene expression via ER-mediated pathways, either directly through EREs or indirectly through interaction of ER with other transcription factors including NF-kB and AP-1 [354, 355]; NF-kB response elements have been found in the promoter of several cytokine genes like IL-6, IL-10, TNF-a IL-1β, IL-12, and IL-2 [356–358]. Thus, estrogens by acting via their receptors and their crosstalk with other transcription factors in immune cells and organs can modulate immunological parameters [359].

Exposure to various classes of EDCs (such as DES, TCDD, PCBs, organochlorides) has been shown to cause immunosuppression and potential disease susceptibility [360, 361] both in humans and animals; dolphins exposed to EDCs (DDT, PCBs) showed impaired immune function [362]; decreased immune function and increased incidence of infections has also been observed among affected people [363]. After the incidence of Japan in 1968 and in Taiwan 1979 from contaminated rice oil, increased incidence of rheumatoid arthritis had been found, while the patients affected by the Yusho disease suffered respiratory infections for a long time [364–366]. Perinatal exposure to estrogenic compounds (i.e., dioxin) has been associated with increased incidence of infections (respiratory infections, otitis) [367], lower white blood cell count during the first years of life; reduced thrombocytes have also been reported to dioxin exposure [368, 369]. Allergic asthma has been associated with phthalate exposure since they induce enhancement of mast cell degranulation and eosinophilic infiltration which are important parts in the early inflammation phase [370]. Exposure to EDCs has also been associated with increased prevalence of thyroid antibodies [371].

PCDDs and related compounds may be related to immune diseases, such as atopic dermatitis. The effects of these compounds on the immune system were very clearly shown on the babies of young Japanese after the oil accident [372]. But important questions of clinical relevance of real-life exposure and identification of molecular targets that can explain the interactions remain to be answered.

1.3.8 Effects on Bones

Estrogens regulate skeletal homeostasis in both men and women. They enhance osteoblast bone formation, and their deficiency has been associated with increased bone resorption and osteoporosis [373, 374]. Exposure to environmental chemicals that are able to disrupt the hormonal equilibrium might represent another risk factor for this disease [254].

Estrogen receptors ERα and ERβ have been found in both osteoclasts and osteoblasts. They are differentially expressed in the growth plate and mineralized bone, ERα is more highly expressed in cortical than in cancellous bone, and ERβ is most evident at cancellous than cortical sites, suggesting that they may have different functions [375–377]. The role of ERα is clearer compared to ERβ in bone formation [378].

The effect of estrogens in bone seems to be age and sex specific [379]. The importance of estrogens in males has been well documented from the fact that a loss of function mutation in ERα gene in a man has been connected with osteopenia [380].

In view of the important role of estrogen deficiency in osteoporosis, EDCs, since they interact with the ERs modulating the estrogen signalling pathway and altering estrogen metabolism, have been implicated in the pathogenesis of osteoporosis. Polychlorinated biphenyls, β-hexachlorocyclohexane, and 2,3,7,8-tetrachlorodibenzo-p-dioxin are among the compounds that have been associated with osteoporosis [381, 382], but the relation between organochlorine exposure and bone quality and osteoporosis is not clear; thus, further studies are needed [383].

1.3.9 Exposure In Utero and During Lactation

The exposure of the embryo to EDCs has been a major concern. The exposure to these chemicals begins from the first days of the in utero life since the placenta easily permits substances with low molecular weight to enter the fetal circulation, suggesting that these compounds can affect organogenesis [384]. Since these chemicals are lipophilic, they tend to accumulate in the adipose tissue of the pregnant women [385] and from there to be transferred to fetuses and infants through the placenta and breastfeeding. Since EDCs cross the placenta, the embryo is exposed to these chemicals and their metabolites; the neonate is further exposed to relatively high EDCs concentrations found in milk.

Even in the absence of epidemiological studies, concern over adverse effects of xenoestrogens is warranted given the unique vulnerability of the developing fetus and child [386]; placenta does not protect the embryo, and the embryo and young children lack the protective mechanisms an adult disposes, including liver metabolism, detoxifying mechanisms, and blood–brain barrier. Maternal exposure to phthalates has been connected to sex steroid hormone status in fetal and newborn

stages. Prenatal exposure to DES has been connected with adverse effects on the reproductive tract both in male and female offspring, including pseudohermaphroditism, genital malformations, and a reduced semen quality [218, 387]. Prenatal BPA exposure has the potential to alter neurodevelopmental, reproductive, and metabolic end points throughout the life span [388–390] at low doses, while at high doses fetal viability is compromised [391].

Although many efforts have been done to establish the lower threshold doses for toxicity, the issue has not been resolved for many products [392]; since there is no threshold in endocrine systems and no safe doses that exist. Actually an effect can be observed in lower doses, while in higher doses little or no effect is shown [393]. One of the reasons the data so far are conflicting is that many methods used lack sensitivity and precision [394]. Furthermore, it is important to take into consideration that the effects on the embryo also depend upon the developmental stage when the exposure happens and they are gender specific. Prenatal and perinatal stages are the most susceptible to the vulnerable effects, but the particular window of exposure during prenatal life makes the whole issue more complicated and the conclusive decisions about the harmful effects difficult. In this context, DDT use has been shown to increase preterm births, which is a major contributor to infant mortality [395].

EDCs can modify gene transcription disrupting the normal signalling systems that determine fetal development, and according to Colborn, they can impose a life sentence on the embryo [396]. Such an example could be considered the higher risk of overweight and obesity later in life that is associated with exposure to EDCs during development.

Further exposure of the neonate is with lactation. Maternal adipose tissue is catabolized and mobilized during lactation to provide 60 % of the fats in milk fat [397]. The adipose tissue catabolism results in the release of persistent EDCs that have been accumulated over the years (i.e., PCDs, DDT) to breast milk. It is interesting that the higher concentrations of the compounds in breast milk are consumed by the first child because the mother's fat stores are depleted with each subsequent child [398]. Human infants are exposed by breastfeeding, on a bodyweight basis, to doses of xenoestrogens that exceed the doses of adults by at least two orders of magnitude. In a group of breastfed children exposed to a PCB home environment, it was shown that the PCB concentrations were markedly increased with the duration of breastfeeding and were about five times higher than in the non-breastfed children [399].

An analysis of human milk revealed increased concentrations of several cosmetic chemicals (i.e., UV filters, synthetic musks, parabens); their concentrations were correlated to the frequency of use of the cosmetics [400]. Human breast milk levels of polybrominated organic compounds have increased 60-fold in the past 30 years and doubled in the last 5 years [401].

In view of the persistency of these chemicals, the ideal solution should be to place the mother and the infant in a protected environment, with no contamination of air, water, and food! But the practical solution for a pregnant and lactating woman should be to avoid consuming contaminated fish, such as fish from freshwater containing PCBS, and minimize exposure to products containing EDCs (cleaning products, paints, etc.). Single products may not be so hazardous but mixtures are [402].

The Greater Boston Physicians for Social Responsibility created a site for lactating women, where one can find information about the contaminated products with EDCs (http://www.igc.org/psr/breastfeeding.htm).

1.3.10 Risk Assessment

It is still not clear what the relationship between observed or assumed effects in humans and wildlife and exposure to man-made estrogenic compounds is; apart from the uses they are designed for, they may have unforeseen adverse effects or synergistic effects.

To make a prediction on human health consequences of the exposure to a range of substances which are suspected of interfering with the endocrine system (i.e., a risk assessment) is a very complex procedure since the following steps are important: identification of the substance, identification of the dose–response relationship, identification of a threshold dose which protects human health, quantification, and qualification of the adverse health effects. Thus, to predict a risk is a time-consuming procedure that needs knowledge of the mechanism of action, identification of the exposed population, and knowledge of the heterogeneity of the population, i.e., genetic predispositions, age and gender, diet, work exposure, and special conditions, such as pregnancy and lactation, route which exposures might occur and estimation of the magnitude, and duration and timing of the doses that people might receive as a result of the exposure. Together these studies indicate variable sensitivity to disruption by environmental chemicals during the developmental period and underscore the complexity of the mechanisms involved in their effects.

In order to examine the acute and chronic toxicity, carcinogenicity, genotoxicity, and effect on reproduction and development (organogenesis and fetal period) caused by EDCs, various pharmacological and toxicological tests have been developed [403].

A major problem in the case of EDCs is that one cannot make accurate predictions about the toxicity of xenoestrogens because of the absence of unambiguous dose–response relationships. Dose–response relationships are known for a number of individual EDCs; they present not a monotonic but a nontraditional dose–response curves such as an inverted "U" or even multiple "U"-shaped curves of their effects. Some effects are dose related, others dose independent or inversed according to the dose; some effects are reversible and others are not [404]. Due to the fact that the threshold model does not exist in the endocrine system, higher doses results are not predictive for lower doses effects [405], i.e., no dose effect has been found in dioxin that caused clear cell adenocarcinoma [406], while in the case of bisphenol A, very small doses have been shown to cause sex reversal in turtles [407]. In Belgium, the dioxin crisis was caused by only roughly 1 g of dioxin contained in 25 l of old PCB transformer oil [12, 13]. Furthermore, the fact that effects of many chemicals across species differ, the dose effect experiments in animals do not permit safe conclusions about the adverse effects in humans.

In view of the exposure to mixtures, the whole procedure becomes even more complex. Little is known about the interactive effects of mixture; the consequences

on human health are multifactorial depending upon the concentration (ppm) of a certain compound in the product: i.e., in cosmetics, the volume of cosmetic used (ml) per application, times of applications per day, and rate of absorbance depending upon route of administration should be determined.

Lower observed effect levels (LOEL) and no effect levels (NOEL) are two of the indexes used, but for many EDCs, the current tests do not provide evidence for the existence of a NOAEL function. Synergistic or antagonistic effects are for the most part unknown; compared to prescribed medication, a medication's license can be withdrawn upon reports on adverse effects, while it is very difficult to revoke the license of a non-pharmaceutical chemical because the data will be partially confounded by the mixture problem and means of effect is almost always lacking.

A major problem is that the exposures to EDCs are involuntary, often chronic in duration; nontoxic effects are known a priori, and the appearance of a new illness or pathology is documented only after high-dose disaster happens [408]. Then, while acute, subacute, and chronic exposure and reproductive and genotoxicity tests in animals are routine for pharmaceuticals for the majority of the chemicals, this does not apply. In general, any chemical with a production level of <1,000 tonnes/year in a country will require little testing. Thus, a considerable amount of research is still required to ascertain the scope and the seriousness of endocrine disruption, including confirmation of epidemiological studies.

The exposure levels that could have deleterious health effects are somewhat difficult to determine and are actively debated [409, 410], but a most critical point is the concentrations at the target organs are most critical. Thus, data on breakdown, excretion, and bioaccumulation are very essential.

Newer methods have been developed in order to measure the amount of EDCs in biological fluids and tissues (i.e., exposure to the phthalates in perfumes and their concentration in hair) [411, 412]. In a recent study, by use of a bioassay, the exposure to EDCs, estimated in total 17β-estradiol equivalents (EEQs), was found increased in occupational exposure to pesticides, disinfectants, and exhaust fumes [413].

Recently, a scalable and statistical method has been developed, in an effort to predict known and novel associations of several chemicals with prostate, lung, and breast cancers, using publicly available data (e.g., on estradiol and bisphenol) [414].

1.4 Importance of Identification of Compounds with Estrogenic Activity

The majority of the EDCs are compounds structurally unrelated, and the prediction of the estrogenic activity is very difficult, and only in a very small number of chemicals can be done. Thus, in view of the continuously increasing number of these compounds, governmental agencies were forced to examine the whole issue [415] setting as first target the identification of the compounds. Several screening tests have been developed; the principal requirement is to assess the potential of

these compounds to interact with the endocrine system of man and wildlife in order to anticipate adverse effects and then to elucidate the mechanism of action. By the in vitro screening methods, the affinity to the nuclear ER (α, β) was evaluated [416–418]. From 58,000 synthetic compounds that were checked in 2002, including synthetic estrogens, natural products, several plasticizers, commercial chemicals, and impurities, 6,903 were found to dispose weak estrogenic activity (at least 1,000-fold less compared to E_2) [419]. Among the bioassays, the E-SCREEN assay is a simple, fast, reproducible, reliable, and quite sensitive assay [109]; it has allowed the identification not only of the chemicals with estrogenic activity but their discrimination into estrogen full and partial agonist and antagonist compounds by measuring the cell proliferation on cell lines as well. Other assays that have been used are the binding receptor assay [109, 420], the cell proliferation assay [421], and the gene expression assay [422], but none of them can distinguish between agonist and antagonist. Although useful, in vitro assays suffer from problems associated with the absence of effective means to metabolize chemicals. Thus, the big problem is that EDCs must be evaluated in intact organisms; in vitro assays are of value just for evaluation of mechanisms of action or prescreening chemicals for potential endocrine-disrupting properties and for setting priorities for in-depth in vivo testing. Big efforts have been made in the USA, EU, Japan, and OECD in establishing appropriate tests and to harmonize the strategy efficiently. A two-tiered program is currently running that includes a combination of in vitro and in vivo assays in order to identify and classify substances in relation to their potential to interact with the endocrine systems (tier 1) and then to develop concentration response curves in animal models (tier two, under validation) [423]. The ultimate goal is to clarify the biological responses of these compounds in whole organism, but in order to test approximately 80,000 compounds, millions of animals should be sacrificed. In view of the ethical and economical problems, animals have been replaced by cell lines or simplified systems (i.e., yeast); compared to the complexity of an organism, often the conclusions drawn are different from in vivo experiments [424, 425].

Pretests of new chemicals before they are marketed and a group classification of the EDCs based on their chemical biochemical and biological activities should be done; the problem is difficult to resolve since there are no adequate tools to test complex mixtures.

References

1. Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, Lucier G, Luster M, Mac MJ, Maczka C, Miller R, Moore J, Rolland R, Scott G, Sheehan DM, Sinks T, Tilson HA (1996) Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the USEPA-sponsored workshop. Environ Health Perspect 104(Suppl 4):715–740
2. Katzenellnbogen JA (1995) The structural pervasiveness of estrogenic activity. Environ Health Perspect 103(suppl 7):99–101
3. Hammond GL (1995) Potential functions of plasma steroid-binding proteins. Trends Endocrinol Metab 6:298–304

4. Apter D, Bolton NJ, Hammond GL, Vihko R (1984) Serum sex hormone-binding globulin during puberty in girls and in different types of adolescent menstrual cycles. Acta Endocrinol (Copenh) 107:413–419
5. Belgorosky A, Rivarola MA (1986) Progressive decrease in serum sex hormone-binding globulin from infancy to late prepuberty in boys. J Clin Endocrinol Metab 63:510–512
6. Hammond GL, Langley MS, Robinson PA, Nummi S, Lund L (1984) Serum steroid binding protein concentrations, distribution of progestogens, and bioavailability of testosterone during treatment with contraceptives containing desogestrel or levonorgestrel. Fertil Steril 42:44–51
7. Colborn T (2004) Endocrine disruption overview: are males at risk? Adv Exp Med Biol 545:189–201
8. Kinlay M, Plant JA, Bell JN, Voulvoulis N (2008) Endocrine disrupting pesticides: implications for risk assessment. Environ Int 34:168–183
9. Golden RJ, Noller KL, Titus-Ernstoff L, Kaufman RH, Mittendorf R, Stillman R, Reese EA (1998) Environmental endocrine modulators and human health: an assessment of the biological evidence. Crit Rev Toxicol 28:109–227
10. Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ Jr, Jégou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Müller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J, Skakkebaek NE (1996) Male reproductive health and environmental xenoestrogens. Environ Health Perspect 104(Suppl 4):741–803
11. Colborn T, Dumanoski D, Myers JP (1995) Our stolen future. Penguin, New York
12. van Larebeke N, Hens L, Schepens P, Covaci A, Baeyens J, Everaert K, Bernheim JL, Vlietinck R, De Poorter G (2001) The Belgian PCB and dioxin incident of January–June 1999: exposure data and potential impact on health. Environ Health Perspect 109:265–273
13. Carpenter DO, Arcaro KF, Bush B, Niemi WD, Pang S, Vakharia DD (1998) Human health and chemical mixtures: an overview. Environ Health Perspect 106(Suppl 6):1263–1270
14. Damstra T, Barlow S, Bergman A, Kavlock R, Van der Kraak G (eds) (2002) International Programme on Chemical Safety: global assessment of the state-of-the-science on endocrine disruptors. WHO, Geneva. http://www.who.int/pcs/emerg_site/edc/global_edc_TOC.htm. Accessed 20 Oct 2013
15. Welshons WV, Nagel SC, vom Saal FS (2006) Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology 147(6 Suppl):S56–S69
16. Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N (1995) Xenoestrogens released from lacquer coatings in food cans. Environ Health Perspect 103:608–612
17. Muncke J (2009) Exposure to endocrine disrupting compounds via the food chain: is packaging a relevant source? Sci Total Environ 407:4549–4559
18. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV (2007) Human exposure to bisphenol A (BPA). Reprod Toxicol 24:139–177
19. Grund S, Higley E, Schönenberger R, Suter MJF, Giesy JP, Braunbeck T, Hecker M, Hollert H (2010) The endocrine disrupting potential of sediments from the Upper Danube River (Germany) as revealed by in vitro bioassays and chemical analysis. Environ Sci Pollut Res 18:446–460
20. Rodgers-Gray TP, Jobling S, Kelly C, Morris S, Brighty G, Waldock MJ, Sumpter JP, Tyler CR (2001) Exposure of juvenile roach (Rutilus rutilus) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. Environ Sci Technol 35:462–470
21. Ternes TA, Stumpf M, Mueller J, Haberer K, Wilken RD, Servos M (1999) Behavior and occurrence of estrogens in municipal sewage treatment plants–I. Investigations in Germany, Canada and Brazil. Sci Total Environ 225:81–90
22. Jobling S, Williams R, Johnson A, Taylor A, Gross-Sorokin M, Nolan M, Tyler CR, van Aerle R, Santos E, Brighty G (2006) Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. Environ Health Perspect 114(Suppl 1):32–39
23. Keiter S, Rastall A, Kosmehl T, Wurm K, Erdinger L, Braunbeck T, Hollert H (2006) Ecotoxicological assessment of sediment, suspended matter and water samples in the upper

Danube river – a pilot study in search for the causes for the decline of fish catches. Environ Sci Pollut Res 13:308–319
24. Gaido K, Dohme L, Wang F, Chen I, Blankvoort B, Ramamoorthy K, Safe S (1998) Comparative estrogenic activity of wine extracts and organochlorine pesticide residues in food. Environ Health Perspect 106(Suppl 6):1347–1351
25. Borchers A, Teuber SS, Keen CL, Gershwin ME (2010) Food safety. Clin Rev Allergy Immunol 39:95–141
26. Judd N, Griffith WC, Faustman EM (2004) Contribution of PCB exposure from fish consumption to total dioxin-like dietary exposure. Regul Toxicol Pharmacol 40:125–135
27. Grimvall E, Rylander L, Nilsson-Ehle P, Nilsson U, Strömberg U, Hagmar L, Ostman C (1997) Monitoring of polychlorinated biphenyls in human blood plasma: methodological developments and influence of age, lactation, and fish consumption. Arch Environ Contam Toxicol 32:329–336
28. Onn Wong K, Woon Leo L, Seah HL (2005) Dietary exposure assessment of infants to bisphenol A from the use of polycarbonate baby milk bottles. Food Addit Contam 22:280–288
29. Pinto B, Reali D (2009) Screening of estrogen-like activity of mineral water stored in PET bottles. Int J Hyg Environ Health 212:228–232
30. Wagner M, Oehlmann J (2009) Endocrine disruptors in bottled mineral water: total estrogenic burden and migration from plastic bottles. Environ Sci Pollut Res Int 16:278–286
31. Witorsch RJ (2002) Endocrine disruptors: can biological effects and environmental risks be predicted? Regul Toxicol Pharmacol 3:118–130
32. Guo YL, Ryan JJ, Lau BP, Yu ML, Hsu CC (1997) Blood serum levels of PCBs and PCDFs in Yucheng women 14 years after exposure to a toxic rice oil. Arch Environ Contam Toxicol 33:104–108
33. Milbrath MO, Wenger Y, Chang CW, Emond C, Garabrant D, Gillespie BW, Jolliet O (2009) Apparent half-lives of dioxins, furans, and polychlorinated biphenyls as a function of age, body fat, smoking status, and breast-feeding. Environ Health Perspect 117:417–425
34. Garritano S, Pinto B, Calderisi M, Cirillo T, Amodio-Cocchieri R, Reali D (2006) Estrogen-like activity of seafood related to environmental chemical contaminants. Environ Health 30:5–9
35. Kogevinas M (2001) Human health effects of dioxins: cancer, reproductive and endocrine system effects. Hum Reprod Update 7:331–339
36. Giguere V, Yang N, Segui P, Evans RM (1988) Identification of a new class of steroid hormone receptors. Nature 331:91–94
37. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M, Gustafsson JA (1997) Human estrogen receptor b–gene structure, chromosomal localization, expression pattern. J Clin Endocrinol Metab 82:4258–4265
38. Arts J, Kuiper GG, Janssen JM, Gustafsson JA, Löwik CW, Pols HA, van Leeuwen JP (1997) Differential expression of estrogen receptor α and β mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology 138:5067–5070
39. Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson J-A (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 3:863–870
40. Kuiper GGJM, Shughrue PJ, Pelto-Huikko M, Merchenthaler I, Gustafsson J-A (1998) The estrogen receptor β subtype: a novel mediator of estrogen action in neuroendocrine systems. Front Neuroendocrinol 19:253–286
41. Lindner V, Kim SK, Karas RH, Kuiper GGJM, Gustafsson J-A, Mendelsohn ME (1998) Increased expression of estrogen receptor-β mRNA in male blood vessels after vascular injury. Circ Res 83:224–229
42. Osterlund M, Kuiper GJM, Gustafsson J-A, Hurd YL (1998) Differential distribution and regulation of estrogen receptor α and β mRNA within the female rat brain. Mol Brain Res 54:175–180
43. Matthews J, Gustafsson JA (2003) Estrogen signaling: a subtle balance between ERα and Erβ. Mol Interv 3:281–292

44. Moore JT, McKee DD, Slentz-Kesler K, Moore LB, Jones SA, Horne EL, Su JL, Kliewer SA, Lehmann JM, Willson TM (1998) Cloning and characterization of human estrogen receptor β isoforms. Biochem Biophys Res Commun 247:75–78
45. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA et al (1997) Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 389:194–198
46. McKenna NJ, Lanz RB, O'Malley BW (1999) Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 20:321–344
47. Klein-Hitpass L, Tsai SY, Greene GL, Clark JH, Tsai MJ, O'Malley BW (1989) Specific binding of estrogen receptor to the estrogen response element. Mol Cell Biol 9:43–49
48. Vanacker JM, Pettersson K, Gustafsson JA, Laudet V (1999) Transcriptional targets shared by estrogen receptor-related receptors (ERRs) and estrogen receptor (ER)alpha but not by ER beta. EMBO J 18:4270–4279
49. Chen JD, Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457
50. Heery DM, Kalkhoven E, Hoare S, Parker MG (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387:733–736
51. Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer D (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell 89:373–380
52. Lomvardas S, Thanos D (2002) Opening chromatin. Mol Cell 9:209–211
53. Johansson L, Thomsen JS, Damdimopoulos AE, Spyrou G, Gustafsson J-A, Treuter E (1999) The orphan nuclear receptor SHP inhibits agonist-dependent transcriptional activity of estrogen receptors ERα and ERβ. J Biol Chem 247:345–353
54. Seol W, Hanstein B, Brown M, Moore DD (1998) Inhibition of estrogen receptor action by the orphan receptor SHP (Short Heterodimer Partner). Mol Endocrinol 12:1551–1557
55. Brown M, Moore DD (1998) Inhibition of estrogen receptor action by the orphan receptor SHP (Short Heterodimer Partner). Mol Endocrinol 12:1551–1557
56. Warnmark A, Almlof T, Leers J, Gustafsson JA, Treuter E (2001) Differential recruitment of the mammalian mediator subunit TRAP220 by estrogen receptors ERalpha and ERbeta. J Biol Chem 276:23397–23404
57. Wong CW, Komm B, Cheskis BJ (2001) Structure-function evaluation of ERalpha and Erbeta interplay with SRC family coactivators. ER selective ligands. Biochemistry 40:6756–6765
58. Graham JD, Bain DL, Richer JK, Jackson TA, Tung L, Horwitz KB (2000) Nuclear receptor conformation, coregulators, and tamoxifen-resistant breast cancer. Steroids 65:579–584
59. Smith CL, Nawaz Z, O'Malley BW (1997) Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol 11:657–666
60. Zhang X, Jeyakumar M, Petukhov S, Bagchi MK (1998) A nuclear receptor corepressor modulates transcriptional activity of antagonist-occupied steroid hormone receptor. Mol Endocrinol 12:513–524
61. Bramlett KS, Wu Y, Burris TP (2001) Ligands specify coactivator nuclear receptor (NR) box affinity for estrogen receptor subtypes. Mol Endocrinol 15:909–922
62. Kraichely DM, Sun J, Katzenellenbogen JA, Katzenellenbogen BS (2000) Conformational changes and coactivator recruitment by novel ligands for estrogen receptor alpha and estrogen receptor-beta: correlations with biological character and distinct differences among SRC coactivator family members. Endocrinology 141:3534–3545
63. Ko L, Cardona GR, Iwasaki T, Bramlett KS, Burris TP, Chin WW (2002) Ser-884 adjacent to the LXXLL motif of coactivator TRBP defines selectivity for ERs and TRs. Mol Endocrinol 16:128–140
64. Pettersson K, Grandien K, Kuiper GGJM, Gustafsson J-A (1997) Mouse estrogen receptor beta forms estrogen response element binding heterodimers with estrogen receptor alpha. Mol Endocrinol 11:1486–1496
65. Chang EC, Frasor J, Komm B, Katzenellenbogen BS (2006) Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. Endocrinology 147:4831–4842

66. Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM (2002) Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J Biol Chem 277: 24353–24360
67. Pettersson K, Delaunay F, Gustafsson JA (2000) Estrogen receptor beta acts as a dominant regulator of estrogen signaling. Oncogene 19:4970–4978
68. Monroe DG, Secreto FJ, Subramaniam M, Getz BJ, Khosla S, Spelsberg TC (2005) Estrogen receptor alpha and beta heterodimers exert unique effects on estrogen- and tamoxifen-dependent gene expression in human U2OS osteosarcoma cells. Mol Endocrinol 19:1555–1568
69. Stossi F, Barnett DH, Frasor J, Komm B, Lyttle CR, Katzenellenbogen BS (2004) Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) alpha or ER beta in human osteosarcoma cells: distinct and common target genes for these receptors. Endocrinology 145:3473–3486
70. Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P (2000) Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74:311–317
71. Safe S (2001) Transcriptional activation of genes by 17 b-estradiol through estrogen receptor-Sp1 interactions. Vitam Horm 62:231–252
72. Gaub MP, Bellard M, Scheuer I, Chambon P, Sassone-Corsi P (1990) Activation of the ovalbumin gene by the estrogen receptor involves the fos–jun complex. Cell 63:1267–1276
73. Li C, Briggs MR, Ahlborn TE, Kraemer FB, Liu J (2001) Requirement of Sp1 and estrogen receptor alpha interaction in 17beta estradiol-mediated transcriptional activation of the low density lipoprotein receptor gene expression. Endocrinology 142:1546–1553
74. Schmitt M, Bausero P, Simoni P, Queuche D, Geoffroy V, Marschal C, Kempf J, Quirin-Stricker C (1995) Positive and negative effects of nuclear receptors on transcription activation by AP-1 of the human choline acetyl-transferase proximal promoter. J Neurosci Res 40:152–164
75. Umayahara Y, Kawamori R, Watada H, Imano E, Iwama N, Morishima T, Yamasaki Y, Kajimoto Y, Kamada T (1994) Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J Biol Chem 269:16433–16442
76. Aronica SM, Katzenellenbogen BS (1993) Stimulation of estrogen receptor mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. Mol Endocrinol 7:743–752
77. Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA, Korach KS (1992) Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. Proc Natl Acad Sci U S A 89:4658–4662
78. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270:1491–1494
79. Bunone G, Briand PA, Miksicek RJ, Picard D (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 15:2174–2183
80. Gottlicher M, Heck S, Herrlich P (1998) Transcriptional cross-talk, the second mode of steroid hormone receptor action. J Mol Med 76:480–489
81. O'Lone R, Frith MC, Karlsson EK, Hansen U (2004) Genomic targets of nuclear estrogen receptors. Mol Endocrinol 18:1859–1875
82. Porter W, Saville B, Hoivik D, Safe S (1997) Functional synergy between the transcription factor Sp1 and the estrogen receptor. Mol Endocrinol 11:1569–1580
83. Stein B, Yang MX (1995) Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta. Mol Cell Biol 15:4971–4979
84. Razandi M, Pedram A, Greene GL, Levin ER (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. Mol Endocrinol 13:307–319
85. Improta-Brears T, Whorton AR, Codazzi F, York JD, Meyer T, McDonnell DP (1999) Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proc Natl Acad Sci U S A 96:4686–4691

86. Ahmad S, Singh N, Glazer RI (1999) Role of AKT1 in 17beta estradiol- and insulin-like growth factor I (IGF-I)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells. Biochem Pharmacol 58:425–430
87. Castoria G, Migliaccio A, Bilancio A, Di Domenico M, deFalco A, Lombardi M, Fiorentino R et al (2001) PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. EMBO J 20:6050–6059
88. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW (1999) Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. J Clin Invest 103:401–406
89. Jessop HL, Sjoberg M, Cheng MZ, Zaman G, Wheeler- Jones CP, Lanyon LE (2001) Mechanical strain and estrogen activate estrogen receptor a in bone cells. J Bone Miner Res 16:1045–1055
90. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC (2001) Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 104:719–730
91. Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E, Auricchio F (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 15:1292–1300
92. Watters JJ, Campbell JS, Cunningham MJ, Krebs EG, Dorsa DM (1997) Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. Endocrinology 138:4030–4033
93. Ordonez-Moran P, Munoz A (2009) Nuclear receptors genomic and non-genomic effects converge. Cell Cycle 8:1675–1680
94. Pappas TC, Gametchu B, Watson CS (1995) Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. FASEB J 9:404–410
95. Ding Q, Gros R, Limbird L, Chorazyczewski J, Feldman RD (2009) Estradiol-mediated ERK phosphorylation and apoptosis in vascular smooth muscle cells requires GPR 30. Am J Physiol Cell Physiol 297:C1178–C1187
96. Filardo EJ, Quinn JA, Bland KI, Frackleton AR (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 14:1649–1660
97. Migliaccio A, Piccolo D, Castoria G, Di Domenico M, Bilancio A, Lombardi M, Gong W, Beato M, Auricchio F (1998) Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. EMBO J 17:2008–2018
98. Razandi M, Pedram A, Park ST, Levin ER, Oh P, Schnitzer J (2003) Proximal events in signaling by plasma membrane estrogen receptors ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. J Biol Chem 278:2701–2712
99. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK (2000) Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 407:538–541
100. Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R, Santen RJ (2002) Linkage of rapid estrogen action to MAPK activation by ERalpha-Shc association and Shc pathway activation. Mol Endocrinol 16:116–127
101. Wyckoff MH, Chambliss KL, Mineo C, Yuhanna IS, Mendelsohn ME, Mumby SM, Shaul PW (2001) Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through Gs(i). J Biol Chem 276:27071–27076
102. Tremblay A, Tremblay GB, Labrie F, Giguere V (1999) Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1. Mol Cell 3:513–519
103. Singleton DW, Feng Y, Burd CJ, Khan SA (2003) Non-genomic activity and subsequent c-fos induction by estrogen receptor ligands are not sufficient to promote deoxyribonucleic acid synthesis in human endometrial adenocarcinoma cells. Endocrinology 144:121–128

104. Stancel GM, Boettger-Tong HL, Chiappetta C, Hyder SM, Kirkland JL, Murthy L, Loose-Mitchell DS (1995) Toxicity of endogenous and environmental estrogens: what is the role of elemental interactions? Environ Health Perspect 103(Suppl 7):29–33
105. Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Bur B, Gustafsson J-A (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252–4263
106. Gulden M, Turan A, Seibert H (1997) Texte 46/97 Substanzen mit endokriner Wirkung in Oberflachengewassern Umweltbundesamt (German Environmental Agency) Berichtsnummer UBA–FB 97-068, ISSN 0722-186X
107. Jorgensen M, Vendelbo B, Skakkebaek NE, Leffers H (2000) Assaying estrogenicity by quantitating the expression levels of endogenous estrogen-regulated genes. Environ Health Perspect 108:403–412
108. Diel P, Olff S, Schmidt S, Michna H (2002) Effects of the environmental estrogens bisphenol A, o, p′-DDT, p-tert-octylphenol and coumestrol on apoptosis induction cell proliferation and the expression of estrogen sensitive molecular parameters in the human breast cancer cell line MCF-7. J Steroid Biochem Mol Biol 80:61–70
109. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO (1995) The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 103(Suppl 7):113–122
110. Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, Wilson EM (1995) Persistent DDT metabolite p, p′-DDE is a potent androgen receptor antagonist. Nature 375:581–585
111. Payne J, Scholze M, Kortenkamp A (2001) Mixtures of four organochlorines enhance human breast cancer cell proliferation. Environ Health Perspect 109:391–397
112. Bulayeva NN, Watson CS (2004) Xenoestrogen-induced ERK- and ERK-2 activation via multiple membrane-initiated signaling pathways. Environ Health Perspect 112:1481–1487
113. Ruehlmann DO, Steinert JR, Valverde MA, Jacob R, Mann GE (1998) Environmental estrogenic pollutants induce acute vascular relaxation by inhibiting L-type Ca^{2+} channels in smooth muscle cells. FASEB J 12:613–619
114. Nadal A, Ropero AB, Laribi O, Maillet M, Fuentes E, Soria B (2000) Nongenomic actions of estrogens and xenoestrogens by binding at a plasma membrane receptor unrelated to estrogen receptor alpha and estrogen receptor beta. Proc Natl Acad Sci U S A 97:11603–11608
115. Zivadinovic D, Gametchu B, Watson CS (2005) Membrane estrogen receptor-alpha levels in MCF-7 breast cancer cells predict cAMP and proliferation responses. Breast Cancer Res 7:R101–R112
116. Zivadinovic D, Watson CS (2005) Membrane estrogen receptor-alpha levels predict estrogen-induced ERK1/2 activation in MCF-7 cells. Breast Cancer Res 7:R130–R144
117. Narita S, Goldblum RM, Watson CS, Brooks EG, Estes DM, Curran EM, Midoro-Horiuti T (2007) Environmental estrogens induce mast cell degranulation and enhance IgE-mediated release of allergic mediators. Environ Health Perspect 115:48–52
118. Alyea RA, Watson CS (2009) Nongenomic mechanisms of physiological estrogen-mediated dopamine efflux. BMC Neurosci 10:59–67
119. Otto C, Fuchs I, Altmann H, Klewer M, Schwarz G, Bohlmann R, Nguyen D, Zorn L, Vonk R, Prelle K, Osterman T, Malmström C, Fritzemeier KH (2008) In vivo characterization of estrogen receptor modulators with reduced genomic versus nongenomic activity in vitro. J Steroid Biochem Mol Biol 111:95–100
120. Silva E, Kabil A, Kortenkamp A (2010) Cross-talk between non-genomic and genomic signalling pathways–distinct effect profiles of environmental estrogens. Toxicol Appl Pharmacol 245:160–170
121. Kochukov MY, Jeng YJ, Watson CS (2009) Alkylphenol xenoestrogens with varying carbon chain lengths differentially and potently activate signaling and functional responses in GH3/B6/F10 somatomammotropes. Environ Health Perspect 117:723–730
122. Kwack SJ, Kwon O, Kim HS, Kim SS, Kim SH, Sohn KH, Lee RD, Park CH, Jeung EB, An BS, Park KL (2002) Comparative evaluation of alkylphenolic compounds on estrogenic activity in vitro and in vivo. J Toxicol Environ Health A 65:419–431

123. Tabira Y, Nakai M, Asai D, Yakabe Y, Tahara Y, Shinmyozu T, Noguchi M, Takatsuki M, Shimohigashi Y (1999) Structural requirements of para-alkylphenols to bind to estrogen receptor. Eur J Biochem 262:240–245
124. Norman AW, Mizwicki MT, Norman DPG (2004) Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. Nat Rev Drug Discov 3:27–34
125. Coosen R, van Velsen FL (1989) Effects of the beta-isomer of hexachlorocyclohexane on estrogen-sensitive human mammary tumor cells. Toxicol Appl Pharmacol 101:310–318
126. Steinmetz R, Young PC, Caperell-Grant A, Gize EA, Madhukar BV, Ben Jonathan N, Bigsby RM (1996) Novel estrogenic action of the pesticide residue beta-hexachlorocyclohexane in human breast cancer cells. Cancer Res 56:5403–5409
127. Silva E, Scholze M, Kortenkamp A (2007) Activity of xenoestrogens at nanomolar concentrations in the E-Screen assay. Environ Health Perspect 115(Suppl 1):91–97
128. Safe S, Wang F, Porter W, Duan R, McDougal A (1998) Ah receptor agonists as endocrine disruptors: antiestrogenic activity and mechanisms. Toxicol Lett 102–103:343–347
129. Gu YZ, Hogenesch JB, Bradfield CA (2000) The PAS superfamily: sensors of environmental and developmental signals. Annu Rev Pharmacol Toxicol 40:519–561
130. Reyes H, Reisz-Porszasz S, Hankinson O (1992) Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science 256:1193–1195
131. Lahvis GP, Bradfield CA (1998) Ahr null alleles: distinctive or different? Biochem Pharmacol 56:781–787
132. Baba T, Mimura J, Nakamura N, Harada N, Yamamoto M, Morohashi K, Fujii-Kuriyama Y (2005) Intrinsic function of the aryl hydrocarbon (dioxin) receptor as a key factor in female reproduction. Mol Cell Biol 25:10040–10051
133. Kociba RJ, Keyes DG, Beyer JE, Carreon RM, Wade CE, Dittenber DA, Kalnins RP, Frauson LE, Park CN, Barnard SD, Hummel RA, Humiston CG (1978) Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats. Toxicol Appl Pharmacol 46:279–303
134. Holcomb M, Safe S (1994) Inhibition of 7,12-dimethylbenzanthracene-induced rat mammary tumor growth by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Cancer Lett 82:43–47
135. Wihlén B, Ahmed S, Inzunza J, Matthews J (2009) Estrogen receptor subtype- and promoter-specific modulation of aryl hydrocarbon receptor-dependent transcription. Mol Cancer Res 7:977–986
136. Safe S, Wormke M (2003) Inhibitory aryl hydrocarbon receptor-estrogen receptor alpha cross-talk and mechanisms of action. Chem Res Toxicol 16:807–816
137. Astroff B, Safe S (1988) Comparative antiestrogenic activities of 2,3,7,8-tetrachlorodibenzo-p-dioxin and 6-methyl-1,3,8-trichlorodibenzofuran in the female rat. Toxicol Appl Pharmacol 95:435–443
138. Astroff B, Safe S (1990) 2,3,7,8-Tetrachlorodibenzo-p-dioxin as an antiestrogen: effect on rat uterine peroxidase activity. Biochem Pharmacol 39:485–488
139. Zacharewski T, Harris M, Safe S (1991) Evidence for the mechanism of action of the 2,3,7,8-tetrachlorodibenzo-p-dioxin-mediated decrease of nuclear estrogen receptor levels in wild-type and mutant mouse Hepa 1c1c7 cells. Biochem Pharmacol 41:1931–1939
140. White TE, Gasiewicz TA (1993) The human estrogen receptor structural gene contains a DNA sequence that binds activated mouse and human Ah receptors: a possible mechanism of estrogen receptor regulation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Biochem Biophys Res Commun 193:956–962
141. Kang KS, Wilson MR, Hayashi T, Chang CC, Trosko JE (1996) Inhibition of gap junctional intercellular communication in normal human breast epithelial cells after treatment with pesticides, PCBs, and PBBs, alone or in mixtures. Environ Health Perspect 104:192–200
142. Kharat I, Saatcioglu F (1996) Antiestrogenic effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin are mediated by direct transcriptional interference with the liganded estrogen receptor. Cross-talk between aryl hydrocarbon- and estrogen-mediated signalling. J Biol Chem 271:10533–10537

143. Jeon MS, Esser C (2000) The murine IL-2 promoter contains distal regulatory elements responsive to the Ah receptor, a member of the evolutionarily conserved bHLH-PAS transcription factor family. J Immunol 165:6975–6983
144. Silverston AE, Frazier DE Jr, Fiore NC, Soults JA, Gasiewicz TA (1994) Dexamethasone, beta-estradiol and 2,3,7,8-tetrachlorodibenzo-p-dioxin elicit thymic atrophy through different cellular targets. Toxicol Appl Pharmacol 126:248–259
145. Kincade PW, Medina KL, Smithson G (1994) Sex hormones as negative regulators of lymphopoiesis. Immunol Rev 137:119–134
146. McMurray RW, Suwannaroj S, Ndebele K, Jenkins JK (2001) Differential effects of sex steroids on T and B lymphocytes: modulation of cell cycling, apoptosis and bcl-2. Pathobiology 69:44–58
147. Nebert DW, Russell DW (2002) Clinical importance of the cytochromes P450. Lancet 360:1155–1162
148. Kawajiri K, Fujii-Kuriyama Y (2007) Cytochrome P450 gene regulation and physiological functions mediated by the arylhydrocarbon receptor. Arch Biochem Biophys 464:207–212
149. Yueh MF, Huang YH, Hiller A, Chen S, Nguyen N, Tukey RH (2003) Involvement of the xenobiotic response element (XRE) in Ah receptor-mediated induction of human UDP-glucuronosyltransferase1A1. J Biol Chem 278:15001–15006
150. Dere E, Boverhof DR, Burgoon LD, Zacharewski TR (2006) In vivo–in vitro toxicogenomic comparison of TCDD-elicited gene expression in Hepa1c1c7 mouse hepatoma cells and C57BL/6 hepatic tissue. BMC Genomics 7:80–97
151. Daujat M, Clair P, Astier C, Fabre I, Pineau T, Yerle M, Gellin J, Maurel P (1991) Induction, regulation and messenger half-life of cytochromes P450 IA1, IA2 and IIIA6 in primary cultures of rabbit hepatocytes. CYP1A1, 1A2 and 3A6 chromosome location in the rabbit and evidence that post-transcriptional control of gene IA2 does not involve mRNA stabilization. Eur J Biochem 200:501–510
152. Nebert DW (1989) The Ah locus: genetic differences in toxicity, cancer, mutation, and birth defects. Crit Rev Toxicol 20:153–174
153. Whitlock JP Jr (1999) Induction of cytochrome P4501A1. Annu Rev Pharmacol Toxicol 39: 103–125
154. Ambrosone CB, Freudenheim JL, Graham S, Marshall JR, Vena JE, Brasure JR, Laughlin R, Nemoto T, Michalek AM, Harrington A et al (1995) Cytochrome P4501A1 and glutathione S-transferase (M1) genetic polymorphisms and postmenopausal breast cancer risk. Cancer Res 55:3483–3485
155. Johnson MR, Carter G, Grint C, Lightman SL (1993) Relationship between ovarian steroids, gonadotrophins and relaxin during the menstrual cycle. Acta Endocrinol (Copenh) 129:121–125
156. Chen M, Hsu I, Wolfe A, Radovick S, Huang KH, Yu SQ, Chang CS, Messing EM, Yeh SY (2009) Defects of prostate development and reproductive system in the estrogen receptor-alpha null male mice. Endocrinology 150:251–259
157. Hess RA, Bunick D, Bahr J (2001) Oestrogen, its receptors and function in the male reproductive tract – a review. Mol Cell Endocrinol 178:29–38
158. McCarthy MM, Schwarz JM, Wright CL, Dean SL (2008) Mechanisms mediating oestradiol modulation of the developing brain. J Neuroendocrinol 20:777–783
159. McCarthy MM (2008) Estradiol and the developing brain. Physiol Rev 88:91–124
160. Cooke PS, Naaz A (2004) Role of estrogens in adipocyte development and function. Exp Biol Med 229:1127–1135
161. Törnwall J, Carey AB, Fox RI, Fox HS (1999) Estrogen in autoimmunity: expression of estrogen receptors in thymic and autoimmune T cells. J Gend Specif Med 2:33–40
162. Vandenput L, Ohlsson C (2009) Estrogens as regulators of bone health in men. Nat Rev Endocrinol 5:437–443
163. Verthelyi D (2001) Sex hormones as immunomodulators in health and disease. Int Immunopharmacol 1:983–993
164. Kristiansson P, Wang JX (2001) Reproductive hormones and blood pressure during pregnancy. Hum Reprod 161:13–17

165. Niino M, Hirotani M, Fukazawa T, Kikuchi S, Sasaki H (2009) Estrogens as potential therapeutic agents in multiple sclerosis. Cent Nerv Syst Agents Med Chem 9:87–94
166. Miller IN, Cronin-Golomb A (2010) Gender differences in Parkinson's disease: clinical characteristics and cognition. Mov Disord 25:2695–2703
167. Hogan AM, Collins D, Baird AW, Winter DC (2009) Estrogen and gastrointestinal malignancy. Mol Cell Endocrinol 307:19–24
168. Harvey PW, Rush KC, Kockburn A (1999) Endocrine hormonal toxicology: an integrated mechanistic and target systems approach. In: Harvey PW, Rush KC, Cockburn A (eds) Endocrine and hormonal toxicology. Wiley, Chichester, pp 3–11
169. Shore LS, Cuneah O, Shemesh M (2010) Effects of environmental estrogens on animals in Israel: implications for effects on humans. Rev Environ Health 25:337–343
170. Lemos MF, van Gestel CA, Soares AM (2010) Developmental toxicity of endocrine disrupters bisphenol A and vinclozolin in a terrestrial isopod. Arch Environ Contam Toxicol 59:274–281
171. Fisher JS (2004) Are all EDC effects mediated via steroid hormone receptors? Toxicology 205:33–41
172. Zheng SJ, Tian HJ, Cao J, Gao YQ (2010) Exposure to di(n-butyl)phthalate and benzo(a) pyrene alters IL-1β secretion and subset expression of testicular macrophages, resulting in decreased testosterone production in rats. Toxicol Appl Pharmacol 248:28–37
173. Homberger E, Reggiani G, Sambeth J, Wipf HK (1979) The Seveso accident: its nature, extent and consequences. Ann Occup Hyg 22:327–367
174. Johnson MH, Everitt BJ (2004) Essential reproduction, 5th edn. Blackwell Publishing, Williston
175. Knobil E, Neill JD (2006) The physiology of reproduction. Elsevier Amsterdam, Boston
176. Herbison AE (1998) Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocr Rev 19:302–330
177. Herbison AE, Pape JR (2001) New evidence for estrogen receptors in gonadotropin-releasing hormone neurons. Front Neuroendocrinol 22:292–308
178. Gu Q, Korach KS, Moss RL (1999) Rapid action of 17b-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors. Endocrinology 140:660–666
179. Abraham IM, Todman MG, Korach KS, Herbison AE (2004) Critical in vivo roles for classical estrogen receptors in rapid estrogen actions on intracellular signalling in mouse brain. Endocrinology 145:3055–3061
180. Rasier G, Parent AS, Gérard A, Denooz R, Lebrethon MC, Charlier C, Bourguignon JP (2008) Mechanisms of interaction of endocrine-disrupting chemicals with glutamate-evoked secretion of gonadotropin-releasing hormone. Toxicol Sci 102:33–41
181. Lee PA, Guo SS, Kulin HE (2001) Age of puberty: data from the United States of America. APMIS 109:81–88
182. Buck Louis GM, Gray LE Jr, Marcus M, Ojeda SR, Pescovitz OH, Witchel SF (2008) Environmental factors and puberty timing: expert panel research needs. Pediatrics 121(Suppl 3):S192–S207
183. Rasier G, Toppari J, Parent AS, Bourguignon JP (2006) Female sexual maturation and reproduction after prepubertal exposure to estrogens and endocrine disrupting chemicals: a review of rodent and human data. Mol Cell Endocrinol 254–255:187–201
184. Gerhard I, Frick A, Monga B, Runnebaum B (1999) Pentachlorophenol exposure in women with gynecological and endocrine dysfunction. Environ Res 80:383–388
185. Mendola P, Buck GM, Sever LE, Zielezny M, Vena JE (1997) Consumption of PCB-contaminated freshwater fish and shortened menstrual cycle length. Am J Epidemiol 146:955–960
186. Windham GC, Lee D, Mitchell P, Anderson M, Petreas M, Lasley B (2005) Exposure to organochlorine compounds and effects on ovarian function. Epidemiology 16:182–190
187. Cummings AM, Hedge JM, Birnbaum LS (1999) Effect of prenatal exposure to TCDD on the promotion of endometriotic lesion growth by TCDD in adult female rats and mice. Toxicol Sci 52:45–49

188. Johnson KL, Cummings AM, Birnbaum LS (1997) Promotion of endometriosis in mice by polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls. Environ Health Perspect 105:750–755
189. Rier SE, Martin DC, Bowman RE, Dmowski WP, Becker JL (1993) Endometriosis in rhesus monkeys (*Macaca mulatta*) following chronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Fundam Appl Toxicol 21:433–441
190. Lang DS, Becker S, Devlin RB, Koren HS (1998) Cell-specific differences in the susceptibility of potential cellular targets of human origin derived from blood and lung following treatment with 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD). Cell Biol Toxicol 14:23–38
191. Mayani A, Barel S, Soback S, Almagor M (1997) Dioxin concentrations in women with endometriosis. Hum Reprod 12:373–375
192. Tsukino H, Hanaoka T, Sasaki H, Motoyama H, Hiroshima M, Tanaka T, Kabuto M, Niskar AS, Rubin C, Patterson DG Jr, Turner W, Needham L, Tsugane S (2005) Associations between serum levels of selected organochlorine compounds and endometriosis in infertile Japanese women. Environ Res 99:118–125
193. Knox SS, Jackson T, Javins B, Frisbee SJ, Shankar A, Ducatman AM (2011) Implications of early menopause in women exposed to perfluorocarbons. J Clin Endocrinol Metab 96: 1747–1753
194. Bermudez DS, Gray LE Jr, Wilson VS (2010) Modeling the interaction of binary and ternary mixtures of estradiol with bisphenol A and bisphenol AF in an in vitro estrogen-mediated transcriptional activation assay (T47D-KBluc). Toxicol Sci 116:477–487
195. Crain DA, Guillette LJ Jr, Rooney AA, Pickford DB (1997) Alterations in steroidogenesis in alligators (Alligator mississippiensis) exposed naturally and experimentally to environmental contaminants. Environ Health Perspect 105:528–533
196. Hayes TB, Case P, Chui S, Chung D, Haeffele C, Haston K, Lee M, Mai VP, Marjuoa Y, Parker J, Tsui M (2006) Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? Environ Health Perspect 114(Suppl 1):40–50
197. Axmon A, Rylander L, Strömberg U, Hagmar L (2000) Miscarriages and stillbirths in women with a high intake of fish contaminated with persistent organochlorine compounds. Int Arch Occup Environ Health 73:204–208
198. De Celis R, Pedrón-Nuevo N, Feria-Velasco A (1996) Toxicology of male reproduction in animals and humans. Arch Androl 37:201–218
199. Jarrell J, Gocmen A, Foster W, Brant R, Chan S, Sevcik M (1998) Evaluation of reproductive outcomes in women inadvertently exposed to hexachlorobenzene in southeastern Turkey in the 1950s. Reprod Toxicol 12:469–476
200. Pathak R, Mustafa M, Ahmed RS, Tripathi AK, Guleria K, Banerjee BD (2010) Association between recurrent miscarriages and organochlorine pesticide levels. Clin Biochem 43:131–135
201. Willis WO, de Peyster A, Molgaard CA, Walker C, MacKendrick T (1993) Pregnancy outcome among women exposed to pesticides through work or residence in an agricultural area. J Occup Med 35:943–949
202. Harris CA, Hamilton PB, Runnalls TJ, Vinciotti V, Henshaw A, Hodgson D, Coe TS, Jobling S, Tyler CR, Sumpter JP (2011) The consequences of feminization in breeding groups of wild fish. Environ Health Perspect 119:306–311
203. Davis DL, Gottlieb MB, Stampnitzky JR (1998) Reduced ratio of male to female births in several industrial countries: a sentinel health indicator? JAMA 279:1018–1023
204. Mocarelli P, Gerthoux PM, Ferrari E, Patterson DG Jr, Kieszak SM, Brambilla P, Vincoli N, Signorini S et al (2000) Paternal concentrations of dioxin and sex ratio of offspring. Lancet 35:1858–1863
205. Taylor HS (2008) Endocrine disruptors affect developmental programming of HOX gene expression. Fertil Steril 89(2 Suppl):e57–e58
206. Fei X, Chung H, Taylor HS (2005) Methoxychlor disrupts uterine Hoxa10 gene expression. Endocrinology 146:3445–3451
207. Filigheddu N, Sampietro S, Chianale F, Porporato PE, Gaggianesi M, Gregnanin I, Rainero E, Ferrara M, Perego B, Riboni F, Baldanzi G, Graziani A, Surico N (2011) Diacylglycerol

kinase α mediates 17-β-estradiol-induced proliferation, motility, and anchorage-independent growth of Hec-1A endometrial cancer cell line through the G protein-coupled estrogen receptor GPR30. Cell Signal 23:1988–1996
208. Cavalieri EL, Stack DE, Devanesan PD, Todorovic R, Dwivedy I, Higginbotham S, Johansson SL, Patil KD, Gross ML, Gooden JK, Ramanathan R, Cerny RL, Rogan EG (1997) Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. Proc Natl Acad Sci 94:10937–10942
209. O'Donnell L, Robertson KM, Jones ME, Simpson ER (2001) Estrogen and spermatogenesis. Endocr Rev 22:289–318
210. Shapiro E, Huang H, Masch RJ, McFadden DE, Wu XR, Ostrer H (2005) Immunolocalization of androgen receptor and estrogen receptors alpha and beta in human fetal testis and epididymis. J Urol 174:1695–1698
211. Taylor RA, Cowin P, Couse JF, Korach KS, Risbridger GP (2006) 17beta-estradiol induces apoptosis in the developing rodent prostate independently of ERalpha or ERbeta. Endocrinology 147:191–200
212. Delbès G, Levacher C, Habert R (2006) Estrogen effects on fetal and neonatal testicular development. Reproduction 132:527–538
213. Guillette LJ Jr, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR (1994) Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ Health Perspect 102:680–688
214. Kholkute SD, Rodriguez J, Dukelow WR (1994) Reproductive toxicity of Aroclor-1254: effects on oocyte, spermatozoa, in vitro fertilization, and embryo development in the mouse. Reprod Toxicol 8:487–493
215. Silvestroni L, Palleschi S (1999) Effects of organochlorine xenobiotics on human spermatozoa. Chemosphere 39:1249–1252
216. Barber LB, Lee KE, Swackhamer DL, Schoenfuss HL (2007) Reproductive responses of male fat head minnows exposed to wastewater treatment plant effluent, effluent treated with XAD8 resin, and an environmentally relevant mixture of alkylphenol compounds. Aquat Toxicol 82:36–46
217. McLachlan JA (1977) Prenatal exposure to diethylstilbestrol in mice: toxicological studies. J Toxicol Environ Health 2:527–537
218. McLachlan JA, Newbold RR, Burow ME, Li SF (2001) From malformations to molecular mechanisms in the male: three decades of research on endocrine disrupters. APMIS 109:263–272
219. Penny R (1982) Pediatrics-epitomes of progress: the effect of des on male offspring. West J Med 136:329–330
220. Adamopoulos DA, Pappa A, Nicopoulou S, Andreou E, Karamertzanis M, Michopoulos J, Deligianni V, Simou M (1996) Seminal volume and total sperm number trends in men attending subfertility clinics in the greater Athens area during the period 1977–1993. Hum Reprod 11:1936–1941
221. Sultan C, Balaguer P, Terouanne B, Georget V, Paris F, Jeandel C, Lumbroso S, Nicolas J (2001) Environmental xenoestrogens, antiandrogens and disorders of male sexual differentiation. Mol Cell Endocrinol 178:99–105
222. Luconi M, Bonaccorsi L, Forti G, Baldi E (2001) Effects of estrogenic compounds on human spermatozoa: evidence for interaction with a nongenomic receptor for estrogen on human sperm membrane. Mol Cell Endocrinol 178:39–45
223. Comhaire FH, Mahmoud AM, Schoonjans F (2007) Sperm quality, birth rates and the environment in Flanders (Belgium). Reprod Toxicol 23:133–137
224. Irvine S, Cawood E, Richardson D, MacDonald E, Aitken J (1996) Evidence of deteriorating semen quality in the United Kingdom: birth cohort study in 577 men in Scotland over 11 years. BMJ 312:467–471
225. Slutsky M, Levin JL, Levy BS (1999) Azoospermia and oligospermia among a large cohort of DBCP applicators in 12 countries. Int J Occup Environ Health 5:116–122

226. Egeland GM, Sweeney MH, Fingerhut MA, Wille KK, Schnorr TM, Halperin WE (1994) Total serum testosterone and gonadotropins in workers exposed to dioxin. Am J Epidemiol 139:272–281
227. Giwercman AH, Rignell-Hydbom A, Toft G, Rylander L, Hagmar L, Lindh C, Pedersen HS, Ludwicki JK, Lesovoy V, Shvets M, Spano M, Manicardi GC, Bizzaro D, Bonefeld-Jorgensen EC, Bonde JP (2006) Reproductive hormone levels in men exposed to persistent organohalogen pollutants: a study of inuit and three European cohorts. Environ Health Perspect 114:1348–1353
228. Swan SH, Brazil C, Drobnis EZ, Liu F, Kruse RL, Hatch M, Redmon JB, Wang C, Overstreet JW, Study for Future Families Research Group (2003) Geographic differences in semen quality of fertile U.S. males. Environ Health Perspect 111:414–420
229. Kristensen P, Irgens LM, Andersen A, Bye AS, Sundheim L (1997) Birth defects among offspring of Norwegian farmers, 1967–1991. Epidemiology 8:537–544
230. Weidner IS, Møller H, Jensen TK, Skakkebaek NE (1998) Cryptorchidism and hypospadias in sons of gardeners and farmers. Environ Health Perspect 106:793–796
231. Adami HO, Bergström R, Möhner M, Zatoński W, Storm H, Ekbom A, Tretli S, Teppo L, Ziegler H, Rahu M et al (1994) Testicular cancer in nine northern European countries. Int J Cancer 59:33–38
232. Kalfa N, Philibert P, Baskin LS, Sultan C (2011) Hypospadias: interactions between environment and genetics. Mol Cell Endocrinol 335:89–95
233. Huyghe E, Matsuda T, Thonneau P (2003) Increasing incidence of testicular cancer worldwide: a review. J Urol 170:5–11
234. Sharpe RM, Skakkebaek NE (1993) Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 2341:1392–1395
235. Skakkebæk NE, Rajpert-De Meyts E, Malm KE (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 16:972–978
236. McGlynn KA, Quraishi SM, Graubard BI, Weber JP, Rubertone MV, Erickson RL (2008) Persistent organochlorine pesticides and risk of testicular germ cell tumors. J Natl Cancer Inst 100:663–671
237. Moline JM, Golden AL, Bar-Chama N, Smith E, Rauch ME, Chapin RE, Perreault SD, Schrader SM, Suk WA, Landrigan PJ (2000) Exposure to hazardous substances and male reproductive health: a research framework. Environ Health Perspect 108:803–813
238. Witorsch RJ (2002) Low-dose in utero effects of xenoestrogens in mice and their relevance to humans: an analytical review of the literature. Food Chem Toxicol 407:905–912
239. Giwercman A, Giwercman YL (2011) Environmental factors and testicular function. Best Pract Res Clin Endocrinol Metab 25:391–402
240. Hardell L, van Bavel B, Lindström G, Carlberg M, Dreifaldt AC, Wijkström H, Starkhammar H, Eriksson M, Hallquist A, Kolmert T (2003) Increased concentrations of polychlorinated biphenyls, hexachlorobenzene, and chlordanes in mothers of men with testicular cancer. Environ Health Perspect 11:930–934
241. Hontz AE, Li SA, Lingle WL, Negron V, Bruzek A, Salisbury JL, Li JJ (2007) Aurora A and B overexpression and centrosome amplification in early estrogen-induced tumor foci in the Syrian hamster kidney: implications for chromosomal instability, aneuploidy, and neoplasia. Cancer Res 67:2957–2963
242. Cavalieri E, Frenkel K, Liehr JG, Rogan E, Roy D (2000) Estrogens as endogenous genotoxic agents – DNA adducts and mutations. J Natl Cancer Inst Monogr 27:75–93
243. Quick EL, Parry EM, Parry JM (2008) Do oestrogens induce chromosome specific aneuploidy in vitro, similar to the pattern of aneuploidy seen in breast cancer? Mutat Res 651:46–55
244. Jobling S, Reynolds T, White R, Parker MG, Sumpter JP (1995) A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ Health Perspect 103:582–587
245. Goto N, Hiyoshi H, Ito I, Tsuchiya M, Nakajima Y, Yanagisawa J (2011) Estrogen and antiestrogens alter breast cancer invasiveness by modulating the transforming growth factor-β signaling pathway. Cancer Sci 102:1501–1508

246. Goodman A, Schorge J, Greene MF (2011) The long-term effects of in utero exposures–the DES story. N Engl J Med 36422:2083–2084
247. Beral V (2003) Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet 362:419–427
248. Bernstein L, Ross RK, Pike MC, Brown JB, Henderson BE (1990) Hormone levels in older women: a study of post-menopausal breast cancer patients and healthy population controls. Br J Cancer 61:298–302
249. Henderson BE, Feigelson HS (2000) Hormonal carcinogenesis. Carcinogenesis 21:427–433
250. Li CI, Malone KE, Porter PL, Weiss NS, Tang MT, Cushing-Haugen KL, Daling JR (2003) Relationship between long durations and different regimens of hormone therapy and risk of breast cancer. JAMA 289:3254–3263
251. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J, Writing Group for the Women's Health Initiative Investigators (2002) Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. JAMA 288:321–333
252. Fussell KC, Udasin RG, Smith PJ, Gallo MA, Laskin JD (2011) Catechol metabolites of endogenous estrogens induce redox cycling and generate reactive oxygen species in breast epithelial cells. Carcinogenesis 32:1285–1293
253. Bocchinfuso WP, Korach KS (1997) Mammary gland development and tumorigenesis in estrogen receptor knockout mice. J Mammary Gland Biol Neoplasia 2:323–334
254. Deroo BJ, Korach KS (2006) Exposure to environmental chemicals that are able to disrupt the hormonal equilibrium might represent another risk factor for this disease: estrogen receptors and human disease. J Clin Invest 116:561–570
255. Yaich L, Dupont WD, Cavener DR, Parl FF (1992) Analysis of the PvuII restriction fragment-length polymorphism and exon structure of the estrogen receptor gene in breast cancer and peripheral blood. Cancer Res 52:77–83
256. Levitz M, Banerjee S, Raju U, Toniolo PG, Shore RE, Nachtigall LE (1997) Sex hormone-binding globulin in estrogen-dependent cancer and estrogen replacement therapy. Ann N Y Acad Sci 828:358–365
257. Zeleniuch-Jacquotte A, Shore RE, Koenig KL, Akhmedkhanov A, Afanasyeva Y, Kato I, Kim MY, Rinaldi S, Kaaks R, Toniolo P (2004) Postmenopausal levels of oestrogen, androgen, and SHBG and breast cancer: long-term results of a prospective study. Br J Cancer 90:153–159
258. Henderson AK, Rosen D, Miller GL, Figgs LW, Zahm SH, Sieber SM, Rothman N, Humphrey HE, Sinks T (1995) Breast cancer among women exposed to polybrominated biphenyls. Epidemiology 6:544–546
259. Safe SH (2000) Endocrine disruptors and human health-is there a problem? An update. Environ Health Perspect 108:487–493
260. Kettles MK, Browning SR, Prince TS, Horstman SW (1997) Triazine herbicide exposure and breast cancer incidence: an ecologic study of Kentucky counties. Environ Health Perspect 10511:1222–1227
261. Wolff MS, Toniolo PG, Lee EW, Rivera M, Dubin N (1993) Blood levels of organochlorine residues and risk of breast cancer. J Natl Cancer Inst 85:648–652
262. Høyer AP, Grandjean P, Jørgensen T, Brock JW, Hartvig HB (1998) Organochlorine exposure and risk of breast cancer. Lancet 352:1816–1820
263. Unger M, Kiaer H, Blichert-Toft M, Olsen J, Clausen J (1984) Organochlorine compounds in human breast fat from deceased with and without breast cancer and in a biopsy material from newly diagnosed patients undergoing breast surgery. Environ Res 34:24–28
264. Bertazzi A, Pesatori AC, Consonni D, Tironi A, Landi MT, Zocchetti C (1993) Cancer incidence in a population accidentally exposed to 2,3,7,8-tetrachlorodibenzo-para-dioxin. Epidemiology 4:398–406
265. La Merrill M, Harper R, Birnbaum LS, Cardiff RD, Threadgill DW (2010) Maternal dioxin exposure combined with a diet high in fat increases mammary cancer incidence in mice. Environ Health Perspect 118:596–601

266. Fenton SE, Hamm JT, Birnbaum LS, Youngblood GL (2002) Persistent abnormalities in the rat mammary gland following gestational and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Toxicol Sci 67:63–74
267. Høyer AP, Gerdes AM, Jørgensen T, Rank F, Hartvig HB (2002) Organochlorines, p53 mutations in relation to breast cancer risk and survival. A Danish cohort-nested case-controls study. Breast Cancer Res Treat 71:59–65
268. Badawi AF, Cavalieri EL, Rogan EG (2001) Role of human cytochrome P450 1A1, 1A2, 1B1, and 3A4 in the 2-, 4-, and 16alpha-hydroxylation of 17beta-estradiol. Metabolism 50:1001–1003
269. Cavalieri EL, Kumar S, Todorovic R, Higginbotham S, Badawi AF, Rogan EG (2001) Imbalance of estrogen homeostasis in kidney and liver of hamsters treated with estradiol: implications for estrogen-induced initiation of renal tumors. Chem Res Toxicol 14: 1041–1050
270. Yager JD (2000) Endogenous estrogens as carcinogens through metabolic activation. J Natl Cancer Inst Monogr 27:67–73
271. Safe S (2000) Molecular biology of the Ah receptor and its role in carcinogenesis. Toxicol Lett 120:1–7
272. Darbre PD, Charles AK (2010) Environmental oestrogens and breast cancer: evidence for combined involvement of dietary, household and cosmetic xenoestrogens. Anticancer Res 30:815–827
273. Wang Y, Liang H, Tussing L, Braunschweig C, Caballero B, Flay B (2007) Obesity and related risk factors among low socio-economic status minority students in Chicago. Public Health Nutr 10:927–938
274. Cooke PS, Heine PA, Taylor JA, Lubahn DB (2001) The role of estrogen and estrogen receptor-alpha in male adipose tissue. Mol Cell Endocrinol 178:147–154
275. Couse JF, Korach KS (1999) Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 20:358–417
276. Naaz A, Zakroczymski M, Heine P (2002) Effect of ovariectomy on adipose tissue of mice in the absence of estrogen receptor alpha (ERalpha): a potential role for estrogen receptor beta (ERbeta). Horm Metab Res 34:758–763
277. Penza M, Montani C, Romani A, Vignolini P, Pampaloni B, Tanini A, Brandi ML, Alonso-Magdalena P, Nadal A, Ottobrini L, Parolini O, Bignotti E, Calza S, Maggi A, Grigolato PG, Di Lorenzo D (2006) Genistein affects adipose tissue deposition in a dose-dependent and gender-specific manner. Endocrinology 147:5740–5751
278. Liang YQ, Akishita M, Kim S, Ako J, Hashimoto M, Iijima K, Ohike Y, Watanabe T, Sudoh N, Toba K, Yoshizumi M, Ouchi Y (2002) Estrogen receptor beta is involved in the anorectic action of estrogen. Int J Obes Relat Metab Disord 26:1103–1109
279. Musatov S, Chen W, Pfaff DW, Mobbs CV, Yang XJ, Clegg DJ, Kaplitt MG, Ogawa S (2007) Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome. Proc Natl Acad Sci U S A 104:2501–2506
280. Newbold RR, Padilla-Banks E, Snyder RJ, Jefferson WN (2007) Perinatal exposure to environmental estrogens and the development of obesity. Mol Nutr Food Res 51:912–917
281. Newbold RR, Padilla-Banks E, Snyder RJ, Phillips TM, Jefferson WN (2007) Developmental exposure to endocrine disruptors and the obesity epidemic. Reprod Toxicol 23:290–296
282. Newbold RR, Jefferson WN, Grissom SF, Padilla-Banks E, Snyder RJ, Lobenhofer EK (2007) Developmental exposure to diethylstilbestrol alters uterine gene expression that may be associated with uterine neoplasia later in life. Mol Carcinog 46:783–796
283. McAllister EJ, Dhurandhar NV, Keith SW, Aronne LJ, Barger J, Baskin M, Benca RM, Biggio J, Boggiano MM, Eisenmann JC, Elobeid M, Fontaine KR, Gluckman P, Hanlon EC, Katzmarzyk P, Pietrobelli A, Redden DT, Ruden DM, Wang C, Waterland RA, Wright SM, Allison DB (2009) Ten putative contributors to the obesity epidemic. Crit Rev Food Sci Nutr 49:868–913
284. Collins S (2005) Overview of clinical perspectives and mechanisms of obesity. Birth Defects Res A Clin Mol Teratol 73:470–471

285. Baillie-Hamilton PF (2002) Chemical toxins: a hypothesis to explain the global obesity epidemic. J Altern Complement Med 8:185–192
286. Heindel JJ (2003) Endocrine disruptors and the obesity epidemic. Toxicol Sci 76:247–249
287. Newbold RR (2010) Impact of environmental endocrine disrupting chemicals on the development of obesity. Hormones (Athens) 93:206–217
288. Goncharov A, Haase RF, Santiago-Rivera A, Santiago-Rivera A, Morse G, Akwesasne Task Force on the Environment, McCaffrey RJ, Rej R, Carpenter DO (2008) High serum PCBs are associated with elevation of serum lipids and cardiovascular disease in a Native American population. Environ Res 106:226–239
289. Lee DH, Lee IK, Jin SH, Steffes M, Jacobs DR Jr (2007) Association between serum concentrations of persistent organic pollutants and insulin resistance among nondiabetic adults: results from the National Health and Nutrition Examination Survey 1999–2002. Diabetes Care 30:622–628
290. Lee DH, Steffes MW, Jacobs DR Jr (2008) Can persistent organic pollutants explain the association between serum gamma-glutamyltransferase and type 2 diabetes? Diabetologia 51:402–407
291. Pelletier C, Doucet E, Imbeault P, Tremblay A (2002) Associations between weight loss-induced changes in plasma organochlorine concentrations, serum T(3) concentration, and resting metabolic rate. Toxicol Sci 67:46–51
292. Gladen BC, Ragan NB, Rogan WJ (2000) Pubertal growth and development and prenatal and lactational exposure to polychlorinated biphenyls and dichlorodiphenyl dichloroethene. J Pediatr 136:490–496
293. Vasiliu O, Cameron L, Gardiner J, Deguire P, Karmaus W (2006) Polybrominated biphenyls, polychlorinated biphenyls, body weight, and incidence of adult-onset diabetes mellitus. Epidemiology 17:352–359
294. World Health Organization (2005) Welcome to the diabetes program 2005. http://www.who.int/diabetes/en/
295. Tchernof A, Calles-Escandon J, Sites CK et al (1998) Menopause, central body fatness and insulin resistance: effects of hormone-replacement therapy. Coron Artery Dis 9:503–511
296. Bryzgalova G, Lundholm L, Portwood N, Gustafsson JA, Khan A, Efendic S, Dahlman-Wright K (2008) Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice. Am J Physiol Endocrinol Metab 295:E904–E912
297. Salpeter SR, Walsh JM, Ormiston TM, Greyber E, Buckley NS, Salpeter EE (2006) Meta-analysis: effect of hormone-replacement therapy on components of the metabolic syndrome in postmenopausal women. Diabetes Obes Metab 8:538–554
298. Barros RP, Machado UF, Gustafsson JA (2006) Estrogen receptors: new players in diabetes mellitus. Trends Mol Med 12:425–431
299. Nadal A, Alonso-Magdalena P, Soriano S, Quesada I, Ropero AB (2009) The pancreatic beta-cell as a target of estrogens and xenoestrogens: implications for blood glucose homeostasis and diabetes. Mol Cell Endocrinol 304:63–68
300. Alonso-Magdalena P, Ropero AB, Carrera MP, Cederroth CR, Baquié M, Gauthier BR, Nef S, Stefani E, Nadal A (2008) Pancreatic insulin content regulation by the estrogen receptor ER alpha. PLoS One 3:e2069
301. Le May C, Chu K, Hu M, Ortega CS, Simpson ER, Korach KS, Tsai MJ, Mauvais-Jarvis F (2006) Estrogens protect pancreatic beta-cells from apoptosis and prevent insulin-deficient diabetes mellitus in mice. Proc Natl Acad Sci U S A 103:9232–9237
302. Soriano S, Ropero AB, Alonso-Magdalena P, Ripoll C, Quesada I, Gassner B, Kuhn M, Gustafsson JA, Nadal A (2009) Rapid regulation of K(ATP) channel activity by 17{beta}-estradiol in pancreatic beta}-cells involves the estrogen receptor {beta and the atrial natriuretic peptide receptor. Mol Endocrinol 23:1973–1982
303. Barros RP, Machado UF, Warner M, Gustafsson JA (2006) Muscle GLUT4 regulation by estrogen receptors ERbeta and ERalpha. Proc Natl Acad Sci U S A 103:1605–1608
304. Lang IA, Galloway TS, Scarlett A, Henley WE, Depledge M, Wallace RB, Melzer D (2008) Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. JAMA 300:1303–1310

305. vom Saal FS, Myers JP (2008) Bisphenol A and risk of metabolic disorders. JAMA 300:1353–1355
306. Lee DH, Lee IK, Porta M, Steffes M, Jacobs DR Jr (2007) Relationship between serum concentrations of persistent organic pollutants and the prevalence of metabolic syndrome among non-diabetic adults: results from the National Health and Nutrition Examination Survey 1999–2002. Diabetologia 50:1841–1851
307. Lee DH, Lim JS, Song K, Boo Y, Jacobs DR Jr (2006) Graded associations of blood lead and urinary cadmium concentrations with oxidative-stress-related markers in the U.S. population: results from the third National Health and Nutrition Examination Survey. Environ Health Perspect 114:350–354
308. Sakurai K, Kawazuma M, Adachi T, Harigaya T, Saito Y, Hashimoto N, Mori C (2004) Bisphenol A affects glucose transport in mouse 3T3-F442A adipocytes. Br J Pharmacol 141:209–214
309. Masuno H, Iwanami J, Kidani T, Sakayama K, Honda K (2005) Bisphenol A accelerates terminal differentiation of 3T3-L1 cells into adipocytes through the phosphatidylinositol 3-kinase pathway. Toxicol Sci 84:319–327
310. Masuno H, Kidani T, Sekiya K, Sakayama K, Shiosaka T, Yamamoto H, Honda K (2002) Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. J Lipid Res 43:676–684
311. Hugo ER, Brandebourg TD, Woo JG, Loftus J, Alexander JW, Ben-Jonathan N (2008) Bisphenol A at environmentally relevant doses inhibits adiponectin release from human adipose tissue explants and adipocytes. Environ Health Perspect 116:1642–1647
312. Amantea D, Russo R, Bagetta G, Corasaniti MT (2005) From clinical evidence to molecular mechanisms underlying neuroprotection afforded by estrogens. Pharmacol Res 52:119–132
313. Ardelt AA, McCullough LD, Korach KS, Wang MM, Munzenmaier DH, Hurn PD (2005) Estradiol regulates angiopoietin-1 mRNA expression through estrogen receptor-alpha in a rodent experimental stroke model. Stroke 36:337–341
314. Shulman LM (2002) Is there a connection between estrogen and Parkinson's disease? Parkinsonism Relat Disord 8:289–295
315. Gillies GE, Murray HE, Dexter D, McArthur S (2004) Sex dimorphisms in the neuroprotective effects of estrogen in an animal model of Parkinson's disease. Pharmacol Biochem Behav 78:513–522
316. Quesada A, Micevych PE (2004) Estrogen interacts with the IGF-1 system to protect nigrostriatal dopamine and maintain motoric behavior after 6-hydroxdopamine lesions. J Neurosci Res 75:107–116
317. Luine VN (1985) Estradiol increases choline acetyltransferase activity in specific basal forebrain nuclei and projection areas of female rats. Exp Neurol 89:484–490
318. Behl C, Widmann M, Trapp T, Holsboer F (1995) 17-beta estradiol protects neurons from oxidative stress-induced cell death in vitro. Biochem Biophys Res Commun 216:473–482
319. Pinkerton JV, Henderson VW (2005) Estrogen and cognition with a focus on Alzheimer's disease. Semin Reprod Med 23:172–179
320. Morinaga A, Ono K, Takasaki J, Ikeda T, Hirohata M, Yamada M (2011) Effects of sex hormones on Alzheimer's disease-associated β-amyloid oligomer formation in vitro. Exp Neurol 228:298–302
321. Janicki SC, Schupf N (2010) Hormonal influences on cognition and risk for Alzheimer's disease. Curr Neurol Neurosci Rep 10:359–366
322. Nicoletti A, Arabia G, Pugliese P, Nicoletti G, Torchia G, Condino F, Morgante L, Quattrone A, Zappia M (2007) Hormonal replacement therapy in women with Parkinson disease and levodopa-induced dyskinesia: a crossover trial. Clin Neuropharmacol 30:276–280
323. Saunders-Pullman R (2003) Estrogens and Parkinson disease: neuroprotective, symptomatic, neither, or both? Endocrine 21:81–87
324. Sherwin BB (2003) Estrogen and cognitive functioning in women. Endocr Rev 24:133–151
325. Lebrun CE, van der Schouw YT, de Jong FH, Pols HA, Grobbee DE, Lamberts SW (2005) Endogenous oestrogens are related to cognition in healthy elderly women. Clin Endocrinol (Oxf) 63:50–55

326. Schupf N, Pang D, Patel BN, Silverman W, Schubert R, Lai F, Kline JK, Stern Y, Ferin M, Tycko B, Mayeux R (2003) Onset of dementia is associated with age at menopause in women with Down's syndrome. Ann Neurol 54:433–438
327. Bloch M, Daly RC, Rubinow DR (2003) Endocrine factors in the etiology of postpartum depression. Compr Psychiatry 44:234–246
328. Farage MA, Osborn TW, MacLean AB (2008) Cognitive, sensory, and emotional changes associated with the menstrual cycle: a review. Arch Gynecol Obstet 278:299–307
329. Hampson E (1990) Variations in sex-related cognitive abilities across the menstrual cycle. Brain Cogn 14:26–43
330. Lewinsohn PM, Rohde P, Seeley JR (1998) Major depressive disorder in older adolescents: prevalence, risk factors, and clinical implications. Clin Psychol Rev 18:765–794
331. Steiner M, Dunn E, Born L (2003) Hormones and mood: from menarche to menopause and beyond. J Affect Disord 74:67–83
332. Rubinow DR, Schmidt PJ (2006) Gonadal steroid regulation of mood: the lessons of premenstrual syndrome. Front Neuroendocrinol 27:210–216
333. Dubal DB, Zhu H, Yu J, Rau SW, Shughrue PJ, Merchenthaler I, Kindy MS, Wise PM (2001) Estrogen receptor alpha, not beta, is a critical link in estradiol-mediated protection against brain injury. Proc Natl Acad Sci U S A 98:1952–1957
334. Panzica GC, Viglietti-Panzica C, Mura E, Quinn MJ Jr, Lavoie E, Palanza P, Ottinger MA (2007) Effects of xenoestrogens on the differentiation of behaviorally-relevant neural circuits. Front Neuroendocrinol 28:179–200
335. Shinomiya N, Shinomiya M (2003) Dichlorodiphenyltrichloroethane suppresses neurite outgrowth and induces apoptosis in PC12 pheochromocytoma cells. Toxicol Lett 137:175–183
336. Petersen SL, Krishnan S, Hudgens ED (2006) The aryl hydrocarbon receptor pathway and sexual differentiation of neuroendocrine functions. Endocrinology 147:S33–S42
337. Stump DG, Beck MJ, Radovsky A, Garman RH, Freshwater LL, Sheets LP, Marty MS, Waechter JM Jr, Dimond SS, Van Miller JP, Shiotsuka RN, Beyer D, Chappelle AH, Hentges SG (2010) Developmental neurotoxicity study of dietary bisphenol A in Sprague–Dawley rats. Toxicol Sci 115:167–182
338. Seo BW, Sparks AJ, Medora K, Amin S, Schantz SL (1999) Learning and memory in rats gestationally and lactationally exposed to 2,3,7,8-tetrachlo-rodibenzo-p-dioxin (TCDD). Neurotoxicol Teratol 21:231–239
339. Hojo R, Stern S, Zareba G, Markowski VP, Cox C, Kost JT, Weiss B (2002) Sexually dimorphic behavioral responses to prenatal dioxin exposure. Environ Health Perspect 110: 247–254
340. Holene E, Nafstad I, Skaare JU, Krogh H, Sagvolden T (1999) Behavioural effects in female rats of postnatal exposure to sub-toxic doses of polychlorinated biphenyl congener 153. Acta Paediatr Suppl 88:55–63
341. Masuo Y, Morita M, Oka S, Ishido M (2004) Motor hyperactivity caused by a deficit in dopaminergic neurons and the effects of endocrine disruptors: a study inspired by the physiological roles of PACAP in the brain. Regul Pept 123:225–234
342. Johnson BL, DeRosa CT (1995) Chemical mixtures released from hazardous waste sites: implications for health risk assessment. Toxicology 105:145–156
343. Guo YL, Lambert GH, Hsu CC, Hsu MML (2004) Yucheng: health effects of prenatal exposure to polychlorinated biphenyls and dibenzofurans. Int Arc Occup Environ Health 77:153–158
344. Jacobson SW, Fein GG, Jacobson JL, Schwartz PM, Dowler JK (1985) The effect of intrauterine PCB exposure on visual recognition memory. Child Dev 564:853–860
345. Patandin S, Lanting CI, Mulder PG, Boersma ER, Sauer PJ, Weisglas-Kuperus N (1999) Effects of environmental exposure to polychlorinated biphenyls and dioxins on cognitive abilities in Dutch children at 42 months of age. J Pediatr 1341:33–41
346. Winneke G, Bucholski A, Heinzow B, Krämer U, Schmidt E, Walkowiak J, Wiener JA, Steingrüber HJ (1998) Developmental neurotoxicity of polychlorinated biphenyls (PCBs): cognitive and psychomotor functions in 7-month old children. Toxicol Lett 102–103:423–428

347. Boucher O, Muckle G, Bastien CH (2009) Prenatal exposure to polychlorinated biphenyls: a neuropsychologic analysis. Environ Health Perspect 1171:7–16
348. Schantz SL, Widholm JJ, Rice DC (2003) Effects of PCB exposure on neuropsychological function in children. Environ Health Perspect 111:357–376
349. Cicchetti DV, Kaufman AS, Sparrow SS (2004) The relationship between prenatal and postnatal exposure to polychlorinated biphenyls (PCBs) and cognitive, neuropsychological and behavioral deficits: a critical appraisal. Psychol Sch 41:589–624
350. Ross G (2004) The public health implications of polychlorinated biphenyls (PCBs) in the environment. Ecotoxicol Environ Saf 59:275–291
351. Tanida T, Warita K, Ishihara K, Fukui S, Mitsuhashi T, Sugawara T, Tabuchi Y, Nanmori T, Qi WM, Inamoto T, Yokoyama T, Kitagawa H, Hoshi N (2009) Fetal and neonatal exposure to three typical environmental chemicals with different mechanisms of action: mixed exposure to phenol, phthalate, and dioxin cancels the effects of sole exposure on mouse midbrain dopaminergic nuclei. Toxicol Lett 189:40–47
352. Schuurs AH, Verheul HAJ (1990) Effects of gender and sex steroids on the immune response. J Steroid Biochem 35:157–172
353. Tsokos GC, Kammer GM (2000) Molecular aberrations in human systemic lupus erythematosus. Mol Med Today 6:418–424
354. Cerillo G, Rees A, Manchanda N, Reilly C, Brogan I I, White A, Needham M (1998) The oestrogen receptor regulates NFκB and AP-1 activity in a cell-specific manner. J Steroid Biochem Mol Biol 67:79–88
355. McKay LI, Cidlowski JA (1999) Molecular control of immune/inflammatory responses: interactions between nuclear factor-κB and steroid receptor-signaling pathways. Endocr Rev 20:435–459
356. Baeuerle PA, Henkel T (1994) Function and activation of NF- κB in the immune system. Annu Rev Immunol 12:141–179
357. Liu J, Beller DI (2003) Distinct pathways for NF-κB regulation are associated with aberrant macrophage IL-12 production in lupus- and diabetes-prone mouse strains. J Immunol 9:4489–4496
358. Katsiari CG, Tsokos GC (2006) Transcriptional repression of interleukin-2 in human systemic lupus erythematosus. Autoimmun Rev 5:118–121
359. Dean JH, Cornacoff JB, Haley PJ, Hincks JR (1994) The integration of immunotoxicology in drug discovery and development: investigative and in vitro possibilities. Toxicol In Vitro 8:939–944
360. Koppe J, de Boer P (2001) Immunotoxicity by dioxins and PCBs in the perinatal period. In: Nikolopoulou-Stamati P, Hens L, Howard CV (eds) Endocrine disruptors environmental health and policies. Kluwer Academic Publishers, Dordrecht
361. Lahvis GP, Wells RS, Kuehl DW, Stewart JL, Rhinehart HL, Via CS (1995) Decreased lymphocyte responses in free-ranging bottlenose dolphins (*Tursiops truncatus*) are associated with increased concentrations of PCBs and DDT in peripheral blood. Environ Health Perspect 103(Suppl 4):67–72
362. Smialowicz RJ, DeVito MJ, Williams WC, Birnbaum LS (2008) Relative potency based on hepatic enzyme induction predicts immunosuppressive effects of a mixture of PCDDS/PCDFS and PCBS. Toxicol Appl Pharmacol 227:477–484
363. Pesatori AC, Zocchetti C, Guercilena S, Consonni D, Turrini D, Bertazzi PA (1998) Dioxin exposure and non-malignant health effects: a mortality study. Occup Environ Med 55:126–131
364. Nagayama J, Tsuji H, Iida T, Hirakawa H, Matsueda T, Ohki M (2001) Effects of contamination level of dioxins and related chemicals on thyroid hormone and immune response systems in patients with "Yusho". Chemosphere 43:1005–1010
365. Nakanishi Y, Shigematsu N, Kurita Y, Matsuba K, Kanegae H, Ishimaru S, Kawazoe Y (1985) Respiratory involvement and immune status in Yusho patients. Environ Health Perspect 59:31–36
366. Aoki Y (2001) Polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans as endocrine disrupters–what we have learned from Yusho disease. Environ Res 86:2–11

367. Weisglas-Kuperus N, Patandin S, Berbers GA, Sas TC, Mulder PG, Sauer PJ, Hooijkaas H (2000) Immunologic effects of background exposure to polychlorinated biphenyls and dioxins in Dutch preschool children. Environ Health Perspect 108:1203–1207
368. Leijs MM, Koppe JG, Olie K, van Aalderen WM, de Voogt P, ten Tusscher GW (2009) Effects of dioxins, PCBs, and PBDEs on immunology and hematology in adolescents. Environ Sci Technol 43:7946–7955
369. ten Tusscher GW, Koppe JG (2004) Perinatal dioxin exposure and later effects–a review. Chemosphere 54:1329–1336
370. Bornehag CG, Nanberg E (2010) Phthalate exposure and asthma in children. Int J Androl 33:333–345
371. Langer P (2010) The impacts of organochlorines and other persistent pollutants on thyroid and metabolic health. Front Neuroendocrinol 314:497–518
372. Iida T, Hirakawa H, Matsueda T, Takenaka S, Nagayama J (1999) Polychlorinated dibenzo-p-dioxins and related compounds: the blood levels of young Japanese women. Chemosphere 3815:3497–3502
373. Lane NE (2006) Epidemiology, etiology, and diagnosis of osteoporosis. Am J Obstet Gynecol 194(Suppl 2):S3–S11
374. North American Menopause Society (2006) Management of osteoporosis in postmenopausal women: 2006 position statement of The North American Menopause Society. Menopause 13:340–367
375. Bord S, Horner A, Beavan S, Compston J (2001) Estrogen receptors alpha and beta are differentially expressed in developing human bone. J Clin Endocrinol Metab 86:2309–2314
376. Braidman IP, Hainey L, Batra G, Selby PL, Saunders PT, Hoyland JA (2001) Localization of estrogen receptor beta protein expression in adult human bone. J Bone Miner Res 16:214–220
377. Windahl SH, Vidal O, Andersson G, Gustafsson JA, Ohlsson C (1999) Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERbeta (-/-) mice. J Clin Invest 104:895–901
378. McDougall KE, Perry MJ, Gibson RL, Colley SM, Korach KS, Tobias JH (2003) Estrogen receptor-alpha dependency of estrogen's stimulatory action on cancellous bone formation in male mice. Endocrinology 144:1994–1999
379. Windahl SH, Andersson G, Gustafsson JA (2002) Elucidation of estrogen receptor function in bone with the use of mouse models. Trends Endocrinol Metab 13:195–200
380. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS (1994) Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056–1061
381. Alveblom AK, Rylander L, Johnell O, Hagmar L (2003) Incidence of hospitalized osteoporotic fractures in cohorts with high dietary intake of persistent organochlorine compounds. Int Arch Occup Environ Health 76:246–248
382. Beard J, Marshall S, Jong K, Newton R, Tripplett-McBride T, Humphries B, Bronks R (2000) 1,1,1-trichloro-2,2-bis (p-chlorophenyl)-ethane (DDT) and reduced bone mineral density. Arch Environ Health 55:177–180
383. Côté S, Ayotte P, Dodin S, Blanchet C, Mulvad G, Petersen HS, Gingras S, Dewailly E (2006) Plasma organochlorine concentrations and bone ultrasound measurements: a cross-sectional study in peri-and postmenopausal Inuit women from Greenland. Environ Health 21:5–33
384. Wang SL, Lin CY, Guo YL, Lin LY, Chou WL, Chang LW (2004) Infant exposure to polychlorinated dibenzo-p-dioxins, dibenzofurans and biphenyls (PCDD/Fs, PCBs)–correlation between prenatal and postnatal exposure. Chemosphere 54:1459–1473
385. Tan J, Li QQ, Loganath A, Chong YS, Xiao M, Obbard JP (2008) Multivariate data analyses of persistent organic pollutants in maternal adipose tissue in Singapore. Environ Sci Technol 42:2681–2687
386. Braun JM, Hauser R (2011) Bisphenol A and children's health. Curr Opin Pediatr 23:233–239
387. Steinhardt GF (2004) Endocrine disruption and hypospadias. Adv Exp Med Biol 545:203–215

388. Palanza P, Gioiosa L, vom Saal FS, Parmigiani S (2008) Effects of developmental exposure to bisphenol A on brain and behavior in mice. Environ Res 108:150–157
389. Schönfelder G, Friedrich K, Paul M, Chahoud I (2004) Developmental effects of prenatal exposure to bisphenol a on the uterus of rat offspring. Neoplasia 6:584–594
390. Fernández M, Bourguignon N, Lux-Lantos V, Libertun C (2010) Neonatal exposure to bisphenol a and reproductive and endocrine alterations resembling the polycystic ovarian syndrome in adult rats. Environ Health Perspect 118:1217–1222
391. vom Saal FS, Akingbemi BT, Belcher SM, Birnbaum LS, Crain DA, Eriksen M, Farabollini F, Guillette LJ Jr, Hauser R, Heindel JJ, Ho SM, Hunt PA, Iguchi T, Jobling S, Kanno J, Keri RA, Knudsen KE, Laufer H, LeBlanc GA, Marcus M, McLachlan JA, Myers JP, Nadal A, Newbold RR, Olea N, Prins GS, Richter CA, Rubin BS, Sonnenschein C, Soto AM, Talsness CE, Vandenbergh JG, Vandenberg LN, Walser-Kuntz DR, Watson CS, Welshons WV, Wetherill Y, Zoeller RT (2007) Chapel Hill bisphenol A expert panel consensus statement: integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure. Reprod Toxicol 24:131–138
392. Golub MS, Wu KL, Kaufman FL, Li LH, Moran-Messen F, Zeise L, Alexeeff GV, Donald JM (2010) Bisphenol A: developmental toxicity from early prenatal exposure. Birth Defects Res B Dev Reprod Toxicol 89:441–466
393. Welshons WV, Nagel SC, Thayer KA, Judy BM, vom Saal FS (1999) Low-dose bioactivity of xenoestrogens in animals: fetal exposure to low doses of methoxychlor and other xenoestrogens increases adult prostate size in mice. Toxicol Ind Health 15:12–25
394. Baker VA (2001) Endocrine disrupters–testing strategies to assess human hazard. Toxicol In Vitro 15:413–419
395. Longnecker MP, Klebanoff MA, Zhou H, Brock JW (2001) Association between maternal serum concentration of the DDT metabolite DDE and preterm and small-for-gestational-age babies at birth. Lancet 358:110–114
396. Colborn T (2004) Neurodevelopment and endocrine disruption. Environ Health Perspect 112:944–949
397. Schreiber J (1997) Transport of organic chemicals to breast milk: tetrachloroethene case study. In: Kacew S, Lambert G (eds) Environmental toxicology and pharmacology of human development. Taylor & Francis, Washington, DC
398. Iida T, Hirakawa H, Matsueda T, Takenaka S, Nagayama J (1999) Polychlorinated dibenzo-p-dioxins and related compounds in breast milk of Japanese primiparas and multiparas. Chemosphere 38:2461–2466
399. Walkowiak J, Wiener JA, Fastabend A, Heinzow B, Krämer U, Schmidt E, Steingrüber HJ, Wundram S, Winneke GO (2001) Environmental exposure to polychlorinated biphenyls and quality of the home environment: effects on psychodevelopment in early childhood. Lancet 358:1602–1607
400. Schlumpf M, Kypke K, Wittassek M, Angerer J, Mascher H, Mascher D, Vökt C, Birchler M, Lichtensteiger W (2010) Exposure patterns of UV filters, fragrances, parabens, phthalates, organochlor pesticides, PBDEs, and PCBs in human milk: correlation of UV filters with use of cosmetics. Chemosphere 81:1171–1183
401. Hooper K, McDonald TA (2000) The PBDEs: an emerging environmental challenge and another reason for breast-milk monitoring programs. Environ Health Perspect 108:387–392
402. Howdeshell KL, Wilson VS, Furr J, Lambright CR, Rider CV, Blystone CR, Hotchkiss AK, Gray LE Jr (2008) A mixture of five phthalate esters inhibits fetal testicular testosterone production in the Sprague–Dawley rat in a cumulative, dose-additive manner. Toxicol Sci 105:153–165
403. Harvey PW, Everett DJ (2006) Regulation of endocrine-disrupting chemicals: critical overview and deficiencies in toxicology and risk assessment for human health. Best Pract Res Clin Endocrinol Metab 20:145–165
404. Bignert A, Olsson M, Persson W, Jensen S, Zakrisson S, Litzén K, Eriksson U, Häggberg L, Alsberg T (1998) Temporal trends of organochlorines in Northern Europe, 1967–1995. Relation to global fractionation, leakage from sediments and international measures. Environ Pollut 99:177–198

405. Rubin BS, Murray MK, Damassa DA, King JC, Soto AM (2001) Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. Environ Health Perspect 109:675–680
406. Palmer JR, Anderson D, Helmrich SP, Herbst AL (2000) Risk factors for diethylstilbestrol-associated clear cell adenocarcinoma. Obstet Gynecol 95:814–820
407. Sheehan DM, Willingham E, Gaylor D, Bergeron JM, Crews D (1999) No threshold dose for estradiol-induced sex reversal of turtle embryos: how little is too much? Environ Health Perspect 107:155–159
408. Laporte JR (1978) Multinationals and health: reflections on the Seveso catastrophe. Int J Health Serv 8:619–632
409. Myers JP, Zoeller RT, vom Saal FS (2009) A clash of old and new scientific concepts in toxicity, with important implications for public health. Environ Health Perspect 117:1652–1655
410. Watson CS, Jeng YJ, Kochukov MY (2010) Nongenomic signaling pathways of estrogen toxicity. Toxicol Sci 115:1–11
411. Koo HJ, Lee BM (2004) Estimated exposure to phthalates in cosmetics and risk assessment. J Toxicol Environ Health A 67:1901–1914
412. Yang SH, Morgan AA, Nguyen HP, Moore H, Figard BJ, Schug KA (2011) Quantitative determination of bisphenol A from human saliva using bulk derivatization and trap-and-elute liquid chromatography coupled to electrospray ionization mass spectrometry. Environ Toxicol Chem 30:1243–1251
413. Brouwers MM, Besselink H, Bretveld RW, Anzion R, Scheepers PT, Brouwer A, Roeleveld N (2011) Estrogenic and androgenic activities in total plasma measured with reporter-gene bioassays: relevant exposure measures for endocrine disruptors in epidemiologic studies? Environ Int 37:557–564
414. Patel CJ, Butte AJ (2010) Predicting environmental chemical factors associated with disease-related gene expression data. BMC Med Genomics 6:3–17
415. Hubal EA, Sheldon LS, Zufall MJ, Burke JM, Thomas KW (2000) The challenge of assessing children's residential exposure to pesticides. J Expo Anal Environ Epidemiol 10:638–649
416. Bonefeld-Jørgensen EC, Andersen HR, Rasmussen TH, Vinggaard AM (2001) Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. Toxicology 158:141–153
417. Fang H, Tong W, Perkins R, Soto AM, Prechtl NV, Sheehan DM (2002) Quantitative comparisons of in vitro assays for estrogenic activities. Environ Health Perspect 108:723–729
418. Legler J, Zeinstra LM, Schuitemaker F, Lanser PH, Bogerd J, Brouwer A, Vethaak AD, De Voogt P, Murk AJ, Van der Burg B (2002) Comparison of in vivo and in vitro reporter gene assays for short-term screening of estrogenic activity. Environ Sci Technol 36:4410–4415
419. Hong H, Tong W, Fang H, Shi L, Xie Q, Wu J, Perkins R, Walker JD, Branham W, Sheehan DM (2002) Prediction of estrogen receptor binding for 58,000 chemicals using an integrated system of a tree-based model with structural alerts. Environ Health Perspect 110:29–36
420. Bolger R, Wiese TE, Ervin K, Nestich S, Checovich W (1998) Rapid screening of environmental chemicals for estrogen receptor binding capacity. Environ Health Perspect 106:551–557
421. Soto AM, Justicia H, Wray JW, Sonnenschein C (1991) p-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. Environ Health Perspect 92:167–173
422. Jeltsch JM, Roberts M, Schatz C, Garnier JM, Brown AM, Chambon P (1987) Structure of the human oestrogen-responsive gene pS2. Nucleic Acids Res 15:1401–1414
423. EDSTAC (1998) Endocrine disruptor screening and testing advisory committee final report U.S. Environmental Protection Agency. http://www.epa.gov/endo/pubs/edspoverview/finalrpt.htm
424. Charles GD, Kan HL, Schisler MR, Bhaskar Gollapudi B, Sue Marty M (2005) A comparison of in vitro and in vivo EDSTAC test battery results for detecting antiandrogenic activity. Toxicol Appl Pharmacol 202:108–120
425. Folmar LC, Hemmer MJ, Denslow ND, Kroll K, Chen J, Cheek A, Richman H, Meredith H, Grau EG (2002) A comparison of the estrogenic potencies of estradiol, ethynylestradiol, diethylstilbestrol, nonylphenol and methoxychlor in vivo and in vitro. Aquat Toxicol 60:101–110

Part II
Methodology of Measuring BPA and Its Effects

Chapter 2
Analytical Methods for Determination of Bisphenol A

Dimitra Voutsa

2.1 Introduction

Bisphenol A (2,2-bis-(4-hydroxyphenyl)propane, BPA) is an industrial chemical used in a wide range of applications. It is formed through an acid-catalyzed condensation reaction of phenol and acetone [1]. The chemical structure and the physicochemical properties of BPA are shown in Table 2.1. BPA is a moderately water-soluble compound, with low volatility that is easily biodegraded.

Bisphenol A is a chemical manufactured in large quantities. It is estimated that 1,150 tonnes/year are produced and used in Western Europe. Almost 96 % of BPA is used as a monomer for the production of polycarbonate and epoxy resins. Other applications include its use as stabilizing agent in plastics, as antioxidant in tire production, and as basic chemical in the production of certain flame retardants. The BPA-based materials are used in food and beverage containers, protective coating, automotive lenses, optical lenses, adhesives, powder paints, building materials, compact disks, thermal paper, paper coatings, dental, surgical, and prosthetical materials [3, 4]. The production and extensive use of these materials result in the release of this compound into the environment during processing, handling, and transportation of final products. It was estimated that 39.5 % of the total environmental release of BPA comprised total air release, 1 % water release, 54 % land release, and 5.4 % underground injection [3].

Various in vitro and in vivo assays showed that BPA presents estrogenic activity, and consequently, it is considered as important organic pollutant [3]. BPA may cause a variety of adverse effects on reproduction and development of exposed organisms, being more striking and irreversible during embryonic development. These effects may occur even at doses of BPA well below those showing adverse effects in routine toxicity studies [3–7].

D. Voutsa
Environmental Pollution Control Laboratory, Department of Chemistry,
Aristotle University of Thessaloniki, 54 124 Thessaloniki, Greece
e-mail: dvoutsa@chem.auth.gr

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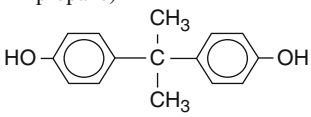
Table 2.1 Physicochemical properties of BPA [2]

CAS no.	80-05-7
Organic compound	Bisphenol A (2,2-bis-(4hydroxyphenyl) propane)
Chemical structure	
Formula	$C_{15}H_{16}O_2$
Molecular weight	228.29 g/mol
Boiling point	398 °C at 760 mmHg
Melting point	153–157 °C
pKa	9.6–11.3
Water solubility	120–300 mg/L
Vapor pressure	0.2 mmHg at 170 °C
Log Kow	2.20–3.82
Henry's constant	1.0×10^{-10} atm m^3/mol

The US-EPA, under the Toxic Substances Control Act, indents to consider initiating rulemaking to identify BPA on the Concern List as a substance that may present an unreasonable risk to the environment on the basis of its potential for long-term adverse effects on growth, reproduction, and development in aquatic species at concentrations similar to those found in the environment [4]. BPA is candidate to be among the first substances to go through Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) in EU registration (EU Regulation No 1907/2006). Canada was the first country that has classified BPA as toxic substance and announced restriction of imports, sales, and advertising of polycarbonate baby bottles containing BPA. Recently, the European Commission published a new directive (2011/8/EU) to restrict bisphenol A in feeding bottles that are intended for use by infants under the age of 12 months [8]. According to this directive, member states are required to prohibit the manufacture of polycarbonate feeding bottles containing BPA as well as their import and sale in EU.

The study of the occurrence of BPA in various environmental compartments, in food, in dental materials, and in biological fluids contributes to the knowledge on the environmental fate of this compound, the possible pathways of exposure, the biotransformation mechanisms, and the possible risks. In order to identify and determine trace levels of BPA in complex matrices, sensitive analytical methods are required.

A number of analytical methods have been developed for the determination of BPA. The general scheme of analysis usually comprises isolation from samples through extraction, cleanup steps, and determination by employing a sensitive analytical technique. The major problems associated with the analysis are possible loss or contamination during sampling and storage, the need of preconcentration and, possibly, of cleanup, as well as the need for highly efficient separation procedures

and selective detection techniques. Reliable analytical procedures require detailed method validation and careful evaluation. In addition, sampling and sample preparation should be considered integrally with the characterization of an analytical procedure.

2.2 Sampling and Storage

The first step in the measurement of BPA involves representative sampling and maintaining sample integrity prior to analysis. The sampling strategy should reflect the known or expected variability of the system.

All the equipment that may come into contact with sample or the extract should be free from interfering compounds. The sampling containers should be made of materials that do not change the sample during the contact time. Plastics and other organic materials should be avoided during sampling, sample storage, or extraction. Glass brown bottles with glass stoppers or with PTFE-lined screw caps, carefully precleaned, are recommended for sampling and storage of samples. Rinsing with acetone is recommended for all glassware used in the analysis. Alternatively, non-volumetric glassware may be heated to at least 200 °C for a minimum of 2 h. The samples should be analyzed as soon as possible; otherwise, they can be stored at 2–5 °C for 2 weeks [9].

2.3 Extraction Techniques

Sample preparation is an important stage in the analytical process when trace analyte determination is needed. The analysis of pollutants at low concentrations in complex matrices requires the elimination of interferences and the reduction of final extract volumes to attain higher preconcentration of target analytes. Generally these pretreatment methods are necessary in order to improve the detection and quantification limits, avoid matrix implications, limit background noise, and extend the life of the analytical column.

The analysis of BPA in environmental, food, and biological liquid samples employs a wide range of sample extraction techniques. Solid-phase extraction is frequently used for isolation and preconcentration of BPA (Tables 2.2, 2.3, 2.4, and 2.5). Moreover, other techniques such as the traditional liquid–liquid extraction, solid-phase microextraction, and stir bar sorptive extraction have been used.

Liquid–liquid extraction (LLE) is a reliable technique for the extraction of BPA from liquid samples. LLE is proposed for the recovery of BPA along with other compounds (NP, tOP, NPE1EO, NPE2EO) from environmental waters in ASTM D7065-06 method. LLE has the advantage of low equipment costs, but there is

Table 2.2 Analytical methods and concentration range of BPA in dental materials

Dental materials	Samples analyzed/ pretreatment	Analytical method	LOD	Concentrations of BPA	Reference
Core build-up materials	Eluates in ethanol 75 %	LC-MS/MS Column: CC 125/4 Nucleodur 100-5 C18 Mobile phase: 0.1 % formic acid/ acetonitrile Diagnostic ions: *m/z* 227	0.5 μg/mL	BDL-6.14 μg/mL	Brenn-Struckhofova and Cichna-Markl [43]
Orthodontic adhesives	Eluates in alcohol 99 %	HPLC-UV/Vis Column: C18 Mobile phase: acetonitrile/water (60:40 v/v) Wavelength: 228 nm	0.1 ppm	BDL	Fontana et al. [57]
Dental sealants	Eluates in ethanol 95 %	HPLC-UV Column: Nova Pak C18 Mobile phase: A acetonitrile/water (50:50 v/v) B acetonitrile Wavelength: 215 nm	0.0001 μg/ mg	BDL	Cunha et al. [58]
Dental sealants	Eluates in distilled water	HPLC-UV/Vis Column: C18 resolved column Mobile phase: isocratic 70 % methanol Wavelength: 215 nm	–	BDL	Salafranca et al. [59]
Orthodontic adhesives	Eluates in distilled water SPE (Oasis HLB) Elution with acetone Derivatization with BSTFA	GC-MS, EI, SIM Column: 5 % diphenyl-95 % dimethyl polysiloxane Diagnostic ions: *m/z* 357.2, 358.2 Internal standard BPA-*d16*	2.3 ng/L	0.16–2.90 μg/L	Maragou et al. [44]

Composites/sealants	Vigorous agitation with distilled water (37 °C) at various pH values (1–12)	HPLC-UV Column: C18 Mobile phase: gradient A acetonitrile/water (1:1 v/v) and B acetonitrile Wavelength: 280 nm GC-MS Column methyl silica Diagnostic ions: *m/z* 213, 228	0.20 µg/mL	0.3–116.1 µg/mL in polymerized materials <0.2–179.5 µg/mL in unpolymerized materials	[60]
Dental sealants/adhesive resins	Eluates in water/acetonitrile (43/57)	HPLC-UV/Vis Reversed phase column Mobile phase: water/acetonitrile (43/57) Wavelength: 215 nm GC-MS Column: methyl silicon DB-1	100 pg	BDL	[60]
Dental sealants	Saliva Urine SPE C18 Elution with methanol Derivatization: pentafluoro-benzyl bromide	GC-MD, NCI Column: 5 % phenyl-methyl-polysiloxane Diagnostic ions: *m/z* 407, 299 Internal standard $^{13}C_{12}$-BPA	0.1 ng/mL	0.17–96.2 ng/mL 0.6–112.2 ng/m	Kawaguchi et al. [46]
Composite resins	Saliva	ELISA "EIKEN"kit		0.3–100 ng/L	Shao et al. [39]

(continued)

Table 2.2 (continued)

Dental materials	Samples analyzed/ pretreatment	Analytical method	LOD	Concentrations of BPA	Reference
Dental sealants	Saline solution (37 °C) Saliva Serum SPE C18 Elution with acetonitrile	HPLC-FLD Column: Supelcosil LC-C18 Mobile phase: acetonitrile/water (50:50 v/v) Exc/Emis wv: 278/315 nm	5 ppb	BDL BDL	Chang et al. [45]
Restorative composites Dental sealants	Eluates in ethanol Saliva	HPLC-diode array Column: S5 ODS Mobile phase: gradient A acetonitrile/water (1:1) and B acetonitrile Wavelength: 235 nm GC-MS confirmation		BDL-84.4 μg/100 mg 3.5–30 μg/mL	Kawaguchi et al. [61]

BDL below detection limit

Table 2.3 Analytical methods and levels of BPA in environmental samples

Samples/Country	Pretreatment	Analytical method	LOD	Concentration of BPA	Reference
River water	LLE with dichloromethane	GC-MS, EI, SIM	0.5 pg/μL	17–776 ng/L	Heemken et al. [21]
Sea water	HPLC clean up	Column: 5 % phenylmethyl silicon Diagnostic ions: *m/z* 315, 331, 407		BDL-249 ng/L	
Germany	Derivatization with HFBA	Internal standard BPA-*d16*			
Freshwater	Filtration	GC-MS, EI, Full-scan	20 ng/L	BDL-1, 924 ng/L	Quednow and Püttmann [22]
	SPE (Bod Elute OOL)	Column: BP-X5 Diagnostic ions: *m/z* 213, 228			
Germany	Elution with methanol/acetonitrile				
Surface waters	SPE (LiChrolut)	GC-MS/MS, EI	0.1 ng/L	0.5–410 ng/L	Fromme et al. [23]
Sewage effluents	Elution with acetone	RP-HPLC-FLD (Exc/Emis wv 228/310 nm) Mobile phase: gradient A hexane	2.0 ng/L	18–702 ng/L	
Germany		B hexane/methanol/isopropanol (40/45/15)			
Surface waters	Filtration	GC-MS, EI, SIM	2.4 ng/L	9–76 ng/L	Voutsa et al. [24]
	SPE (Oasis HLB)	Column: DB-5 Diagnostic ions: *m/z* 357.2, 358.3			
	Elution with acetone	Internal standard BPA-*d16*			
Switzerland	Derivatization with MSTFA/2 % Sylon BTZ				
Surface water	Filtration	LC-MS/MS, ESI, NI, MRM	1.1 ng/L	2–46 ng/L 1.3–1, 640 ng/L	Jonkers et al. [25]
		Column: 100 RP18ec			
Wastewaters	SPE (Oasis HLB)	Mobile phase: gradient A water 4 mM ammonium acetate B methanol			
		Precursor ion: *m/z* 227.02			
		Product ion: *m/z* 211.8			
Switzerland	Elution with MTBE/2-propanol	Internal standard BPA-*d16*			

(continued)

Table 2.3 (continued)

Samples	Pretreatment	Analytical method	LOD	Concentration of BPA	Reference
Wastewaters	SPE (Oasis HLB)	GC-MS, EI, SIM	0.5 ng/L	450 ng/L	Jeannot et al. [26]
	Elution with methanol-diethyl ether (10:90 v/v)	Column 95 % dimethyl-5 % phenylpolysiloxane			
		Identification ion: *m/z* 358			
		GC-MS/MS, EI			
		Precursor ion: *m/z* 358			
France	Derivatization with BSTFA	Product ions: *m/z* 191, 267, 357			
Surface waters	Filtration	GC-MS, EI, SIM	2.3 ng/L	15–460 ng/L	Arditsoglou and Voutsa [27, 28]
	SPE (Oasis HLB)	Column: 5 % diphenyl-95 % dimethyl polysiloxane		15–56 ng/L	Pothitou and Voutsa [29]
	Elution with acetone	Diagnostic ions: *m/z* 357.2, 358.2		15–2, 358 ng/L	Arditsoglou and Voutsa [30]
Coastal waters	Derivatization BSTFA	Internal standard BPA-*d16*			
Wastewaters					
Greece					
Surface waters	Decantation	LC-MS/MS, RP, ESI, API	2 ng/L	3–175 ng/L	Loos et al. [31]
	SPE (Oasis HLB)	Column: Synergi Polar RP			
Wastewaters	Elution with ethanol/acetone/ ethyl-acetate (2:2:1)	Mobile phase: water/acetonitrile			
Italy-Belgium		Precursor ion: *m/z* 227			
		Product ions: *m/z* 133, 212			

Lagoon water Italy	SPE (ENVI-18) Elution with acetonitrile, methanol, water	HPLC-MS, ESI Column: C8-2 Mobile phase: gradient A acetonitrile B water Internal standard BPA-*d16*	1 ng/L	BDL-145 ng/L	Pojana et al. [32]
Precipitation The Netherlands	LLE with dichloromethane	LC-MS, ESI, NI Column: Symmetry C18	5 ng/L	bdl-357 ng/L	Peters et al. [33]
Surface waters The Netherlands	Filtration SPE (SDV-XC disks) Elution with methanol Derivatization with SIL A	GC-MS Column: SGE BPX5 Diagnostic ions: *m/z* 357 Internal standard: BPA-*d16*	14 ng/L	BDL-330 ng/L	Belfoid et al. [34]
Wastewater Portugal	Filtration SPE (Oasis HLB) Elution with dichloromethane	LC-MS/MS, NI, MRM Column: Purospher STAR RP-18 Mobile phase: gradient A methanol B water Precursor ion: *m/z* 227 Product ions: *m/z* 133, 211 Internal standard: oxybenzoic acid ELISA	2 ng/L 5 µg/L	0.15–1.55 µg/L	Mauricio et al. [35]
Surface waters Wastewater Spain	Filtration SPE (Lichrolut RP-18) Elution with acetonitrile	HPLC-MS, ESI, NI Column: 100RP-18 Mobile phase: gradient A methanol B water Diagnostic ions: *m/z* 227 Internal standard: 4-heptylpheno	0.09 µg/L	BDL-2.97 µg/L 0.06–1.51 µg/L	Céspedes et al. [36]

BDL below detection limit

Table 2.4 Analytical methods and levels of BPA in food samples

Samples	Pretreatment	Analytical method	LOD	Concentration of BPA	Reference
Bottled water	LLE with dichloromethane Derivatization BSTFA	GC-MS, EI Column: 5 % diphenyl-95 % dimethyl polysiloxane Diagnostic ions: *m/z* 357.2, 358.2 Internal standard BPA-*d16*	2.3 ng/L	3.5–150 ng/L	Nathanson et al. [12]
Bottled water	SPE (OASIS HLB or C18) Elution two steps A dichloromethane/hexane (4:1 v/v) B ethanol/dichloromethane (9:1 v/v)	GC-MS, EI Column: HP 5MS Diagnostic ions: *m/z* 213, 119, 228 Internal standard: 4nNP	0.005 µg/mL	bdl-0.011 µg/L	Inoue et al. [76]
Mineral water Soda beverages	SPE (OASIS HLB) Elution with methanol/dichloromethane	LC-MS/MS, ESI, NI, MRM Column: A symmetry C-18 Mobile phase: A methanol and B water Precursor ion: *m/z* 227.2 Product ions: *m/z* 93.1, 133.4, 212.4	0.01 ng/L 0.60 ng/L	BDL	Gallart-Ayala et al. [77]
Canned soft drink products	SPE (C18) Elution acetonitrile-water (1:1 v/v)	GC-MS, EI Column: HP 5MS Diagnostic ions: *m/z* 213, 228, 270, 312 Internal standard: BPA-*d16*	27–74 ng/L	0.032–4.5 µg/L	Hennion [51]
Soft drinks/beers	DLLME Derivatization acetic anhydrite	GC-MS, EI Column: HP 5HT, HP 5MS Diagnostic ions: *m/z* 213, 228, 270, 312 Internal standard: BPA-*d16*	5 ng/L	BDL-4.7 µg/L	Joskow et al. [17]

Milk	Mixing with C18 SPE clean up	LC-MS/MS, ESI, NI, MRM Column: A symmetry C-18 Mobile phase: A methanol and B water Precursor ion: *m/z* 227.2 Product ions: *m/z* 93.1, 133.4, 212.4	0.1 µg/kg	bdl-0.49 µg/kg	Qubiňa et al. [78]
Milk	Dilution with methanol SPE (C-18) Elution with ethylacetate	GC-MS, EI Column: HP 5MS Diagnostic ions: *m/z* 213, 119, 228 Internal standard: 4nNP	0.15 µg/kg	0.28–2.64 µg/kg	[79]
Milk infant formula	DLLME Derivatization acetic anhydrite	GC-MS, EI Column: HP 5HT, HP 5MS Diagnostic ions: *m/z* 213, 228, 270, 312 Internal standard: BPA-*d16*	60 ng/L	BDL-0.40 µg/L	Joskow et al. [17]
Wine	SPE (C18) Elution with acetonitrile Clean up sol-gel	HPLC-FLD Column: LiChrosper 60 Mobile phase: Acetonitrile/water (30:70 v/v) Exc/Emis wv: 275/305 nm	0.1 ng/mL	bdl-2.1 ng/mL	Mohapatra et al. [80]
Food simulants – water and 3 % w/v acetic acid (leachates from baby bottles)	SPME	HPLC-FLD Column: ODS-2 C18-bonded Mobile phase: methanol/water (70:30 v/v) Exc/Emis wv: 235/317 nm	1.8 ng/mL 2.4 ng/mL	2.4–14.3 µg/kg	[81]
Food simulants – hot water (leachates from commercially available bottles)	Derivatization with BSTFA/1 % TMCS	GC-MS, EI, SIM Column DB-5MS Diagnostic ions: *m/z* 357, 372 Internal standard BPA-*d16*	0.4 ng/L	0.7–78.5 µg/L	

BDL below detection limit

Table 2.5 Analytical methods and levels of BPA in urine samples

Samples	Pretreatment	Analytical method	LOD	Concentration of BPA	Reference
Human urine	HF-LPME	GC-MS, EI, SIM Column DB-5MS	0.02 ng/mL	0.1–0.4 ng/mL	Kawaguchi et al. [46]
	In situ derivatization with acetic acid anhydride	Diagnostic ions: *m/z* 213, 228 Internal standard BPA-^{13}C12 Analyte determined: BPA-glucuronide			
Human urine	Treatment with β-glucuronidase/ sulfatase	LC-MS/MS, APCI, Column: a symmetry C-18 Mobile phase: A methanol and B water	0.4 μg/L	0.5–15.9 μg/L (10–95th percentiles)	Calafat et al. [47]
	Online SPE	Precursor ion: *m/z* 227.2 Product ions: *m/z* 93.1, 133.4, 212.4 Analyte determined: total BPA		2.6 μg/L (geometric mean)	
Human urine	Treatment with β-glucuronidase	HPLC-ED Column: Inertsil ODS-3V	0.2 ng/mL	1.92 ng/mL (total BPA)	Fukata et al. [48]
	SPE	Mobile phase: phosphate buffer/ethanol/ acetonitrile (11 :1 :7 v/v/v) Analyte determined: total and free BPA			
	Dilution with methanol	Confirmation with LC-MS/MS, MRM Analyte determined: BPA-glucuronide			
Human urine	Online SPE cleanup	LC-MS/MS, APCI, NI with column switching Column: LiChrospher RP-18 Mobile phase: A methanol and B water	0.4 ng/mL	BDL–11.5 ng/mL (10–95th percentiles)	Ye et al. [49]
	With and without glucuronidase and sulfatase treatment	Precursor ion: *m/z* 227 Product ions: *m/z* 133, 212 Analyte determined: BPA, BPA-glucuronide		3.5 ng/mL (mean)	

Human urine	Treatment with β-glucuronidase	HPLC-FLD Column: symmetry C-18 Mobile phase: A acetonitrile/methanol +0.1 mM octanesulfonic acid and B acetonitrile/water+0.1 mM octanesulfonic acid Exc/emm wv 275/300 nm Analyte determined: total BPA	0.28 ng/mL	0.85–9.83 ng/mL (men) 1.00–7.64 ng/mL (women)	Kim et al. [50]

BDL below detection limit

concern associated with the relatively large volumes of organic solvents used. The selection of suitable solvent is based on its extraction efficiency and selectivity, its inertness, and its boiling point. Other factors which are usually considered are the toxicity of the extracting solvent, relative densities of the two phases, and its tendency to form emulsions [51, 52]. The solvents that have been used for the recovery of BPA are dichloromethane, ethyl acetate, and chloroform. Repeated extractions are necessary to ensure complete recovery of BPA [37, 53]. Solvent microextraction techniques representing the miniaturization of liquid–liquid extraction have received major attention, because they resulted in a more efficient analyte enrichment, faster sample preparation, and lower solvent consumption. For the determination of BPA, various techniques such as dispersive liquid–liquid microextraction (DLLME), vortex-assisted liquid–liquid microextraction (VALLME), and ultrasound-assisted emulsification microextraction (USAEME) have been recently introduced [54–58].

Solid-phase extraction (SPE) is the technique usually employed for the recovery of BPA from liquid samples (Tables 2.2, 2.3, 2.4, and 2.5). SPE is the extraction proposed for isolation of BPA from waters in the methods ISO 18857-2:2009 and ASTM D7574-09. SPE does not require large volume of toxic organic solvents, analysis times can decrease significantly, and online and/or automated procedures are easily designed. SPE can be performed with commercially available extraction cartridges with the suitable sorbent [51, 52]. The divinylbenzene/N-vinylpyrrolidone copolymer (Oasis HLB) has been the most used sorbent (Table 2.3). Chemically bonded reversed-phase silica (C18) and PS-DVB have been also proposed as SPE sorbents.

Solid-phase microextraction (SPME) is another technique for isolation of BPA from various types of samples. The advantages of the method are the absence of solvents and the relatively small volumes of sample required compared to other methods. SPME utilizes a small fused-silica fiber, coated with a suitable polymeric stationary phase for isolation and preconcentration of analyte. The extraction can be performed with direct immersion of the fiber to the liquid sample or through headspace by suspending the fiber in the vapor phase. The extraction efficiency of SPME depends on many factors such as the matrix, the stationary phase, the exposure time, and the desorption temperature [51, 52]. SPME followed by GC-MS has been applied to the determination of BPA in aqueous samples. Different stationary phases have been used for isolation of BPA such as polydimethylsiloxane (PDMS), polyacrylate (PA), carbowax/divinylbenzene (CW/DVB), and carboxen/polydimethylsiloxane (CAR/PDMS) with PA showing better extraction efficiency [59, 60].

A stir bar sorptive extraction (SBSE) is another technique lately used for the isolation of various analytes from environmental and biological samples. SBSE uses a stir bar into a sealed glass tube that is coated with suitable stationary-phase sorbent. The stir bar can be immersed into the liquid sample or can be held in the headspace above the liquid sample. Removal of the analyte from the bar is achieved by GC thermal desorption or by a suitable solvent. Stationary phase such as PDMS or β-cyclodextrin (PDMS/β-CD) has been used for the extraction of BPA from waters, saliva samples, and biological fluids (human urine and plasma) [61–63].

The demand of highly selective sorbent materials for the determination of traces contaminants in complex samples leads to development of molecularly imprinted polymers (MIPs). MIPs are synthetic polymers having molecular recognition ability for a target analyte. MIPs offer stability against organic solvents, strong acids or bases, and elevated temperatures and pressures. Furthermore, they permit larger sample volumes and reusability [64]. MIPs for isolation of BPA from various types of samples have been developed and used as SPE sorbent materials, as SPME fibers coatings, and as online pretreatment devices [65–69].

2.4 Analytical Techniques

Chromatographic based analytical techniques are used to identify and quantify BPA in environmental, food, and biological samples. Gas chromatography and liquid chromatography coupled with mass spectrometry are the most widely employed techniques.

2.4.1 Gas Chromatography-Mass Spectrometry

Gas chromatography coupled with mass spectrometry (GC-MS) has often been applied for determination of BPA (Tables 2.2, 2.3, 2.4, and 2.5). The analytical columns are phenyl-methyl polysiloxane. The electron impact (EI) is the most common ionization source, although negative ion chemical ionization (NICI) has been applied [70]. The EI mass spectra of BPA are shown in Fig. 2.1. The spectra are characterized by a molecular ion at *m*/*z* 228 ($[C_{15}H_{16}O_2]^+$), whereas the most abundant fragment ion at *m*/*z* 213 ($[C_{14}H_{13}O_2]^+$) corresponds to the loss of methyl group. An alternative minor fragmentation pathway involves the loss of one of the aryl groups from the molecular ion to give *tert*-benzylic carbocation ($[C_9H_8O]^+$) at *m*/*z* 135 and the subsequent loss of methane to give a fragment ion at *m*/*z* 119. GC-MS is employed for the determination of BPA in environmental waters along with other compounds (NP, tOP, NPE1EO, NPE2EO) in ASTM D7065-06 method. This method adheres to selected ion monitoring mass (SIM) spectrometry, but full-scan mass spectrometry has also been shown to work well under these conditions. The method detection limit for BPA is 0.9 μg/L.

Tandem mass spectrometry (GC-MS/MS) can be also used for determination of BPA. In this case the most intense fragment ions on the EI spectrum are those corresponding to the loss of a methyl group ($[M\text{-}15]^+$) and part of the aliphatic chain ($[M\text{-}83]^+$) and are considered as precursors ions resulting in daughter ions (198, 119, 165) which are indicative of the structure of the compound [71].

In order to overcome the drawbacks of low volatility polar characteristics of BPA and improve the selectivity, sensitivity, and performance of gas chromatography, a derivatization procedure is usually employed. Derivatization approaches such as silylation, acetylation, and methylation have been used for determination of low

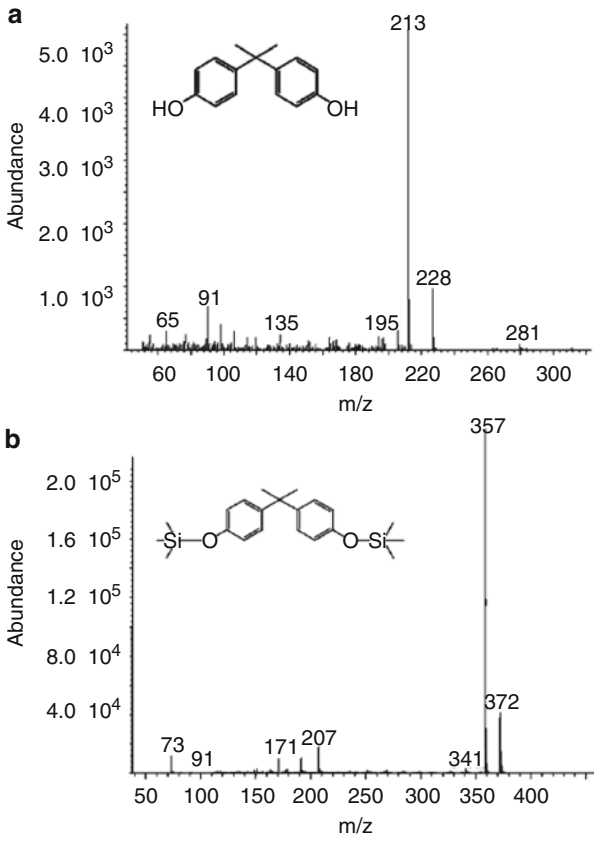


Fig. 2.1 Mass spectra of bisphenol A (**a**) and bisphenol A trimethylsilylated derivative (**b**) by employing GC-EI-MS [60]

concentrations of BPA in various matrices. Silylation is the method most commonly applied (Tables 2.2, 2.3, 2.4, and 2.5) because the derivatization reaction is fast and quantitative and yields thermally stable and highly volatile derivatives. The most popular silylation reagent is *N*-*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Moreover, BSTFA containing 1 % of trimethylchlorosilane (TMCS) or *N*′-*N*′-methyl-(trimethylsilyl)trifluoroacetamide (MSTFA) has been also used. The EI mass spectra and the fragmentation pattern of BPA silylated derivative are characterized by the base peak at *m*/*z* 357 that corresponds to the loss of methyl group ($[C_{20}H_{29}Si_2O_2]^+$) from the molecular ion ($[C_{21}H_{32}Si_2O_2]^+$) at *m*/*z* 372 (Fig. 2.1). The ISO 18857-2:2009 method specifies GC-MS determination of bisphenol A and selected alkylphenols and their ethoxylates in drinking, ground-, surface, and wastewaters following solid-phase extraction and derivatization with MSTFA.

Acetylation of hydroxyl groups of BPA by using acetic anhydride or trifluoroacetic anhydride as derivatizing reagents is also used. Fluoro-derivatizing reagents are also used to analyze phenolic compounds. The mass spectra of *O*-bis(trifluoroacetyl) derivatives of BPA have the base peak at *m*/*z* 405 that corresponds to the loss of methyl group $[M-15]^+$ from molecular ion at *m*/*z* 420. The mass spectra of diacetate BPA have the base peak at *m*/*z* 213 [53, 62, 72, 73]. In order to

minimize possible interferences or loss of BPA in situ, derivatization has been proposed [46, 61].

2.4.2 Liquid Chromatography

Liquid chromatography (LC) has been employed for the determination of BPA in various samples (Tables 2.2, 2.3, 2.4, and 2.5). LC is usually carried out in reverse-phase C18 columns. The detectors that have been used are ultraviolet (UV), fluorescence (FLD), electrochemical (ED), and mass spectrometry (MS). Solvents in mobile phase include water, acetonitrile, and methanol. Gradient elution is usually performed since BPA is often determined simultaneously with other phenolic endocrine-disrupting compounds.

2.4.2.1 Liquid Chromatography-Ultraviolet Detection

Reverse-phase liquid chromatography coupled with UV detector (HPLC-UV) at various wavelengths (215–280 nm) has been applied for the determination of BPA (Table 2.2). UV detector exhibits relative low sensitivity for BPA. This method offers poor selectivity for the determination of traces of BPA in complex matrices such as environmental and biological samples [72].

2.4.2.2 Liquid Chromatography-Fluorescence Detection

Reverse-phase liquid chromatography coupled with fluorescence detector (HPLC-FLD) has been also employed for the determination of BPA (Tables 2.4 and 2.5). After excitation at 275 nm, BPA shows fluorescence at emission wavelengths range 300–320 nm. The fluorescence intensity is much higher in organic media (methanol and acetonitrile), and thus, the sensitivity is dependent on the composition of the mobile phase [53].

2.4.2.3 Liquid Chromatography-Electrochemical Detection

Liquid chromatography coupled with electrochemical detection (HPLC-ED) has been used for the determination of BPA (Table 2.5). It is a sensitive and selective method that presents low detection limits which are 3,000 and 200 times lower than those obtained with UV and FLD detectors [74]. The comparatively high selectivity of electrochemical detector is due to the electroactivity of the phenolic groups of BPA. The pH and electrolyte content of mobile phase influence the electron transfer rate constants, so they have to be optimized in order to get maximum sensitivity. Isocratic elution is preferred otherwise rather large equilibrium time is required [53].

2.4.2.4 Liquid Chromatography-Mass Spectrometry

Liquid chromatography coupled with mass spectrometry (LC-MS, LC-MS/MS) is a valuable tool for determination of BPA since it combines high selectivity and sensitivity (Tables 2.2, 2.3, 2.4, and 2.5). Mass spectrometry offers structural confirmation resulting in higher confidence in identification than LC-UV, LC-FLD, and LC-ED. Moreover, LC/MS has the advantage over GC-MS that derivatization is not required. The most common ionization sources in LC-MS are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), both in negative mode. ESI is more frequently used than APCI because it generally provides better sensitivity. The mass spectra of BPA exhibit the ion *m*/*z* 227 that corresponds to deprotonated molecule ion $[M–H]^-$. Under MS/MS conditions the characteristic fragments of product ion mass spectra are shown in Fig. 2.2. The most abundant fragment at *m*/*z* 212 can be attributed to the loss of methyl group $[M–H–CH3]^-$. Another product ion at *m*/*z* 133 results from the cleavage of the hydroxybenzyl group $[M–H–C_6H_5OH]^-$ [70, 75–77]. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is proposed for the determination of BPA in environmental waters according to the ASTM D7574-09 method. The detection limit of this method is 5 ng/L.

2.5 Immunoassays

Immunoassays are analytical tests that utilize antibodies to selectively bind organic compounds and have been employed for the determination of various organic micropollutants. They provide unique selectivity on the basis of molecular recognition, which is particularly suited to complex matrices [78]. The application of immunoassays to the determination of BPA is rather recent. Several enzyme-linked immunosorbent assays (ELISAs) have been developed for the determination of BPA in various media [18, 35, 53]. ELISAs are simple, rapid, and cost-efficient assays. However, special attention has to be given when ELISAs are applied in complex matrices with low concentrations of BPA, due to the cross-reactivity phenomena and matrix effects that may reduce the precision of the method [72, 78].

2.6 Quality Assurance and Quality Control

Analytical methods for the determination of BPA have to lead in reliable measurements of trace levels in complex matrices. At these levels, many factors may affect the reliability of the results. Therefore, the analytical procedure should be subjected to detailed evaluation regarding efficiency.

Samples shall be obtained, handled, and processed in such a way that avoids possible contamination or loss of BPA. The analytical accuracy of the method is normally measured directly by analysis of certified reference materials (CRM).

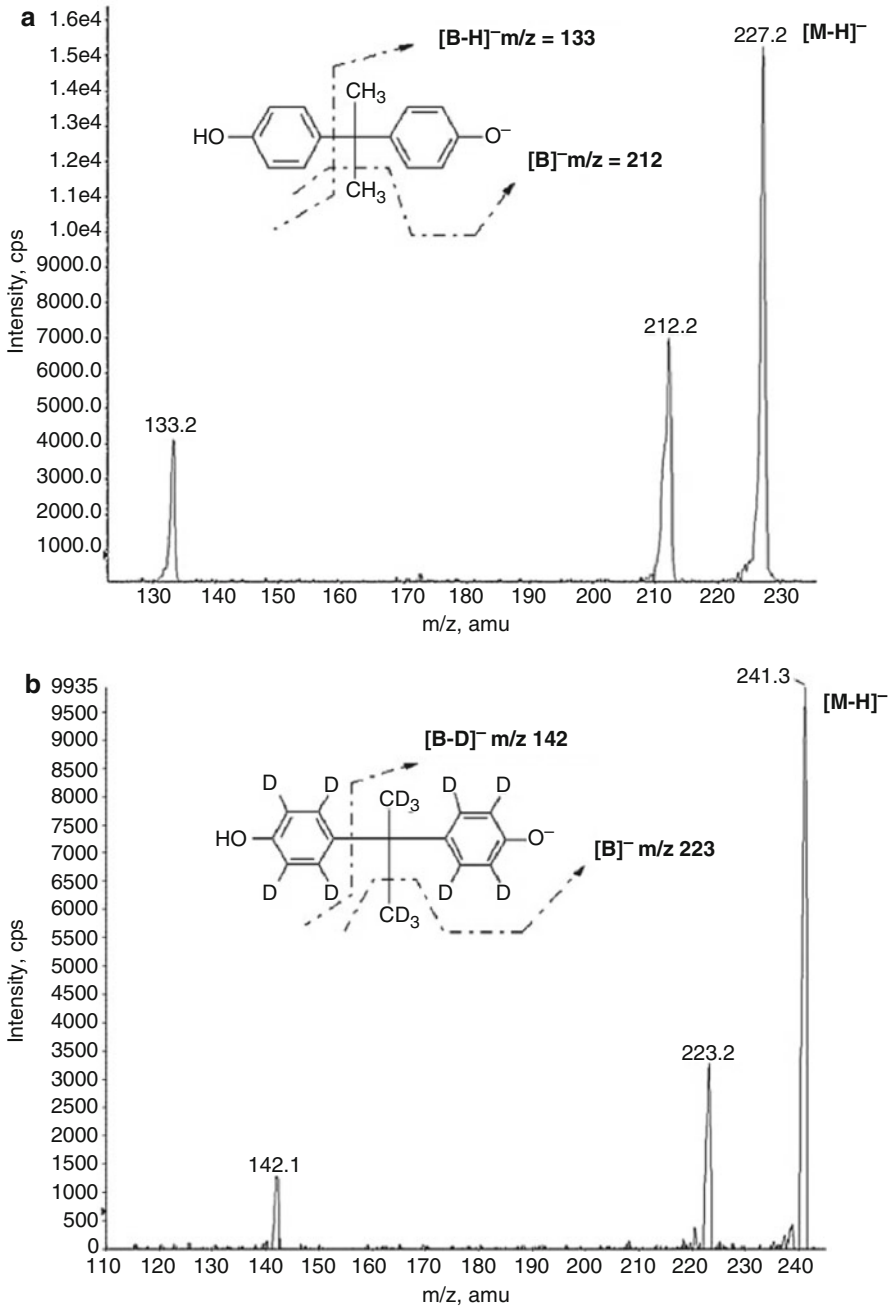


Fig. 2.2 Product ion mass spectra of (**a**) BPA (precursor ion *m/z* 227) and (**b**) BPA-*d16* (IS) (precursor ion *m/z* 241) by employing HPLC/ESI-MS/MS in negative ion mode [75]

In the case of BPA, there is no certified reference material currently available. Instead the recovery can be determined using fortified matrix samples containing known amounts of BPA at least two or more concentration levels. Thus, the recovery efficiency of BPA can be established and appropriate correction can be performed. Fortified samples are also useful to determine whether the sample matrix contributes bias to the results.

One of the most important points in analysis of BPA is the use of appropriate internal standard (IS) to compensate for possible loss of the analyte during sample processing and variations in instrumental performance. The addition of IS at the beginning of the extraction procedure is recommended. Depending on the availability, either stable isotope-labeled forms of BPA, which is particularly suited for mass-spectrometric detection, or compounds that are structurally related to the analyte are used. The isotope-labeled internal standards mostly used in the analysis of BPA by employing mass spectrometry are BPA-*d16* and $^{13}C_{12}$-BPA (Fig. 2.2).

The identification of BPA in chromatographic techniques is based on relative retention time of the eluted peak. The retention time of BPA in the samples has to match that of calibration standard within of specific retention time window, i.e., the retention time of the analyte to that of the internal standard shall correspond to that of calibration solution at a tolerance of ±0.5 for GC and of ±2.5 for LC [79]. When mass spectrometric detection is employed (GC-MS and LC-MS methods), additional criteria for identification of BPA, besides retention time, are the characteristic diagnostic ions (molecular ion, characteristic adducts of the molecular ion, characteristic fragment ions, and all their isotope ions) and their relative abundance. The relative intensities of the diagnostic ions of BPA, expressed as a percentage intensity of the most intense ion or transition, shall correspond to those of the calibration standard, at an acceptable tolerance (i.e., ±15 % in GC-MS and ±25 % in LC-MS) [9, 79].

Cross-checks involving reagent, procedural and field blanks, calibration standards, quality control samples, standard additions on samples, should be carried out through the entire procedure simultaneously with samples in order to ensure the quality of the analytical results.

2.7 Sources and Occurrence of Bisphenol A

2.7.1 Dental Restorative Materials

Bisphenol A is a common ingredient in the resin-based restorative materials used in dentistry. BPA is a precursor to the resin monomer of bisphenol A diglycidyl ether methacrylate (Bis-GMA) and bisphenol A dimethylacrylate (Bis-DMA) that are the main constituents of most commercially available composites and sealants used in dentistry. The resin matrix is initially a fluid containing a monomer that is cured or converted into a rigid polymer by chemical or photo-initiated polymerization reaction.

Various studies reported that BPA is leached from dental materials (Table 2.2). The leaching of BPA could be attributed to (a) unreacted BPA impurities in resins due to incomplete polymerization process, (b) chemical and/or mechanical degradation of these materials, (c) hydrolysis of carbonate linkages of BPA-based epoxy resins at high temperatures, and (d) enzymatic degradation through esterase enzymes present in saliva which can hydrolyze the susceptible ester bond in Bis-DMA monomers.

The leachable BPA concentrations greatly depend on the type of dental material, the polymerization conditions, elution media (i.e., water, methanol, ethanol), and exposure conditions (i.e., pH values, time of elution) [10, 14, 15]. The occurrence of BPA in saliva of patients after treatment with BPA-based dental sealants or composites has been often reported (Table 2.2). The higher concentrations of BPA immediately after placement of dental materials, being decreased within the next hours [17, 19]. However, the magnitude of these exposures, the long-term potential for sealant leaching, and the potential for adverse effects are still controversial.

2.7.2 Environmental Samples

In the analysis of environmental samples, BPA is usually determined along with other xenoestrogens such as nonylphenol and octylphenol, thus the methods employed aiming at the simultaneous extraction and determination of all target compounds. Due to widespread application of BPA, it is commonly found in sewage effluents, industrial wastewaters, and surface waters (Table 2.3). BPA occurred at high concentrations in raw wastewaters. Treatment of wastewaters through the conventional or advanced methods results in elimination of BPA in treated effluents [29, 80]. Surface waters usually exhibited relatively low concentrations. However, higher concentrations of BPA have been determined in surface waters directly impacted from specific sources and/or occasional discharges. BPA is subjected for possible identification as priority hazardous substance in water, due to possible adverse effects to aquatic environment [81]. For the protection of aquatic life in freshwaters, a predicted no-effect concentration of 1.6 μg/L is proposed, whereas this value in marine waters is 0.15 μg/L [82].

2.7.3 Food Samples

Bisphenol A can be present in foods as a result of migration from epoxy resin coatings used to lacquer-coat the interior of food cans, wine storage vats, water containers, and water pipes. The other main source is polycarbonate plastics used in a wide range of applications such as water carboys, reusable milk containers, food storage vessels, and baby bottles. Incomplete polymerization of these materials during manufacture and increased temperatures imposed during heating result in migration of BPA from these materials into food [3, 72].

The concentration ranges of BPA in various products (water, beverages, wine, milk, and food simulants) are shown in Table 2.4. The reported concentrations of BPA are relatively low, although variations have been observed. The occurrence of BPA in food products depends on the coating/packing materials, type of food, handling, and storage conditions [7, 37, 40].

Food is considered as the major pathway of human exposure to BPA. The tendency of this compound to migrate from food contact materials has been acknowledged in European Union food law. A specific migration limit of 3 mg BPA/kg food has been initially set. However, in 2004, a lower limit value of 0.6 mg/kg food has been proposed in the amending document relating to plastic materials and articles intended to come into contact with foodstuff [83, 84]. The European Food Safety Authority established a tolerable daily intake (TDI) of 0.050 mg/kg bw, which represents a safe level for daily exposure over a lifetime [85]. Similarly, the Integrated Risk Information System (IRIS) of US EPA proposed a reference dose of 0.050 mg/kg bw/day for chronic oral exposure (RfD) [86].

2.7.4 Biological Samples

The widespread human exposure to BPA has been highlighted by measurements in human fluids and tissues. The presence of BPA or its metabolites in urine, blood, or various tissues is an indication of human daily or cumulative exposure to this compound. However, an estimation of daily exposure to BPA based on the concentrations found in biological samples requires a detailed knowledge of its biotransformation pathways and toxicokinetics. Based on the known human toxicokinetics of BPA, measurement of urinary concentrations of bisphenol A-glucuronide is the most appropriate and feasible way to assess daily exposure to BPA in humans.

The occurrence of BPA in urine samples along with analytical methodology used is shown in Table 2.5. The biomonitoring data demonstrate that the average concentrations of BPA in urine samples from the general population are relatively low and confirm that BPA is mainly present as glucuronide in human urine [72].

References

1. Rykowska I, Wasiak W (2006) Properties, threats, and methods of analysis of bisphenol A and its derivatives. Acta Chromatogr 16:7–27
2. Verschueren K (2009) Handbook of environmental data on organic chemicals, 5th edn. Wiley, New York
3. Markey CM, Michaelson CL, Sonnenschein C, Soto AM (2001) Alkylphenols and bisphenol A as environmental estrogens. In: Metzler M (ed) The handbook of environmental chemistry, vol 3, Endocrine disruptors, part I. Springer, Berlin/Heidelberg
4. EPA (2010) Bisphenol A Action Plan U.S. Environmental Protection Agency, 29 Mar 2010
5. Vom Saal FS, Richter CA, Ruhlen RR, Nagel SC, Timms BG, Welshons WV (2005) The importance of appropriate controls, animal feed, and animal models in interpreting results from low-dose studies of bisphenol A. Birth Defects Res A Clin Mol Teratol 73:140–145

6. Vom Saal FS, Hughes C (2005) An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. Environ Health Perspect 113:926–933
7. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV (2007) Human exposure to bisphenol A (BPA). Reprod Toxicol 24:139–177
8. Commission Directive 2011/8/EU of 28 January 2011 amending Directive 2002/72/EC as regards the restriction of use of Bisphenol A in plastic infant feeding bottles OJ L 26/11, 29 Jan 2011
9. ISO 18857-2 (2009) Water quality-determination of selected alkylphenols-part 2: gas chromatographic-mass spectrometric determination of alkylphenols, their ethoxylates and bisphenol A in non-filtered samples following solid-phase extraction and derivatization
10. Polydorou O, Hammad M, König A, Hellwig E, Kümmerer K (2000) Release of monomers from different core build-up materials. Dent Mater 25:1090–1095
11. Eliades T, Hiskia A, Eliades G, Athanasiou AE (2007) Assessment of bisphenol A release from orthodontic adhesives. Am J Orthod Dentofacial Orthop 131(1):72–75
12. Nathanson D, Lertpitayakun P, Lamkin M, Edalatpour M, Chou L (1997) In vitro elution of leachable components from dental sealants. J Am Dent Assoc 128:1517–1523
13. Hamid A, Hume WR (1997) A study of component release from resin pit and fissure sealants in vitro. Dent Mater J 13:98–102
14. Eliades T, Voutsa D, Sifakakis I, Makou M, Katsaros C (2011) Release of bisphenol-A from light-cured adhesive bonded to lingual fixed retainers. Am J Orthod Dentofacial Orthop 139:192–195
15. Pulgar R, Olea-Serrano F, Novillo-Fertell A, Rivas A, Pazos P, Pedraza V, Navajas JM, Olea N (2000) Determination of bisphenol A and related aromatic compounds released from Bis-GMA-based composites and sealants by High Performance Liquid Chromatography. Environ Health Perspect 108(1):21–27
16. Tarumi H, Imazato S, Narimatsu M, Mtsuo M, Ebisu S (2000) Estrogenicity of fissure sealants and adhesive resins determined by reporter gene assay. J Dent Res 79(11):1838–1843
17. Joskow R, Barr DB, Barr JR, Calafat AM, Needham LL, Rubin C (2006) Exposure to bisphenol A from bis-glycidyl dimethacrylate-based dental sealants. J Am Dent Assoc 137: 353–362
18. Sasaki N, Okuda H, Kato T, Kakishima H, Okuma H, Abe K, Tachino H, Tuchida K, Kubono K (2005) Salivary bisphenol A levels detected by ELISA after restoration with composite resin. J Mater Sci Mater Med 16:297–300
19. Fung EYK, Ewoldsen NO, St Germain HA Jr, Marx DB, Miaw CL, Siew C, Chou HN, Gruninger SE, Meyer DM (2000) Pharmacokinetics of bisphenol-A released from a dental sealant. J Am Dent Assoc 131:51–58
20. Olea N, Pulgar R, Perez P, Olea-Serrano F, Rivas A, Novillo-Fertell A, Pedraza V, Soto AM, Sonnenschein C (1996) Estrogenicity of resin-based composites and sealants used in dentistry. Environ Health Perspect 104:298–305
21. Heemken OP, Reincke H, Stache B, Theobald N (2001) The occurrence of xenoestrogens in Elbe River and North Sea. Chemosphere 45:245–259
22. Quednow K, Püttmann W (2008) Endocrine disruptors in freshwater streams of Hesse, Germany: changes in concentration levels in the time span from 2003 to 2005. Environ Pollut 152:476–483
23. Fromme H, Kuchler T, Otto T, Pilz K, Muller J, Wenzel A (2002) Occurrence of phthalates and bisphenol A and F in the environment. Water Res 36:1429–1438
24. Voutsa D, Hartmann P, Schaffer C, Giger W (2006) Benzotriazols, alkylphenols and bisphenol A in municipal wastewaters and in the Glatt River, Switzerland. Environ Sci Pollut Res 13:333–341
25. Jonkers N, Kohler HP, Dammshäuser A, Giger W (2009) Mass flows of endocrine disruptors in Glatt River during varying weather conditions. Environ Pollut 157:714–723
26. Jeannot R, Sabik H, Sauvard E, Dagnc T, Dohrendorf K (2002) Determination of endocrine-disrupting compounds in environmental samples using gas and liquid chromatography with mass spectrometry. J Chromatogr A 974:143–159

27. Arditsoglou A, Voutsa D (2008) Determination of phenolic and steroid endocrine disrupting compounds in environmental matrices. Environ Sci Pollut Res 15(3):228–236
28. Arditsoglou A, Voutsa D (2008) Passive sampling of selected endocrine disrupting compounds using polar organic chemical integrative samplers. Environ Pollut 156:316–324
29. Pothitou P, Voutsa D (2008) Endocrine disrupting compounds in municipal and industrial wastewater treatment plants in Northern Greece. Chemosphere 73:1716–1723
30. Arditsoglou A, Voutsa D (2010) Partitioning of endocrine disrupting compounds in waters and wastewaters discharged into coastal area of Thessaloniki, Northern Greece. Environ Sci Pollut Res 17:529–538
31. Loos R, Hanke G, Umlauf G, Eisenreich SJ (2007) LC-MS-MS analysis and occurrence of octyl- and nonylphenol, their ethoxylates and their carboxylates in Belgian and Italian textile industry, wastewater treatment plant effluents and surface waters. Chemosphere 66(4): 690–699
32. Pojana G, Gomiero A, Jonkers N, Marcomini A (2007) Natural and synthetic endocrine disrupting compounds (EDCs) in water, sediment and biota of a coastal lagoon. Environ Int 33:929–936
33. Peters RJB, Beeltje H, van Delft R (2008) Xeno-estrogenic compounds in precipitation. J Environ Monit 10:760–769
34. Belfoid A, van Velzen M, van der Horst B, Vethaak D (2002) Occurrence of bisphenol A in surface water and uptake in fish: evaluation of field measurements. Chemosphere 49:97–103
35. Mauricio E, Diniz M, Petrovic M, Amaral L, Peres I, Barcelo D, Santana F (2006) A characterization of selected endocrine disruptor compounds in a Portuguese wastewater treatment plant. Environ Monit Assess 118:75–87
36. Céspedes R, Lacorte S, Raldúa D, Ginebreda A, Barceló D, Piña B (2005) Distribution of endocrine disruptors in the Llobregat River basin (Catalonia, NE Spain). Chemosphere 61: 1710–1719
37. Amiridou D, Voutsa D (2011) Alkylphenols and phthalates in bottled waters. J Hazard Mater 185:281–285
38. Casajuana N, Lacorte S (2003) Presence and release of phthalic esters and other endocrine disrupting compounds in drinking water. Chromatographia 57:649–655
39. Shao B, Han H, Hu J, Zhao J, Wu G, Xue Y, Ma Y, Zhang S (2005) Determination of alkylphenol and bisphenol A in beverages using liquid chromatography/electrospray ionization tandem mass spectrometry. Anal Chem Acta 530:245–252
40. Cao XL, Corriveau J, Popovic S (2009) Levels of bisphenol A in canned soft drink products in Canadian markets. J Agric Food Chem 57:1307–1311
41. Shao B, Han H, Tu X, Huang L (2007) Analysis of alkylphenol and bisphenol A in eggs and milk by matrix solid phase dispersion extraction and liquid chromatography with tandem mass spectrometry. J Chromatogr B 850:412–416
42. Casajuana N, Lacorte S (2004) New methodology for the determination of phthalate esters, bisphenol A, bisphenol A diglycidyl ether, and nonylphenol in commercial whole milk samples. J Agric Food Chem 52:3702–3707
43. Brenn-Struckhofova Z, Cichna-Markl M (2006) Determination of bisphenol A in wine by sol-gel immunoaffinity chromatography, HPLC and fluorescence detector. Food Addit Contam 23:1227–1235
44. Maragou NC, Makri A, Lampi EN, Thomaidis NS, Koupparis MA (2008) Migration of bisphenol A from polycarbonate baby bottles under real use conditions. Food Addit Contam 25:373–383
45. Chang CM, Chou CC, Lee MR (2005) Determining leaching of bisphenol A from plastic containers by solid-phase microextraction and gas chromatography-mass spectrometry. Anal Chim Acta 539:41–47
46. Kawaguchi M, Ito R, Okanouchi N, Saito K, Nakazawa HJ (2008) Miniaturized hollow fiber assisted liquid–phase microextraction with in situ derivatization and gas chromatography-mass spectrometry for analysis of bisphenol a in human urine sample. J Chromatogr B 870: 98–102

47. Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL (2008) Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003–2004. Environ Health Perspect 116:39–44
48. Fukata H, Miyagawa H, Yamazaki N, Mori C (2006) Comparison of Elisa- and LC-MS-based methodologies for the exposure assessment of bisphenol A. Toxicol Mech Method 16:427–430
49. Ye XY, Kuklenyik Z, Needham LL, Calafat AM (2005) Automated on-line column-switching JPLC-MS/Ms method with peak focusing for the determination of nine environmental phenols in urine. Anal Chem 77:5407–5413
50. Kim YH, Kim CS, Park S, Han SY, Pyo MY, Yang M (2003) Gender differences in the levels of bisphenol A metabolites in urine. Biochem Biophys Res Commun 312:441–448
51. Hennion MC (2000) Sample handling strategies for the analysis of organic compounds in environmental water samples. In: Barceló D (ed) Sample handling and trace analysis of pollutants. Techniques, applications and quality assurance, 1st edn. Elsevier, Amsterdam
52. Reemtsma T, Quintana JB (2006) Analytical methods for polar pollutants. In: Reemtsma T, Jekel M (eds) Organic pollutants in the water cycle, 1st edn. Wiley-VCHW, Weinheim
53. Ballesteros-Gómez A, Rubio S, Perez-Bendito D (2009) Analytical methods for the determination of bisphenol A in food. J Chromatogr A 1216:449–469
54. Rezaee M, Yamini Y, Shariati S, Esrafili A, Shamsipur M (2009) Dispersive liquid–liquid micro-extraction combined with high-performance liquid chromatography-UV detection as a very simple, rapid and sensitive method for the determination of bisphenol A in water samples. J Chromatogr A 1216:1511–1514
55. Wang X, Diao C-P, Zhao R-S (2009) Rapid determination of bisphenol A in drinking water using dispersive liquid-phase micro-extraction with in situ derivatization prior to GC-MS. J Sep Sci 32:154–159
56. Yiantzi E, Psillakis E, Tyrovola K, Kalogerakis N (2010) Vortex-assisted liquid-liquid microextraction of octylphenol, nonylphenol and bisphenol-A. Talanta 80:2057–2062
57. Fontana AR, Munoz de Toro M, Altamirano JC (2011) One-step derivatization and preconcentration microextraction technique for determination of bisphenol A in beverage samples by gas chromatography-mass spectrometry. J Agric Food Chem 59:3559–3565
58. Cunha SC, Almeida C, Mendes E, Fernandes JO (2011) Simultaneous determination of bisphenol A and bisphenol B in beverages and powdered infant formula by dispersive liquid–liquid micro-extraction and heartcutting multidimensional gas chromatography-mass spectrometry. Food Addit Contam 28(4):513–526
59. Salafranca J, Balle R, Nerin C (1999) Use of solid–phase microextraction for the analysis of bisphenol A and bisphenol A diglycidyl ether in food simulants. J Chromatogr A 864:137–144
60. NIST/EPA/NIH (2002) Mass Spectral Library, version 2.0
61. Kawaguchi M, Inoue K, Yoshimura M, Ito R, Sakui N, Okanouchi N, Nakazawa H (2004) Determination of bisphenol A in river water and body fluid samples by stir bar sorptive extraction with in situ derivatization and thermal desorption-gas chromatography-mass spectrometry. J Chromatogr B 805:41–48
62. Nakamura S, Daishima S (2004) Simultaneous determination of alkylphenols and bisphenol A in river water by stir bar sorptive extraction with in situ acetylation and thermal desorption-gas chromatography-mass spectrometry. J Chromatogr A 1038:291–294
63. Hu Y, Zheng Y, Zhu F, Li G (2007) Sol-gel coated polydimethylsiloxane/b-cyclodextrin as novel stationary phase for stir bar sorptive extraction and its application to analysis of estrogens and bisphenol A. J Chromatogr A 1148:16–22
64. Beltran A, Borrull F, Cormack PAG, Marcé RM (2010) Molecularly imprinted polymers: useful sorbents for selective extractions. TrAC 29(11):1363–1375
65. Watabe Y, Kondo T, Morita M, Tanaka N, Haginaka J, Hosoya KJ (2004) Determination of bisphenol A in environmental water at ultra-low level by high-performance liquid chromatography with a effective on-line pretreatment device. J Chromatogr A 1032:45–49
66. Watabe Y, Hosoya K, Tanaka N, Kondo T, Morita M, Kubo T (2005) LC/MS determination of bisphenol A in river water using a surface-modified molecularly-imprinted polymer as an on-line pretreatment device. Anal Bioanal Chem 381(6):1193–1198

67. Alexiadou DK, Maragou NC, Thomaidis NS, Theodoridis GA, Koupparis MA (2008) Molecularly imprinted polymers for bisphenol A for HPLC and SPE from water and milk. J Sep Sci 31:2272–2282
68. Lin Y, Shi Y, Jiang M, Jin Y, Peng Y, Lu B, Dai K (2008) Removal of phenolic estrogen pollutants from different sources of water using molecularly imprinted polymeric microspheres. Environ Pollut 153:483–491
69. Tan F, Zhao H, Li X, Quan X, Chen J, Xiang X, Zhang X (2009) Preparation and evaluation of molecularly imprinted solid-phase microextraction fibers for selective extraction of bisphenol A in complex samples. J Chromatogr A 1216:5647–5654
70. Gallart-Ayala H, Moyano E, Galceran MT (2010) Recent advances in mass spectrometry analysis of phenolic endocrine disruptors and related compounds. Mass Spectrom Rev 29: 776–805
71. Hernando MD, Mezcua M, Gómez MJ, Malato O, Agüera A, Fernández-Alba AR (2004) Comparative study of analytical methods involving gas chromatography-mass spectrometry after derivatization and gas chromatography-tandem mass spectrometry for the determination of selected endocrine disrupting compounds in wastewaters. J Chromatogr A 1047(1): 129–135
72. Dekant W, Völkel W (2008) Human exposure to bisphenol A by biomonitoring: methods, results and assessment of environmental exposures. Toxicol Appl Pharmacol 228:114–134
73. Geens T, Neels H, Covaci A (2009) Sensitive and selective method for the determination of bisphenol-A and triclosan in serum and urine as pentafluorobenzoate-derivatives using GC-ECNI/MS. J Chromatogr B 877:4042–4046
74. Inoue K, Kato K, Yoshimura Y, Makino T, Nakazawa H (2000) Determination of bisphenol A in human serum by high-performance liquid chromatography with multi-electrode electrochemical detection. J Chromatogr B 749:17–23
75. Sabatini L, Barbieri A, Violante FS (2005) Development and validation of a capillary high-performance liquid chromatography/electrospray tandem mass spectrometric method for the quantification of bisphenol A in air samples. Rapid Commun Mass Spectrom 19:3468–3472
76. Inoue K, Kawaguchi M, Funakoshi Y, Nakazawa H (2003) Size-exclusion flow extraction of bisphenol A in human urine for liquid chromatography-mass spectrometry. J Chromatogr B 798:17–23
77. Gallart-Ayala H, Moyano E, Galceran MT (2007) Liquid chromatography /multi-stage mass spectrometry of bisphenol A and its halogenated derivatives. Rapid Commun Mass Spectrom 21:4039–4048
78. Qubiňa A, Ballesteros B, Bou Crrasco PB, Galve R, Gascón J, Inglesias F, Snvicens N, Marco MP (2000) Immunoassays for environmental analysis. In: Barceló D (ed) Sample handling and trace analysis of pollutants. Techniques, applications and quality assurance, 1st edn. Elsevier, Amsterdam
79. Commission decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. OJ L221/8, 17 Aug 2002
80. Mohapatra DP, Brar SK, Tyagi RD, Surampalli RY (2010) Physico-chemical pre-treatment and biotransformation of wastewater and wastewater sludge-fate of bisphenol A. Chemosphere 78:923–941
81. Commission Directive 2008/105/EC on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council OJ L 348/84, 24 Dec 2008
82. European Commission (2010) European Union risk assessment report. 4,4-isopropylidenediphenol (Bisphenol A)
83. Commission Directive 2002/72/EC relating to plastic materials and articles intended to come into contact with foodstuffs OJ L220/18, 15 Aug 2002

84. Commission Directive 2004/19/EC amending Directive 2002/72/EC relating to plastic materials and articles intended to come into contact with foodstuffs. OJ L71/8, 10 Mar 2004
85. EFSA (2006) Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the Commission related to 2,2-bis(4-hydroxyphenyl)-propane (bisphenol A). EFSA J 428:1–75
86. EPA (2009) Integrated Risk Information System, U.S. Environmental Protection Agency

Chapter 3
In Vitro Assay Systems for the Assessment of Oestrogenicity

Harris Pratsinis and Dimitris Kletsas

17β-Estradiol (or simply estradiol) is the predominant sex hormone present in the female mammals, and its impact is vital not only on reproductive and sexual functions but also for many other tissues, most notably the bones. Estradiol acts on target cells through its interaction with two types of specific receptors (oestrogen receptors, ERs) called ER-α and ER-β, which reside in the cytoplasm but upon binding of oestrogen migrate in the nucleus to regulate the transcription of target genes [1, 2]. Recently, membrane-bound receptors for estradiol have also been identified [3].

During the last 50 years, substantial evidence has been accumulated on many exogenous compounds that behave similarly to the endogenous oestrogens, hence termed phytoestrogens – when they are of plant origin – or xenoestrogens, a term mainly referring to chemicals produced industrially [4]. Xenoestrogens belong to a wider group of compounds called "endocrine disruptors" due to the fact that upon their intake by humans or animals, they interfere with the normal hormonal balance of the organism, causing among others reduction in sperm counts and fertility, developmental and/or congenital birth defects, increased incidence of testicular and/or breast cancer in humans as well as gross birth deformities, behavioural abnormalities and both feminisation and masculinisation in animals [4–7].

Xenoestrogens usually are constituents, chemical intermediates or derivatives of industrial products with a huge variety of uses, such as agrochemicals and pesticides, food additives and supplements and medical and pharmaceutical products, to name only a few. Especially in dental practice, there are many products such as restorative materials, liners, adhesives, oral prosthetic devices, tissue substitutes and rebase materials, which possess – or there are reasonable suspicions that they possess – oestrogenic activity [8, 9]. Most important among them is bisphenol-A (BPA),

H. Pratsinis • D. Kletsas (✉)
Laboratory for Cell Proliferation and Ageing, Institute of Biosciences and Applications, NCSR "Demokritos", 15310 Athens, Greece
e-mail: dkletsas@bio.demokritos.gr

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a molecule with established oestrogenicity [10, 11] and endocrine disruptive properties [12]. Beyond BPA, there are other bisphenols, e.g. bisphenol-A dimethacrylate (Bis-DMA), bisphenol-A glycidyl dimethacrylate (Bis-GMA) or BPA diglycidyl-ether (BADGE), and phthalates, e.g. n-butyl benzyl phthalate (BBP) or dibutyl phthalate (DBP), in various dental materials that raise suspicions for endocrine disruptive behaviour [9, 13].

As a consequence of all these undesirable effects of xenoestrogens, a whole battery of assays has been developed for the evaluation of the oestrogenic properties of natural or synthetic compounds. This chapter will focus on the presentation of the in vitro assays used in the literature for the assessment of the oestrogenicity of various compounds with an emphasis on those used in dental practice.

Generally, these assays can be categorised according to the use or not of various cellular types, as follows [14, 15]:

1. Cell-free assay systems
2. Yeast assay systems
3. Mammalian cell assay systems

3.1 Cell-Free Assay Systems

Cell-free assay systems are based on a chemical reaction and can be performed in a test tube. Typically, they assess the affinity of a test compound for oestrogen receptors. As stated above, the formation of a hormone-receptor complex is required for the manifestation of the hormone effects. Many xenoestrogens bind also to oestrogen receptors; hence, the assessment of their receptor-binding affinity (RBA; also stands for relative binding affinity) may provide a first indication of their ability to mimic the endogenous hormones or to interfere with their activities, as well as an indication of their potency.

Originally, the RBA of a xenoestrogen was calculated based on its capacity to compete with radiolabelled estradiol molecules for binding to the ER, and for this reason, sources rich in ER were used, such as cytosolic extracts from breast tumours [16] or murine uterus extracts [17]. More recently, after the cloning of ER-β, recombinant ER-α and ER-β molecules replaced the crude extracts, and high-affinity fluorescent ligands are being used instead of radioactive hormones for competition experiments [18, 19], thus improving the reproducibility of the assay and allowing for screening compounds that may bind only weakly to ERs and have limited aqueous solubility.

A further variation of this type of assays is the use of fluorescein-labelled synthetic oligonucleotide oestrogen response elements (EREs) of various target genes for the assessment of the xenoestrogen-dependent binding of ER to the ERE [20]. Finally, a fusion protein of the ER with glutathione S-transferase (GST) can be used to study the interaction of the xenoestrogen with ER and a radiolabelled coactivator (such as steroid receptor coactivator-1a, SRC-1a or transcriptional intermediary factor-2, TIF2) by autoradiography [21, 22]. In an analogous approach, fluorescently labelled coactivators and ER can be used to assess the

interaction with the xenoestrogen by fluorescence resonance energy transfer (FRET) [23].

The above-mentioned cell-free assays have the advantage that they are easy to perform, thus allowing for a high-throughput screening of test compounds, except from those that include coactivators. However, they only provide information on the chemical affinity of a compound to the ER, without any clue on the biological phenomena triggered by their interaction, and especially without distinguishing between agonistic and antagonistic activity [14]. Hence, typically the RBA assay is used in combination with other assays that are more informative, such as the yeast two-hybrid or the E-screen (see below) [9, 24, 25].

3.2 Yeast Assay Systems

More biologically relevant are the yeast assay systems, which have been made possible through transfection techniques. These systems are based on the artificial expression in the yeast (Saccharomyces cerevisiae) of ERs of human or animal origin or other parts of the molecular machinery conveying the oestrogenic signals. Since it is an artificial system, there is usually a convenient reporter gene included in it, such as β-galactosidase or chloramphenicol acetyltransferase (CAT) or luciferase. For example, an approach similar to the cell-free GST pull-down systems described above is the so-called yeast two-hybrid assay. In this case, the ligand binding domain of the ER and a fusion of galactosidase activation domain with the receptor interaction domain of a coactivator (in most cases TIF2, see above) are subcloned in yeast expression plasmids, in order to transform the appropriate yeast strains, so that the interaction of ER with its ligands can be detected by a chromatic reaction, which can be quantitated measuring absorbance or chemiluminescence [24–27]. In a more focused approach, yeast strains are co-transfected with an ER cDNA and an artificial reporter gene containing the ERE of a known oestrogen target gene linked usually to β-galactosidase [28].

Yeast assay systems are popular to many researchers because yeast is a well-characterised model organism, it is widely accessible, it is readily transformed and it has a broad range of suitable plasmids and promoters available, while the experiments can be performed easily and rapidly. Furthermore, the transfected yeast model is capable of high levels of sensitivity [28], and it is "pure" in the sense that neither ER mechanisms nor other molecules known to interact with them exist in the untransformed organism. However, exactly this lack of the mammalian cell context makes the system highly artificial; hence, the responses observed may not reflect the physiological response in human. It has been reported, for example, that such a system was highly specific for estradiol compared to other molecules with known oestrogenic activity, such as diethylstilbestrol (DES) or mestranol [29]. Moreover – in comparison with a mammalian cell assay system – in the yeast, one could not detect various putative anti-oestrogenic molecules, most probably because some molecules active on mammalian cells cannot cross into the yeast cell through its specialised cell walls [28, 30].

3.3 Mammalian Cell Assay Systems

The vast majority of the studies testing in vitro the oestrogenicity of xenoestrogens or phytoestrogens are using at least one mammalian cell assay system alone or in combination with some of the methods described above. The mammalian cell assay systems can be categorised (a) according to the tissue the cells are originating from (usually breast or endometrium, although pituitary cells have also been used), (b) according to the use or not of genetically engineered cell strains and (c) according to the end point assessed by the method, which can correspond to a very broad range of cellular activities: expression of certain genes or proteins, steroidogenesis and activity of marker enzymes as well as DNA synthesis and cell proliferation [14, 15, 31, 32].

Among the various alternatives of the mammalian cell assay systems, the most popular is by far the so-called E-screen [33], i.e. the assessment of the proliferation of the human breast adenocarcinoma cell line MCF-7. This is true also among the researchers studying potential xenoestrogens especially among the materials used in dental practice [10, 11, 25, 34–41]. MCF-7 cell proliferation during the E-screen can be assessed by direct cell counting, usually by a Coulter counter [42] – by estimating DNA synthesis rate through the incorporation of tritiated thymidine [36] or 5-bromo-2′-deoxyuridine [14] into DNA, by measuring total DNA content fluorometrically after binding of an appropriate dye [43] but most often by chromatometric methods such as the MTT [3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide] assay [33, 34], or the sulforhodamine-B (SRB) assay [25, 37–39, 41], or the utilisation of the neutral red vital stain [11, 44].

The E-screen is appropriate for quantitative analysis of both oestrogenic and anti-oestrogenic activities [15]; it is very sensitive [45] and easy to perform, allowing for high-throughput experimental approaches. Moreover, the fact that it measures a physiological end point of oestrogen action of high biological complexity, i.e. the proliferation of ER-bearing breast cancer cells, affords the opportunity to identify factors that may impact on mixture effect predictability [32].

A scepticism regarding the specificity of E-screen has been expressed, since progesterone and certain 19-nortestosterone derivatives, as well as caffeine, ethanol and various growth factors, have been reported to induce MCF-7 cell proliferation [31, 46]. In contrast, the team of Soto has shown that when the assay is performed in charcoal-dextran stripped serum or plasma, in order to remove endogenous oestrogen – a plasma-borne specific inhibitory activity of ER-bearing breast cancer cell proliferation (termed estrocolyone-I and sharing properties with human serum albumin) remains, and only oestrogens can reverse this inhibition [47, 48]; hence, the assay is absolutely oestrogen specific. Nevertheless, a simple way of identifying molecules stimulating or inhibiting MCF-7 cell proliferation non-specifically is to test them in parallel on an oestrogen-insensitive breast cancer cell line, such as MDA-MB-231 (Fig. 3.1) [34, 36].

Scepticism regarding the E-screen has also been expressed because different clones of MCF-7 cultured in identical conditions showed distinct differences in the proliferative response to estradiol and to the xenoestrogens, *p*-nonyl-phenol and

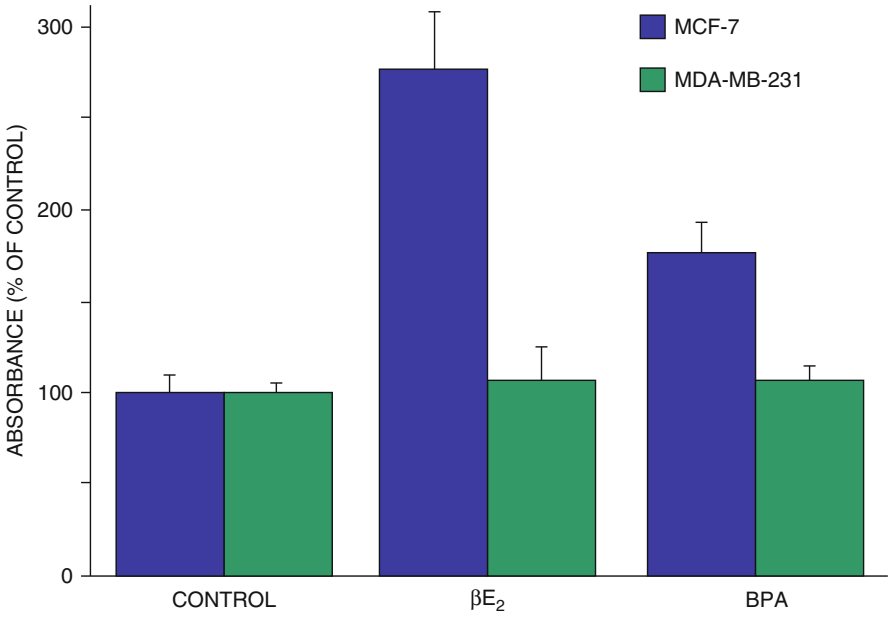

Fig. 3.1 E-screen assay. MCF-7 and MDA-MB-231 cells were grown for 6 days in the absence (control) or presence of 10^{-9} M 17**β**-estradiol (**β**E_2) or 10^{-8} M bisphenol-A (BPA), and their viability was assessed using the MTT assay

bisphenol-A [49], as well as to commercial resin-based dental restorative materials [41]. However, this is a common problem when working with cancer cells, and one can overcome it through meticulously uniform cell stocks. Furthermore, apart from MCF-7 cells, other oestrogen-responsive breast cancer cell lines have been used in the E-screen assay, such as T-47D [43] or ZR-75-1 [50].

The proliferation of the Ishikawa human endometrial cancer cell line has also been proposed to be used for the evaluation of oestrogenic activity [51], but there were indications that the response of this cell line is not specific for oestrogenic molecules [52] in contrast to breast cancer ones. However, Ishikawa cells have been shown to respond to oestrogen and phytoestrogens with a potent induction of alkaline phosphatase (ALP) activity, which is oestrogen specific [53, 54]; hence, it can be used for the screening of potentially oestrogenic compounds [19, 55].

Probably, the most important drawback to the use of both the E-screen and the ALP-induction assays as rapid screening tools is that they are time consuming (the assessment can take from 3 to 6 days, depending on the protocol variation). Accordingly, analysis of oestrogen-regulated gene or protein expression in various cell types can be used as an alternative. For example, expression in MCF-7 cells of the genes coding for the progesterone receptor [56] and for the trefoil peptide pS2 [57, 58] or prolactin production by rat pituitary cells [59, 60] has been proposed as tools to study an oestrogen-specific response. However, these assays are not always

as sensitive as the E-screen [32], and they require the use of laborious and/or expensive techniques such as northern blotting or real-time PCR; hence, they are not appropriate for high-throughput screening.

The use of genetically engineered mammalian cell systems was intended to solve some of the above problems. In most of the cases, cells are transfected with an oestrogen-inducible reporter gene, or they are co-transfected with an ER-construct and an ERE-containing reporter gene, similarly to the approaches described above in yeast. The reporter genes usually are designed for measuring CAT or luciferase activity, which due to their high sensitivity offer the possibility to identify even weak oestrogens [15]. The transfection can be transient [61] or stable [19], the latter being more advantageous in terms of reproducibility, as well as rapidity, once the stable line is ready for use [32]. The parental cells used for transfection can be either ER negative, such as HeLa [61] or HEK-293 [19], or ER responsive, like MCF-7 or MG-63 [62]. Consequently, it is clear that apart from the high-throughput capability and the rapidity of these assays (typically gene expression can be assessed within 24 h), their main advantage is their versatility, allowing for separate tests for the various ER subtypes and EREs, recognising both oestrogens and anti-oestrogens and giving the choice of selecting an ER-naive cell context, such as in the case of HeLa cells, or a more physiological context, such as that of MCF-7 cells [15]. Still, these assay systems are artificial, and there are reports regarding the irreversible silencing of the reporter gene after treatment with anti-oestrogens, such as 4-hydroxy-tamoxifen [63, 64].

3.4 Conclusion

In this chapter, a battery of in vitro assays for the evaluation of the oestrogenic properties of natural or synthetic compounds was presented. One should not forget that the evidence for in vitro oestrogenicity of a test molecule cannot always be conclusive without the knowledge of in vivo data regarding its metabolism and bioavailability. However, only in vitro testing can respond to the urgent need for screening the huge amount of novel materials produced every day in the industrialised societies. Specifically in dental practice, most often, the E-screen, the RBA and various yeast assay systems are being used, a fact probably reflecting their credibility and/or their simplicity.

References

1. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 93(12):5925–5930
2. Moggs JG, Orphanides G (2001) Estrogen receptors: orchestrators of pleiotropic cellular responses. EMBO Rep 2(9):775–781
3. Maggiolini M, Picard D (2010) The unfolding stories of GPR30, a new membrane-bound estrogen receptor. J Endocrinol 204(2):105–114

4. Singleton DW, Khan SA (2003) Xenoestrogen exposure and mechanisms of endocrine disruption. Front Biosci 8:s110–s118
5. Allinson M, Shiraishi F, Salzman SA, Allinson G (2010) In vitro and immunological assessment of the estrogenic activity and concentrations of 17beta-estradiol, estrone, and ethinyl estradiol in treated effluent from 45 wastewater treatment plants in Victoria, Australia. Arch Environ Contam Toxicol 58(3):576–586
6. Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM (2009) Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. Endocr Rev 30(1):75–95
7. Maffini MV, Rubin BS, Sonnenschein C, Soto AM (2006) Endocrine disruptors and reproductive health: the case of bisphenol-A. Mol Cell Endocrinol 254–255:179–186
8. Eliades T (2007) Orthodontic materials research and applications: part 2. Current status and projected future developments in materials and biocompatibility. Am J Orthod Dentofacial Orthop 131(2):253–262
9. Hashimoto Y, Moriguchi Y, Oshima H, Nishikawa J, Nishihara T, Nakamura M (2000) Estrogenic activity of chemicals for dental and similar use in vitro. J Mater Sci Mater Med 11(8):465–468
10. Olea N, Pulgar R, Perez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A et al (1996) Estrogenicity of resin-based composites and sealants used in dentistry. Environ Health Perspect 104(3):298–305
11. Hashimoto Y, Nakamura M (2000) Estrogenic activity of dental materials and bisphenol-A related chemicals in vitro. Dent Mater J 19(3):245–262
12. Tsai WT (2006) Human health risk on environmental exposure to Bisphenol-A: a review. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 24(2):225–255
13. Fleisch AF, Sheffield PE, Chinn C, Edelstein BL, Landrigan PJ (2010) Bisphenol A and related compounds in dental materials. Pediatrics 126(4):760–768
14. Diel P, Smolnikar K, Michna H (1999) In vitro test systems for the evaluation of the estrogenic activity of natural products. Planta Med 65(3):197–203
15. Mueller SO (2002) Overview of in vitro tools to assess the estrogenic and antiestrogenic activity of phytoestrogens. J Chromatogr B Analyt Technol Biomed Life Sci 777(1–2):155–165
16. Van Aswegen CH, Purdy RH, Wittliff JL (1989) Binding of 2-hydroxyestradiol and 4-hydroxyestradiol to estrogen receptors from human breast cancers. J Steroid Biochem 32(4):485–492
17. Shelby MD, Newbold RR, Tully DB, Chae K, Davis VL (1996) Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays. Environ Health Perspect 104(12):1296–1300
18. Bolger R, Wiese TE, Ervin K, Nestich S, Checovich W (1998) Rapid screening of environmental chemicals for estrogen receptor binding capacity. Environ Health Perspect 106(9):551–557
19. Fokialakis N, Lambrinidis G, Mitsiou DJ, Aligiannis N, Mitakou S, Skaltsounis AL et al (2004) A new class of phytoestrogens; evaluation of the estrogenic activity of deoxybenzoins. Chem Biol 11(3):397–406
20. Nikov GN, Hopkins NE, Boue S, Alworth WL (2000) Interactions of dietary estrogens with human estrogen receptors and the effect on estrogen receptor-estrogen response element complex formation. Environ Health Perspect 108(9):867–872
21. Kraichely DM, Sun J, Katzenellenbogen JA, Katzenellenbogen BS (2000) Conformational changes and coactivator recruitment by novel ligands for estrogen receptor-alpha and estrogen receptor-beta: correlations with biological character and distinct differences among SRC coactivator family members. Endocrinology 141(10):3534–3545
22. Routledge EJ, White R, Parker MG, Sumpter JP (2000) Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) alpha and ERbeta. J Biol Chem 275(46):35986–35993
23. Zhou G, Cummings R, Li Y, Mitra S, Wilkinson HA, Elbrecht A et al (1998) Nuclear receptors have distinct affinities for coactivators: characterization by fluorescence resonance energy transfer. Mol Endocrinol 12(10):1594–1604

24. Hashimoto Y, Moriguchi Y, Oshima H, Kawaguchi M, Miyazaki K, Nakamura M (2001) Measurement of estrogenic activity of chemicals for the development of new dental polymers. Toxicol In Vitro 15(4–5):421–425
25. Nishijima M, Hashimoto Y, Nakamura M (2002) Cytocompatibility of new phthalate ester-free tissue conditioners in vitro. Dent Mater J 21(2):118–132
26. Nishikawa J, Saito K, Goto J, Dakeyama F, Matsuo M, Nishihara T (1999) New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicol Appl Pharmacol 154(1):76–83
27. Nomura Y, Ishibashi H, Miyahara M, Shinohara R, Shiraishi F, Arizono K (2003) Effects of dental resin metabolites on estrogenic activity in vitro. J Mater Sci Mater Med 14(4):307–310
28. Wrenn CK, Katzenellenbogen BS (1993) Structure-function analysis of the hormone binding domain of the human estrogen receptor by region-specific mutagenesis and phenotypic screening in yeast. J Biol Chem 268(32):24089–24098
29. Klein KO, Baron J, Colli MJ, McDonnell DP, Cutler GB Jr (1994) Estrogen levels in childhood determined by an ultrasensitive recombinant cell bioassay. J Clin Invest 94(6):2475–2480
30. Berry M, Metzger D, Chambon P (1990) Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. EMBO J 9(9):2811–2818
31. Reel JR, Lamb IJ, Neal BH (1996) Survey and assessment of mammalian estrogen biological assays for hazard characterization. Fundam Appl Toxicol 34(2):288–305
32. Soto AM, Maffini MV, Schaeberle CM, Sonnenschein C (2006) Strengths and weaknesses of in vitro assays for estrogenic and androgenic activity. Best Pract Res Clin Endocrinol Metab 20(1):15–33
33. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO (1995) The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 103(Suppl 7):113–122
34. Eliades T, Gioni V, Kletsas D, Athanasiou A, Eliades G (2007) Oestrogenicity of orthodontic adhesive resins. Eur J Orthod 29(4):404–407
35. Eliades T, Pratsinis H, Athanasiou AE, Eliades G, Kletsas D (2009) Cytotoxicity and estrogenicity of Invisalign appliances. Am J Orthod Dentofacial Orthop 136(1):100–103
36. Gioka C, Eliades T, Zinelis S, Pratsinis H, Athanasiou AE, Eliades G et al (2009) Characterization and in vitro estrogenicity of orthodontic adhesive particulates produced by simulated debonding. Dent Mater 25(3):376–382
37. Hashimoto Y, Kawaguchi M, Miyazaki K, Nakamura M (2003) Estrogenic activity of tissue conditioners in vitro. Dent Mater 19(4):341–346
38. Hashimoto Y, Nakamura M (2004) Cytocompatibility and viscoelastic properties of phthalate ester-free tissue conditioners. Dent Mater J 23(3):412–418
39. Hashimoto Y, Tanaka J, Suzuki K, Nakamura M (2007) Cytocompatibility of a tissue conditioner containing vinyl ester as a plasticizer. Dent Mater J 26(6):785–791
40. Kostoryz EL, Eick JD, Glaros AG, Judy BM, Welshons WV, Burmaster S et al (2003) Biocompatibility of hydroxylated metabolites of BISGMA and BFDGE. J Dent Res 82(5):367–371
41. Lewis JB, Rueggeberg FA, Lapp CA, Ergle JW, Schuster GS (1999) Identification and characterization of estrogen-like components in commercial resin-based dental restorative materials. Clin Oral Investig 3(3):107–113
42. Berthois Y, Pons M, Dussert C, Crastes de Paulet A, Martin PM (1994) Agonist–antagonist activity of anti-estrogens in the human breast cancer cell line MCF-7: an hypothesis for the interaction with a site distinct from the estrogen binding site. Mol Cell Endocrinol 99(2):259–268
43. Welshons WV, Murphy CS, Koch R, Calaf G, Jordan VC (1987) Stimulation of breast cancer cells in vitro by the environmental estrogen enterolactone and the phytoestrogen equol. Breast Cancer Res Treat 10(2):169–175

44. Borenfreund E, Babich H, Martin-Alguacil N (1990) Rapid chemosensitivity assay with human normal and tumor cells in vitro. In Vitro Cell Dev Biol 26(11):1030–1034
45. Welshons WV, Rottinghaus GE, Nonneman DJ, Dolan-Timpe M, Ross PF (1990) A sensitive bioassay for detection of dietary estrogens in animal feeds. J Vet Diagn Invest 2(4):268–273
46. Jones PA, Baker VA, Irwin AJ, Earl LK (1998) Interpretation of the in vitro proliferation response of mcf-7 cells to potential oestrogens and non-oestrogenic substances. Toxicol In Vitro 12(4):373–382
47. Sonnenschein C, Soto AM, Michaelson CL (1996) Human serum albumin shares the properties of estrocolyone-I, the inhibitor of the proliferation of estrogen-target cells. J Steroid Biochem Mol Biol 59(2):147–154
48. Soto AM, Silvia RM, Sonnenschein C (1992) A plasma-borne specific inhibitor of the proliferation of human estrogen-sensitive breast tumor cells (estrocolyone-I). J Steroid Biochem Mol Biol 43(7):703–712
49. Villalobos M, Olea N, Brotons JA, Olea-Serrano MF, Ruiz de Almodovar JM, Pedraza V (1995) The E-screen assay: a comparison of different MCF7 cell stocks. Environ Health Perspect 103(9):844–850
50. Poulin R, Baker D, Poirier D, Labrie F (1991) Multiple actions of synthetic 'progestins' on the growth of ZR-75-1 human breast cancer cells: an in vitro model for the simultaneous assay of androgen, progestin, estrogen, and glucocorticoid agonistic and antagonistic activities of steroids. Breast Cancer Res Treat 17(3):197–210
51. Holinka CF, Anzai Y, Hata H, Kimmel N, Kuramoto H, Gurpide E (1989) Proliferation and responsiveness to estrogen of human endometrial cancer cells under serum-free culture conditions. Cancer Res 49(12):3297–3301
52. Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA (1993) Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. Mol Endocrinol 7(8):992–998
53. Littlefield BA, Gurpide E, Markiewicz L, McKinley B, Hochberg RB (1990) A simple and sensitive microtiter plate estrogen bioassay based on stimulation of alkaline phosphatase in Ishikawa cells: estrogenic action of delta 5 adrenal steroids. Endocrinology 127(6):2757–2762
54. Markiewicz L, Garey J, Adlercreutz H, Gurpide E (1993) In vitro bioassays of non-steroidal phytoestrogens. J Steroid Biochem Mol Biol 45(5):399–405
55. Halabalaki M, Alexi X, Aligiannis N, Lambrinidis G, Pratsinis H, Florentin I et al (2006) Estrogenic activity of isoflavonoids from Onobrychis ebenoides. Planta Med 72(6):488–493
56. Reiner GC, Katzenellenbogen BS, Bindal RD, Katzenellenbogen JA (1984) Biological activity and receptor binding of a strongly interacting estrogen in human breast cancer cells. Cancer Res 44(6):2302–2308
57. Balleine RL, Clarke CL (1999) Expression of the oestrogen responsive protein pS2 in human breast cancer. Histol Histopathol 14(2):571–578
58. Jeltsch JM, Roberts M, Schatz C, Garnier JM, Brown AM, Chambon P (1987) Structure of the human oestrogen-responsive gene pS2. Nucleic Acids Res 15(4):1401–1414
59. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258(8):4741–4745
60. Lieberman ME, Maurer RA, Gorski J (1978) Estrogen control of prolactin synthesis in vitro. Proc Natl Acad Sci U S A 75(12):5946–5949
61. Tarumi H, Imazato S, Narimatsu M, Matsuo M, Ebisu S (2000) Estrogenicity of fissure sealants and adhesive resins determined by reporter gene assay. J Dent Res 79(11):1838–1843
62. Jones PS, Parrott E, White IN (1999) Activation of transcription by estrogen receptor alpha and beta is cell type- and promoter-dependent. J Biol Chem 274(45):32008–32014
63. Badia E, Duchesne MJ, Fournier-Bidoz S, Simar-Blanchet AE, Terouanne B, Nicolas JC et al (1994) Hydroxytamoxifen induces a rapid and irreversible inactivation of an estrogenic response in an MCF-7-derived cell line. Cancer Res 54(22):5860–5866
64. Oliva J, El Messaoudi S, Pellestor F, Fuentes M, Georget V, Balaguer P et al (2005) Involvement of HP1alpha protein in irreversible transcriptional inactivation by antiestrogens in breast cancer cells. FEBS Lett 579(20):4278–4286

Chapter 4
BPA Effects In Vivo: Evidence from Animal Studies

Efthymia Kitraki

4.1 Introduction

Bisphenol A (2,2-bis-4-hydroxyphenyl-propane, BPA), is a well-known endocrine disruptor that is used as a monomer in the manufacture of dental sealants, epoxy resins and polycarbonate plastics that have extensive use in dentistry or medicine, in food packaging industry and in plastics' production. BPA is contained in many everyday life items, such as house plasticware and baby bottles, from where it is released, for example, by heating, resulting in food or drink contamination. Leached components from dental composites and sealants in the oral cavity are also considered a possible source of human exposure. BPA exposure can also occur by inhalation of contaminated air, for example, from decomposed monomers during medical or dental practice [1].

Animal studies confer a valuable tool for the assessment of BPA effects in vivo. The easiness of experimentation with laboratory animals has allowed a variety of in vivo approaches, summarised in comprehensive recent reviews [2, 3]. Aquatic organisms such as fishes or amphibians have been widely used to assess the effects of BPA in the ecosystem. However, the effects of BPA on animals' physiology have been mainly explored in laboratory animals that are closer to humans. Advantages from the use of small rodents include their genomic similarity to humans, suitability for genetic studies and offspring follow-up, as well as a less stringent legislation, compared to that of non-human primates. Disadvantages include their differences in metabolism from humans that may interfere with BPA degradation kinetics. Intrauterine growth also differs significantly between rodents and humans, and position into the bicornate rodent uterus may differentiate the impact of BPA in each embryo [3]. Furthermore, in contrast to humans, developmental maturation in rodents takes place mainly after birth.

E. Kitraki
Department of Basic Sciences, School of Dentistry,
University of Athens, Thivon 2 str., 11527 Athens, Greece
e-mail: ekitraki@dent.uoa.gr

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Although translation from animals to humans should be cautious, given the aforementioned differences in their physiology, growing evidence from animal studies suggests that environmental exposure to BPA may adversely impact human health as well. Initial toxicology studies have exposed animals to rather high doses of BPA and have reported numerous dysfunctions in animals' reproductive physiology. The dose of 50 mg/kg bw/day was set as the LOAEL (lowest-observable-adverse-effect level) dose, based on observations from the reproductive system and tumour growth. To further simulate the low daily exposure of humans, a safe reference dose was determined at 50 μg/kg bw/day. During the last decade, however, many studies have shown that exposures below the safe dose can still affect animals' physiology and behaviour.

Nowadays, there is an ongoing vivid debate on the potential risks for human populations from exposure to low BPA doses. On one side, the majority of independent basic research laboratories emphasise the existence of adverse BPA effects on animals' and subsequently humans' health within the 'safe' exposure [4]. On the other side, governmental agents (US National Toxicology Program and Food and Drug Administration) based on few risk assessment studies [5, 6] assure that there is no risk for human health at current exposures. They only express some concern for possible effects in neural and prostate physiology upon perinatal exposures [7]. The main argument from the side of the scientific community is that the risk assessment studies were not designed to detect delicate developmental effects but were rather focusing on gross BPA-induced changes including mortality, fertility and tumorigenesis. Additional arguments relay on the different mechanisms of BPA actions that do not allow linear extrapolations from high doses to very low ones [8, 9]. Properly designed and reproducible studies have so far provided sound evidence for adverse effects of ecologically relevant BPA exposures during development.

The aim of this chapter is to summarise evidence from rodent studies on the effects of BPA upon exposures that are relevant to humans, that is, exposures around or below the 'safe' daily uptake, estimated at 50 μg/kg bw. The chapter is divided in two parts: In part I, important issues on the design of an animal study will be addressed. In part II, evidence from low-exposure rodent studies will be presented, with an emphasis in the nervous system that appears highly susceptible to low BPA actions.

4.2 Part I: Issues on Experimental Design

Research on the effects of BPA in mammals has produced a wealth of data showing diverse actions of this xenoestrogen in several systems. These effects of BPA often vary significantly, even within the same system/organism, and make it difficult to draw a definite conclusion. The main reason for these discrepancies is the variation of experimental protocols that does not allow direct comparisons from study to study. The aim in the following paragraphs of Part I is to shed light on parameters of the experimental design that may confer diversity in the obtained results.

4.2.1 Route of Exposure

Humans are exposed to BPA via both oral and nonoral routes. These include consumption of BPA-containing foods or drinks, leakage from medical/dental devices as well as inhalation of BPA-contaminated air. Animal studies mimicking the above routes are thus all appropriate in evaluating human effects, given that the dosage used is kept within relevant human exposure. In most rodent studies, oral administration is preferred, because it is considered to represent the most common way of human exposure. Oral administration in rodents is met in several variations: provided into the drinking water, by gastric gavages, dissolved in oil or combined with food. Other approaches bypass the digestive track by applying subcutaneous, intravenous, intracisternal, intramuscular or pumping methods. None of these paradigms is ideal however, as they may occasionally preclude inaccurate dosing, variations in exposure over time, psychological stress or vehicle contaminations [3].

In order to mimic the effect of BPA-containing leached substances from dental sealants and resins, Al-Hiyasat et al. [10, 11] have eluted bisphenolic compounds from dental composites and provided the solution in mice by gastric gavages. In such paradigms, using a mixture of compounds, it is important to precisely analyse the composition and concentration of the active components in the starting material. Even so, it is still difficult to attribute a certain effect to a particular component.

The route of exposure may also differently affect the pharmacokinetics and active levels of BPA. Oral administration results in earlier metabolic inactivation of BPA, compared to SC or IV routes, due to the direct passage from gut and liver before entering circulation. This could possibly differentiate its biological effect, although no definite conclusion has yet been reached, since some but not all studies support this possibility [3, 12].

4.2.2 Pharmacokinetics

BPA is rapidly metabolised in glucuronide and sulphate compounds that show low estrogenic activity and cannot bind to estrogen receptors. The liver is the major site of conjugation of free BPA to inert metabolites. Intravenously injected BPA in rodents can quickly reach all organs (it peaks at 20–30 min) and is also rapidly transferred across the placenta to the fetuses. Efficient conjugation is witnessed with a decline of active BPA concentration after 2 h [13]. Upon oral administration in rodents, it is estimated that approximately 95 % of BPA is soon inactivated through metabolism in the liver or intestine before reaching the general circulation. It is thus possible that rodents receiving orally BPA are exposed for a shorter time in the active compound, compared to the injected animals [14]. Sex differences may also influence the pharmacokinetics and availability of free BPA in both rodents and humans. Higher active BPA concentrations are detected in males that can be

explained by the lower expression of the main BPA glucuronidating enzyme [UDP-glucuronosyltransferase 2B1 (UGT2B1)] in their liver [15, 16].

In humans, pharmacokinetic studies performed in adult volunteers showed that the ingested BPA is metabolised to inactive compounds more rapidly compared to rodents. The kinetic profile of inactive metabolite d-BPA glucuronide showed a rapid peak and urinary elimination with a half-life of approximately 5 h [17].

Based on the rapid metabolic clearance of BPA that is more effective in humans compared to rodents, the European Food Safety Authority (EFSA) concluded in 2008 that rodent toxicity data are not directly relevant for human risk assessment and that perinatal exposure of humans has a negligible risk [18]. However, there are several arguments against this conclusion, summarised as follows:

(a) Bio-monitoring studies have detected free BPA in the rat or human placenta and in fetuses, implying that human exposure to BPA is frequent and not negligible.
(b) The metabolic detoxifying mechanisms are not similarly effective in all tissues, for example, are less potent in the brain.
(c) There are counteracting mechanisms of de-conjugation that re-provide free BPA. Indeed, extensive de-conjugation of BPA glucuronide in utero and BPA sulphate in neonates has been reported [19].
(d) The counteracting mechanisms appear particularly effective during the perinatal period. The enzyme activity required for the de-conjugation is higher in the placenta of rodents, and the concentration of BPA in this tissue is higher than that in the maternal or fetal circulation [15], indicating a higher exposure of fetuses to active BPA [20].
(e) There are evidences for BPA actions (non-genomic) that require very low concentrations of the xenoestrogen and do not depend on receptor binding [21].

4.2.3 Dosage

The dosage of BPA used in animal studies varies from high pharmacological to very low ones that are below the safe limit. Toxicology studies have determined the maximum tolerable dose for BPA at 1,000 mg/kg bw/day. A dose of 50 mg/kg bw/day was set as the LOAEL (lowest-observable-adverse-effect level) dose, concerning effects in the reproductive system and tumorigenesis. The European Food Safety Authority has set the tolerable daily intake (TDI) of BPA for the European Union at 0.05 mg/kg/day [18]. This dose, also termed ‘safe dose’, is however higher (more than 20 times) than doses reported to cause adverse effects in rodents [3]. This discrepancy could be explained as follows: Initial toxicology studies have used threshold-based or linear non-threshold models to estimate the biological effect of different BPA doses that assume effects over a threshold and increasing number of effects by increasing dose, respectively. However, most hormones appear to follow non-linear biphasic dose responses. According to such biphasic models, the highest

effects can be seen in very low and very high concentrations of the hormone (U-shaped) or in the intermediate doses (inverted U-shaped). The biological effects of BPA in cultured cells appear to follow this biphasic model. In in vivo studies, it is more difficult to have a complete confirmation of the model, because data on end point effects at different dosages are missing. Nevertheless, there is some evidence showing a non-linear mechanism of BPA actions [22, 23] that should be taken into consideration when comparing effects of different BPA doses on the same biological system.

4.2.4 Timing and Duration of Exposure

Gonadal steroids exhibit both organisational and activational actions. Organisational actions, taking place mainly during fetal life, refer to the ability of these hormones to program functions of the adult organism. Activational actions exerted in the pubertal and adult organism are driven by the gonadal hormones and regulate relevant physiology and behaviour. Apparently, BPA exposure during development may critically interfere with fetal and neonatal programming. The developing organism is more sensitive to BPA for the additional reason that it lacks fully functional detoxifying and immune systems.

Exposures of adult animals have been used to address the effects of BPA on the mature reproductive system and to study the interactions of this xenoestrogen with the endogenous gonadal steroids. Developmental exposures apply BPA during the whole gestation and/or lactation or during critical time windows within this period that vary depending on the timing of each system's development. BPA is provided to the mother and reaches offspring through the placenta and/or milk. Most developmental studies use long-term exposures to mimic situations in humans. Given the rapid metabolism of BPA, daily exposures for a long time are preferred from acute treatments. In studies with adult exposures, however, BPA is usally administered for shorter periods of time.

4.2.5 Choice of Rodent Species

The choice of rodent species is of importance for the reproducibility of the results obtained, since there are many differences between rats and mice, as well as among strains. In general, mice are considered more sensitive than rats to BPA actions. However, this must be further delineated in light of the specific question to be addressed. For example, mice are preferable for genetic studies, while rats for behavioural testing. Species' differences in the sensitivity of certain tissues also exist. For example, the mammary gland of rats is more susceptible to BPA than that of mice [3, 24].

Attention should be also paid on the strain of rat or mouse, as not all strains show the same sensitivity to BPA [2]. Most reports on strain differences have so far examined effects in the female reproductive system. Fischer 344 rats are considered more sensitive than Sprague–Dawley female rats in the effects of high BPA dose (37.5 mg/kg bw) on vaginal epithelium proliferation [25]. In another study [26], the estrogenic potency of BPA was evaluated in three different rat strains (Sprague–Dawley, Wistar and DA/Han) by determining the uterine weights of adult females exposed for 3 days to high levels of BPA (200 mg/kg bw/day). In contrast to the previous studies, only small differences were observed among the strains, and their blood concentration of BPA did not differ 24 h after the last dose. At this point, it should be noted that the dose used was quite high and that the uterotrophic assay applied has been questioned as to its sensitivity at human relevant exposures. Others have reported reduced sensitivity of the CR–Sprague–Dawley rat strain in the estrogenic actions of BPA, based on the effects of a positive control (estradiol or another potent estrogen) included in the study [2]. However, the ideal positive control to compare BPA actions is still a matter of debate, as xenoestrogens vary significantly in their properties and potential specificity.

Future studies addressing strain sensitivity should take into consideration that animals may differ depending on the biological end point and the dose of BPA used. Furthermore, food content and housing conditions can greatly influence the biological outcome even within a certain laboratory, so it is advised to include all strains to be compared in the same experimental protocol.

4.2.6 Choice of Sex to Study

During the last decade, there is an increasing trend in science towards studying both sexes in basic and clinical research, based on the accumulating gender differences in physiology and disease. The need to study both males and females is more obvious when assessing the biological effects of a xenoestrogen, given the known sex differences in the organism's response to estrogens. Additionally, BPA is acting as a selective estrogen receptor modulator (SERM), and the response of the two sexes cannot always be predicted based on the action of a typical estrogen. Due to the experimental design of most animal studies using developmental exposures, both male and female offspring are available for observation, and there is so far a wealth of evidence concerning sexually dimorphic BPA effects. Special attention should be paid when studying animals in adulthood as to the activational actions of gonadal hormones. Estrous cycle must be monitored and normalised in female subjects, since endogenous estrogens may influence several physiological responses. Similarly, testosterone levels must be measured, and adult males should be individually housed to avoid interference of the testosterone-mediated dominance status in the results.

4.2.7 Appropriate Controls

To assure the effects of BPA on a certain system, one is encouraged to include in the study the appropriate positive controls. These are hormonal compounds, whose properties and biological effects have been well established in the system under study. The use of a positive control is important especially in the cases that there is no observable BPA effect. The positivity of the control compound will then confirm the negative results and the hormonal sensitivity of the rodent species used. 17-β-Estradiol, diethylstilbestrol (DES) and ethinylestradiol have been used as positive controls to verify estrogenic effects of BPA in rodents' reproductive system. DES is a synthetic estrogen often used as a positive control for xenoestrogens. It has a higher activity for ERα and equal affinity for ERβ, compared to estradiol. The selection of positive control must take into consideration the route of BPA administration. For oral exposures, DES and ethinylestradiol are preferable because they retain their activity better than estradiol.

Given that BPA may not only act as an estrogen-mimetic compound, especially outside the reproductive system [2, 27, 28], the a priori selection of a positive estrogenic control may not confer to the complete elucidation of the results. In these cases, incorporation of more than one control substances of different properties (i.e. estrogenic, anti-androgenic or androgenic and antithyroid) could provide a better solution. Furthermore, caution should be paid on the appropriate dose for each control compound used: So far, the doses are adjusted based on hormones' affinities for the classical estrogen receptors. Evidence for non-genomic actions, exerted at much lower concentrations via membrane-bound entities, requires updating of the used rules.

4.3 Part II: Evidence from Low-Exposure Studies

4.3.1 General

Initial toxicological studies for the effects of BPA in vivo have used rather high, pharmacological doses of the agent, close to or higher than 50 mg/kg bw/day, set as the LOAEL dose [29]. These risk assessment studies were focusing on gross BPA-induced changes including fertility, mortality and neoplasia, but were not designed to detect more delicate developmental effects that however may crucially impact individuals' heath. Furthermore, in vivo studies using high levels of BPA may be inappropriate to judge for the harmless of lower doses, since BPA actions often follow a non-linear pattern. In compliance with this, low-dose BPA effects in the reproductive system were not witnessed after exposure to high doses [22, 23].

Given the increasing requirement for animal models that simulate human exposure, most studies conducted during the last years have used human relevant doses

of BPA (around or below the safe reference dose of 50 μg/kg bw/day). In this chapter, only the effects of low BPA exposures will be presented. Information on the impact of high doses can be found in several recent reviews [2, 30]. Most low-dose studies have used perinatal exposures of the animals (during gestation and/or lactation) that preclude the possibility of programming and render the animals more sensitive to BPA actions. In these studies, the biological end points were evaluated either in young offspring or more often in adulthood, to check for possible sustained effects. On the other hand, the number of studies applying adult exposures is limited and focuses on the activational effects of BPA in interaction with the fully developed hormonal system of the animal.

BPA exposure in utero can influence the development of the whole embryo. Imanishi et al. [31] used DNA microarray analysis to define the genes whose expression was altered in the murine placenta at the 18th day of gestation. The daily BPA dose used was only 2 μg/kg bw and was administered in pregnant mice from day 6 to 17 of gestation. Significant alterations, depending on the sex of the embryo, were detected in mRNA levels of several nuclear receptors upon BPA treatment. These included progesterone receptor and estrogen receptor β genes that were upregulated in male, but not female, BPA-treated embryos. Given the critical contribution of ovarian steroids in the maintenance of pregnancy and fetal differentiation, the BPA-induced changes in placenta sensitivity to these hormones may play a role in the normal embryonic development.

Based on the estrogen-mimicking properties of BPA, the tissues initially selected for studies were the well-known targets of estrogens. These included female and male reproductive organs (vagina, uterus, ovaries, testis) and accessories (mammary gland, prostate), as well as central nervous system (CNS) centres (hypothalamus and pituitary) regulating reproductive physiology and behaviour. The end points assessed were related to sexual maturation, fertility and sexually dimorphic behaviours. Recent studies have also investigated possible effects of BPA on thyroid function and metabolism. Search for possible effects in the CNS outside the hypothalamus has recently unravelled several nonreproductive BPA actions upon particularly low exposures. Given the numerous targets of estrogens in the body and the multiple mechanisms of BPA actions, it will not be surprising to detect novel BPA-endangered systems in the near future.

4.3.2 Effects in the Reproductive System

In contrast to the well-described adverse effects of pharmacological doses in the reproductive system of rodents [for review, see 2], BPA exposures to less than 100 μg/kg bw/day have only minor effects.

Oral exposure to 2, 20 or even 200 μg BPA/kg/day from gestational day 7 to postnatal day 18 does not significantly change anogenital distances, vaginal opening, fertility or CNS defeminisation in female rat offspring [32]. In this study, treatment with another estrogen (ethinylestradiol) used as a positive control

significantly affected the above parameters, certifying the lack of effects from low BPA exposure. Previous studies in mice exposed perinatally to BPA, via osmotic mini pumps or releasing pellets, have reported increased antral follicles in the ovaries [33] and chromosomal aberrations in the oocytes [34]. Aberrations in the estrous cycle or para-ovarian cysts have also been reported in adult mice offspring perinatally exposed to BPA via subcutaneous injections at doses exceeding 100 μg/kg [35, 36]. The discrepancies in the aforementioned studies may partly reside in the use of different exposure routes, as nonoral administration that avoids first-pass metabolism in the liver could increase active BPA levels.

Short-term exposure of adult female mice to 20 μg BPA/kg/day increased the likelihood of producing aneuploid gametes [37]. In another study, adult female mice were intragastrically administered 5, 25 and 100 μg BPA/kg bw/day for 28 days and then mated to untreated males. Exposure to 25 and 100 μg BPA significantly increased the number of resorptions and the relative uterine weights. Relative ovarian weights were also significantly increased at the 100 μg dose [11]. In the same study, treatment with the leached components of a dental resin, comprising of tri-(ethylene glycol)-dimethacrylate (TEG-DMA) (5,945 μg/ml), BPA glycerolate dimethacrylate (BIS-GMA) (2,097 μg/ml) and BPA(78 μg/ml) had similar effects on resorptions and additionally led to increased ovarian weights. This study is one of the few examining the effects of leached components from dental sealants in the reproductive system of rodents, and its findings support the adversity of BPA actions.

Developmental exposure to low BPA doses does not have consistent effects on the adult male reproductive system. Decreased levels of testicular [38] or serum [39] testosterone levels were detected in adult male offspring of mothers exposed to 2–2.4 μg BPA/kg bw during gestation or lactation. Similar reductions in circulating testosterone were detected in juvenile male rats exposed to 40 μg BPA/kg bw during puberty [40]. On the other hand, Kato et al. [41] reported no effects on male rat reproductive system upon neonatal injections of low BPA doses. In recent studies, developmental exposure of rats to 2–200 μg BPA /Kg bw did not affect testes weight or sperm production [6, 42, 43], though earlier studies in mice had reported reductions in the weights of epididymis and seminal vesicles [44].

4.3.3 Effects in the Accessory Reproductive Organs

Exposure to low BPA doses can still have adverse effects in the development of mammary and prostate glands of rodents. Several studies associate perinatal BPA exposure with enhanced mammary gland development in females. BPA administered in pregnant mice via implanted mini pumps (0.025 μg/kg bw/day) stimulated mammary gland development in their offspring [45]. Importantly, this treatment enhanced mammary gland sensitivity to estrogens at puberty onset [46] and the appearance of hyperplasias in adult life [24]. Similar observations have been made in rats, considered more appropriate to model mammary gland pathology [3]. More

specifically, Wistar-Furth rats exposed during embryonic life to 2.5, 25, 250 and 1,000 μg BPA/kg bw developed as young adults increased number of hyperplastic ducts (at all doses) that were positive for estrogen receptor and proliferation markers. The hyperplastic ducts were retained in animals exposed to the lowest dose, whereas carcinomas in situ were detected in adult animals exposed to the higher doses. In another study of the same group, Wistar offspring exposed in utero at 25 μg BPA/kg and challenged at puberty with a chemical carcinogen developed in adulthood more hyperplastic ducts and mammary malignancies than the non-BPA treated. These findings correlate BPA exposure with breast cancer and suggest that prenatal BPA can enhance breast cancer susceptibility by sensitising mammary gland to estrogens and environmental carcinogens experienced later in life. Furthermore, a recent in vitro study, using human breast cancer cell lines, showed that BPA at low doses can antagonise the cytotoxic effects of chemotherapeutics such as doxorubicin, cisplatin and vinblastine [47]. It is proposed that BPA increased the expression of antiapoptotic proteins, probably through interactions with alternative ER receptors expressed in cancer cells.

Adverse effects of developmental BPA exposure have been also reported regarding the rodent prostate. One-month-old male rats exposed in utero to a low BPA dose had transient differences in prostate histology, compared to control males, by means of a larger layer of fibroblasts and increased proliferation in the periductal stroma cells [48]. A transient decrease not observed later in adult life was also detected in the expression of androgen receptor and acid phosphatase in the prostatic cells of BPA-treated males. Several other studies conducted in rats and mice report increased adult prostate size in BPA-treated offspring [for a review, see 2]. The increased prostate size and androgen responsiveness upon perinatal BPA exposure have been suggested to increase the susceptibility of prostate to neoplasia in later life [49]. Male rats neonatally injected with BPA (10 μg/kg bw) and retreated as adults with testosterone or estradiol showed increased incidence of prostatic neoplasms. Although BPA is studied for its estrogenic properties, its interaction with androgen receptors (ARs) is also possible. In prostate cancer cells, BPA can act as either AR agonist or antagonist depending on the functional state of the receptors. In the presence of wild-type ARs in these cells, BPA blocks androgen actions, whereas in cases of mutated ARs, BPA promotes cell proliferation [50].

4.3.4 Effects in the Central Nervous System

Beyond their traditional roles in the central regulation of reproductive physiology and behaviour, gonadal steroids influence several other brain functions. Estrogens in particular are implicated in cognitive function, neuroprotection and synaptic plasticity, as well as in the neuropathology of Parkinson's and Alzheimer's diseases.

Most estrogen responses are orchestrated by estrogen receptors (ERs) that are ligand-activated transcription factors. Two types of ERs have been identified in

mammals (ERα and ERβ) that belong to the nuclear receptor superfamily. The two receptors can mediate distinct actions of estradiol, depending on their interactions with the responsive elements in gene promoters, on the combination of the available co-regulators as well as on the ratio of ERα/ERβ in each tissue [51]. ERβ may even inhibit the transcriptional activity of ERα. ERα is the predominant receptor in the hypothalamus and the main regulator of reproduction. ERβ on the other hand is implicated in nonreproductive estrogen actions and is the principal ER subtype in several brain areas including the cerebral cortex, hippocampus and cerebellum. The dynamics of the two receptors and their distribution are important parameters for the final outcome of estrogen actions in the brain.

BPA has approximately 10,000 times lower binding affinity for ERs, compared to 17β-estradiol, and based on this, it is characterised as a weak environmental estrogen. BPA has ten times higher affinity for ERβ than ERα. Importantly, BPA binding to either ER subtype leads to conformational changes that differ from those induced by estradiol binding. This can critically modify the receptor–ligand complex properties in recruiting co-regulators and/or other interacting proteins. In fact, BPA acts as a selective estrogen receptor modulator (SERM), exhibiting either agonist or antagonist behaviour, depending on the target tissue [52]. Thus, the ratio of the two ERs in a given tissue, along with the dynamic equilibrium of co-activators and corepressors, is critical for the nature of BPA effect.

In addition to nuclear ERs, membrane entities binding estradiol have also been described [21], such as the membrane-bound ERα-like receptor (mER) and a transmembrane G-protein-coupled ER (GPR300). By binding to these membrane receptors, BPA can exert rapid non-genomic actions including increases in cellular Ca^{++} or nitric oxide. These two potent intracellular messages can readily alter enzyme activities or membrane potential and permeability in target cells. Studies in MCF-7 breast cancer cells have shown that BPA via membrane receptors can modify cell properties at concentrations 10^{-10}–10^{-12} M that are much lower than those needed for an in vivo effect. However, it is so far technically difficult to distinguish BPA actions mediated via membrane receptors in experimental animals. Notably, in the above-mentioned *ex vivo* studies, BPA can be equally effective with estradiol. In addition, BPA can bind to other receptors, such as the orphan nuclear estrogen-related receptor-γ or the thyroid hormone receptor; however, the in vivo relevance of these interactions has not yet been elucidated.

BPA is able to reach the brain of fetuses or adult rodents shortly upon administration. The estimated time to reach fetal brain is approximately 1 h following subcutaneous injection to the mother [53]. Importantly, the brain does not possess an efficient detoxification system, because the levels of drug-metabolising enzymes are extremely low within this tissue [54], and is thus more susceptible to BPA actions, compared to peripheral tissues.

Perinatal exposure to BPA, as well as to other xenoestrogens, can potentially affect the normal process of sexual differentiation of the brain. In male rodent fetuses, testosterone secreted by the developing testes reaches the brain, where it acts upon conversion to estradiol. This conversion is driven by the enzyme aromatase whose activity increases perinatally, during the critical time windows of brain

masculinisation. Thus, the masculine brain is shaped through the action of estradiol in male fetuses, while at the same time the female fetuses remain hormonally inert [80]. Accordingly, the feminine pattern is the default in a neutral fetus brain. The female fetus is further protected from estradiol of maternal or male littermates' origin by the increased levels of estradiol binding proteins, like alpha-fetoprotein. However, estrogen-binding proteins confer little protection from BPA actions due to its low affinity for them, compared to estradiol.

4.3.4.1 Effects on Brain Structure

Neuronal migration is indispensable during development for the physiological growth of the brain tissue. Migration is particularly important for the formation of multilayered cortex structures. Impaired cortical development and altered connectivity within the brain have been implicated in cognitive deficits and neuropsychiatric disorders in humans [55]. In utero exposure of mice to 20 μg BPA/kg bw results in significant alterations in the migration and differentiation of neocortex precursor neurons [56]. More specifically, BPA enhanced the rate of neuronal migration and differentiation. Furthermore, the expression of neurogenesis-relevant genes (Math3, Ngn2, Hes1) and thyroid receptor-related genes was significantly upregulated in the telencephalus of BPA-treated embryos. When the BPA offspring were examined in later life, they exhibited abnormal positioning of neurons and cortico-thalamic connections. This persistent BPA-induced disturbance in cortical cytoarchitecture resembled the effect of exposure to low ionising radiation [57].

Locus coeruleus is a sexually dimorphic brain area in the brain stem involved in sympathetic stress responses, anxiety and panic. Its dysfunction has been correlated among others with the neurodevelopmental Rett syndrome and the posttraumatic stress disorder (PTSD), while a significant loss of neurons in this area is met in Alzheimer's patients. The volume of this nucleus is larger in females than males, and androgen receptors have been implicated in the emergence of this sex difference in rats, with testosterone lowering neuronal number [58]. BPA treatment of rats at a daily dose of 30 or 300 μg/kg bw, during the fetal and neonatal period, led to the abolishment and inversion of the existing sex difference [59]. The observed increase in locus coeruleus of males has been attributed to the estrogenic action of BPA in this area that harbours both estrogen receptor subtypes.

The anteroventral periventricular (AVPV) preoptic area in the hypothalamus is another sexually dimorphic region that is important for the periodical gonadotropin release and the normal estrous cyclicity. Dopamine-releasing neurons consist the main population in AVPV that is larger in female rodents, compared to males. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of dopamine, and its abundance is also higher in the AVPV of females. The sexual dimorphism of this population of neurons appears to be programmed perinatally by gonadal steroids [60]. Perinatal exposure of mice at low BPA can alter the dimorphic profile of this area. In this study [61], pregnant dams were implanted with osmotic pumps releasing 25 or 250 ng of BPA/kg bw/day from gestational day 8 to

lactation day 16 (in rodents, gestation and lactation last 21 days each). These doses are among the lowest used to date and represent a maximum daily intake of 0.23 μg/kg bw. A significantly reduced number of TH neurons in the above-mentioned area were observed in the female BPA-treated offspring leading to the abolishment of the documented sexual dimorphism. It is worth noting that other dimorphic nuclei in the hypothalamus, like the sexually dimorphic nucleus (SDN), are not sensitive to BPA actions [59, 62, 63].

Reduction in the number of dopamine-synthesising neurons has also been reported in male rats. Male pups were injected with 0.2–20 μg BPA/pup and tested at 1 or 2 months of age [64]. This reduction was witnessed in the midbrain dopaminergic population and was related with the decreased spontaneous motor activity seen in these offspring (discussed below). No females were used in this study to check for a possible sexually dimorphic effect. Nevertheless, several studies have reported effects of perinatal BPA administration on dopaminergic neurons, indicating the increased sensitivity of this neuronal population to the aforementioned endocrine disruptor during development.

4.3.4.2 Effects on Brain Physiology

Changes in Steroidogenesis and Synaptic Plasticity

Steroids synthesised locally in the brain (neurosteroids) play an important role in many physiological responses including neuroprotection and synaptic plasticity. Neurosteroids' actions have been particularly studied in the hippocampus, a brain area that hosts the required synthesising enzymes. Fetal and postnatal exposure of rats to BPA significantly facilitates the local synthesis of estradiol in the hippocampus [65], implying a role of low BPA concentration in the modulation of brain steroidogenesis and consequently synaptic plasticity. However, in other studies, it is apparent that BPA exposure either in adulthood [66] or in early life [67] can adversely influence the synaptic plasticity in rodent brain. MacLusky et al. [66] showed that exposure of adult ovariectomised rats to a low dose of BPA inhibits the rapid synaptogenic response of pyramidal neurons to estradiol. Given that synaptic remodelling has been related to the rapid effects of estrogens on memory, the authors suggest that BPA exposure may modify the existing sex differences in cognitive function, acting in this case as an estrogen antagonist. Accordingly, BPA exposure during aging could exacerbate the impairment in cognitive function caused in females due to the elimination of endogenous estrogens.

The ability of synaptic contacts to change in response to stimuli is indispensable for the processes of learning and memory. A well-established electrophysiological measure of synaptic plasticity concurrent to memory storage is the long-term potentiation (LTP), indicating the strengthening of certain synaptic contacts upon relevant mnemonic stimuli. Deficits in the development of LTP in the striatum of young male rats have been reported in animals perinatally exposed to 20 μg of BPA [67], providing another example of the antiestrogenic effects of low BPA exposure.

The dorsolateral striatum confers the neuroanatomical substrate for motor control, and BPA-treated male offspring in this study exhibit a significant hyper-locomotion that may relate to the function of dopamine receptors and the improper development of synaptic plasticity.

Changes in Neurotransmission

Brain physiology is greatly depended on synaptic activity that in turn is regulated by neurotransmitters. Significant alterations have been reported in neurotransmitter levels and/or receptors upon exposure to low BPA doses. These alterations so far concern dopamine and other monoamines. Reduced population of dopamine neurons in the midbrain, as detected by the reduced immunoreactivity for tyrosine hydroxylase, was reported in the midbrain of 4- and 8-week-old male rats intracisternally injected at postnatal day 5 with 2–20 μg of BPA [64, 68]. The rats had also reduced expression of dopamine receptor D4 and dopamine transporter and exhibited increased spontaneous motor activity. These findings suggest that neonatal low BPA exposure may cause a deficit in the development of dopaminergic neurons that could be causatively linked to the detected molecular and behavioural alterations. Given that the mesocorticolimbic dopamine system has been implicated in the attention deficit hyperactivity disorder (ADHD), a developmental disease characterised by inattention, motor hyperactivity and impulsivity [69], the above results imply a potential contribution of BPA in the appearance of ADHD-like symptomatology. In a recent study using a single intracranial BPA injection (10 μg/pup in 2-day-old male rats), significant alterations were detected 28 days later in norepinephrine, serotonin, dopamine and their metabolites, in the hippocampus, striatum and brain stem [70]. It is noteworthy that although free BPA disappeared from the brain of injected animals 5 h postinjection, BPA effects were well apparent over a period of approximately 1 month.

Nitric oxide (NO) is a gaseous second messenger that also acts as neurotransmitter. It has been implicated in the regulation of several functions including reproductive behaviour. Mice perinatally exposed, through their mothers, to BPA (10–40 μg/kg bw/day) exhibit as adults alterations in the number of cells expressing nitric oxide synthase, the key enzyme for the production of NO, in the medial preoptic area of the hypothalamus and in the bed nucleus of stria terminalis [71]. The observed changes are dose and sex dependent, leading to the loss of normally occurring sex differences or the appearance of novel ones.

Changes in Nuclear Receptors' Levels

Estrogen receptors (ER) are candidate mediators of several BPA actions and at the same time are subjected to modifications induced by this xenoestrogen. In the rodent brain, BPA-induced ER alterations have been detected during adolescence, as well as in the adult life of perinatally exposed animals. Exposure to BPA in utero

resulted in a four-fold increase of ERβ mRNA levels in the preoptic area of male rat offspring examined at 1 and 4 months of age [48]. No alterations were observed at the levels of ERa or ERβ in the medial basal hypothalamus, denoting that BPA effects within the brain can be locally distinct and area specific. In another study, rats exposed to BPA during early puberty showed altered levels of immunohistochemically detected ERa in a number of hypothalamic nuclei. At puberty, control males had more ERa-positive neurons than females in the arcuate nucleus and medial preoptic area, and BPA treatment further enhanced receptor levels in both areas. In adulthood, BPA also increased ERa levels in another hypothalamic nucleus of treated female offspring [72].

In utero exposure of mice to BPA also increased the expression of ERa and ERβ in the dorsal raphe nucleus of male offspring examined at juvenility and adulthood, though variations of the BPA effect were observed at different time periods of adult life [73]. Overall, these results indicate the ability of BPA to change the estrogen responsiveness of neural circuits controlling reproduction during puberty and adulthood, in a sexually and timely distinct way.

Retinoic acid, a vitamin A metabolite, is an essential morphogenetic factor with marked effects on developmental growth and differentiation. Retinoic acid exerts its actions by binding to retinoic acid receptor (RAR) and retinoid X receptor (RXR), belonging to the nuclear receptors' superfamily. In murine embryos exposed to 2 μg BPA/kg bw/day from day 6 to 17 postcoitum, significant changes in gene expression of the aforementioned receptors were detected in the cerebra and cerebellum that differed between male and female fetuses [74].

Steroid receptor co-activators comprise a class of transcription regulators indispensable for the activation of transcription by steroid hormone receptors. Steroid receptor co-activator-1 (SRC-1) is involved in the transcriptional activity of both thyroid and steroid receptors, including estrogen and glucocorticoid receptors [75]. Transiently increased expression of SRC-1 was witnessed [76] in the hippocampus of male rat pups exposed perinatally through their mothers to a very low BPA dose (100 μg BPA per liter of drinking water). Given the critical role of SRC-1 in the transcription of many genes regulated by gonadal hormones, its alterations upon low BPA exposure during development provide an extra mechanism through which this compound may interfere with normal growth.

Alterations in the levels of brain glucocorticoid receptors were also detected upon perinatal exposure to a low BPA dose in rats (presented below as part of the stress response system).

Changes in the Stress Response System

The hypothalamic–pituitary–adrenal (HPA) axis is the neuroendocrine system mediating the organism's central stress response. In this circuit, additional brain areas, such as the prefrontal cortex, the hippocampus and amygdala, have a critical contribution [77]. A stressful event activates the sympathetic nervous system and the HPA axis that mobilise catecholamines and adrenal steroids, respectively.

Glucocorticoids (cortisol in humans, corticosterone in rodents) are secreted by the adrenal cortex at high levels during stress. Initially, they synergise with catecholamines to increase sympathetic arousal, cardiac tone and glucose availability in muscle. Later on, glucocorticoids terminate the stress response by lowering HPA axis activation and subsequently their own increased secretion. These glucocorticoid actions are mediated by two types of receptors: the classical nuclear glucocorticoid receptors (GRs), widely distributed in the brain, and the mineralocorticoid receptors (MRs), selectively located in the limbic system [78]. GRs mediate the negative feedback actions that terminate stress response, while MRs maintain basal HPA axis function. Previous data suggest that HPA axis and the hippocampus are potential targets for estrogens' organisational actions [79, 80]. Both the hypothalamus and hippocampus host glucocorticoid receptors and estrogen and androgen receptors and an interplay between gonadal and adrenal steroids appears critical for the fine tuning of hormonal responses in these areas [81, 82].

We recently investigated whether perinatal exposure to a 'safe' BPA dose can affect components of the stress response system in rats [83]. Wistar rats were orally administered 40 μg BPA/kg bw/day for the entire period of gestation and lactation. The dose used had no effect in anogenital distance of the pups or in body weights at puberty onset, compared to the untreated controls. In accordance with the existing literature for similar exposures, the treatment did not alter the time of vaginal opening and cycling in female offspring or the levels of plasma progesterone and testosterone in adolescent females and males, respectively. BPA treatment altered circulating corticosterone and GR levels in the hippocampus of adolescent rats in a sexually dimorphic manner. Under basal conditions, female BPA offspring had higher hormone levels than control females and BPA males, whereas following a mild stressful experience (a Y maze task), corticosterone levels were increased in BPA offspring of both sexes, compared to untreated stressed animals. Additionally, GR levels were altered only in female BPA offspring: They were reduced under basal conditions but increased following stress. These findings show that prolonged perinatal exposure to a weak estrogen can promote the appearance of sex differences in corticosterone levels that normally arise after puberty in rats (adult females have higher levels than the males [84]). Furthermore, they show that the estrogen-mimicking effects of BPA in the enhancement of stress responsiveness [85, 86] are exerted in a sexually dimorphic way. This is the first study to show intervention of a low BPA exposure to the normal maturation of the neuroendocrine stress response system. Future studies are needed to examine whether the observed hormonal and molecular changes are still present in adulthood, under the activational actions of gonadal steroids and their interplay with adrenal hormones and stressful events.

4.3.4.3 Effects on Behaviour and Cognition

The molecular and cellular changes observed in brain physiology have an immediate impact on behaviour. Several studies have shown that perinatal

exposure to low BPA concentrations reduces exploratory behaviour of female offspring and abolishes the sex differences normally existing in this behaviour [59, 61, 68, 76, 87–91]. On the other hand, BPA-treated male rats exhibit reduced anxiety and even increased spontaneous motor activity [68]. Gestational exposure to BPA increases aggressiveness of male mice in early adulthood, without a concomitant increase of testosterone levels, suggesting that other factors are also implicated [39]. Perinatal BPA exposure (40 μg/kg bw) affects social behaviours as well including play behaviour, social grooming and sociosexual exploration. These behaviours were decreased in young females exposed to BPA [92, 93].

The Morris water maze test is a typical behavioural paradigm to test rodents' ability for spatial learning and memory. Xu et al. [76] have treated rat dams during pregnancy and lactation with 100 μg BPA/liter of drinking water and tested the offspring of both sexes as adults in the water maze. Impaired cognitive performance was detected for male BPA-treated offspring. Using a low dose of BPA (40 μg/kg bw/day), we also detected impairments in the Y maze paradigm of spatial memory in adolescent rats of both sexes perinatally treated [83]. Mice treated with a higher BPA dose (100 μg/kg bw/day) from prenatal day 7 to postnatal day 36 showed also decreased alternation behaviour and decreased novel object recognition [94]. However, the effects of BPA on cognitive abilities may be task specific. Ryan and Vandenbergh [95] reported no effects of perinatal BPA exposure (2 μg/kg bw/day) in the spatial memory of adult mice offspring tested in two different short-term spatial memory tests, the radial-arm maze and Barnes maze. In the same study, a higher dose of BPA (200 μg/kg bw/day) was required for the detection of alterations in the anxiety levels of BPA-treated animals.

BPA administration perinatally, or prior to puberty onset, can also affect the sexual activity of treated offspring in adulthood. Perinatal treatment reduced sexual performance of male rats, in terms of latency and frequency of intromissions. In females, BPA produced a small increase in sexual motivation and receptivity [96]. Similar effects were obtained in males upon juvenile exposure to BPA [40]. Interestingly, these findings did not show a potentiation of male behaviour by BPA, as would be expected based on the classical programming actions of estrogens, implying that this xenoestrogen may act in this instance as an estrogen antagonist.

Alterations in maternal behaviour were detected upon administration of 40 μg BPA/kg bw/day in rat dams during pregnancy. Treatment of mothers with BPA significantly reduced maternal care, in terms of licking–grooming behaviour towards the pups and duration of arched-back posture. Moreover, these behaviours were not influenced by the sex of the pup, as is the case in control dams showing more care towards their male offspring, but were rather equally exerted in all pups [97]. Prenatal and neonatal treatment with BPA (40 μg/kg bw/day in dams) also modified the formalin-induced nociception of treated offspring in adulthood in a sexually dimorphic way [98]. The main effects in the nervous system of rodents from low BPA exposures are summarised in Table 4.1.

Table 4.1 Reported effects of low-dose BPA (<50 μg/kg bw/day) in rodent CNS

Altered neuronal migration. Impaired cortex formation [56, 57]
Reversal of sexual dimorphism in the volume of locus coeruleus [59]
Reduced dopamine neurons in the midbrain and in hypothalamic AVPV (loss of sexual dimorphism). Reduced expression of dopamine receptor D4 and transporter in midbrain [61, 64]
Enhanced brain steroidogenesis [65]
Altered synaptogenesis in hippocampal pyramidal neurons [66]
Deficits in the development of synaptic plasticity in the striatum [67]
Altered gene expression of c-fos, dopamine transporter (Dat1) and Hsp70 [68]
Altered monoamine levels in the brain stem, striatum and hippocampus [70]
Altered nitric oxide synthase expression [71]
Altered ERα and ERβ levels in preoptic area and hypothalamus [48, 72, 73]
Altered expression of retinoic acid receptors (RARa, RXRa) in cerebra and cerebellum, depending on the sex of the embryo [74]
Transiently increased expression of SRC-1 in the male pup hippocampus [76]
Altered basal and stress-induced corticosterone levels. Altered hippocampal GR levels in females [83]
Impaired performance in spatial memory tasks [76, 83]
Reduction of motor activity and explorative behaviour in females. Reduction of anxiety in males. Reduction of sexual activity in males and slight enhancement in females [40, 88, 96]
Increased neophobia and anxiety-like behaviour in pubertal females [83, 87]. Male feminisation of adult impulsive behaviour and reduced activity response to amphetamine [87, 91]
Loss of sex differences in explorative and emotional behaviours (open field, novelty test and elevated plus maze forced swim) [59, 76, 89, 90]
Increased spontaneous motor activity [68]
Decrease of playful social interactions in females [92, 93]
Enhanced aggression in males [39]
Alterations in maternal behaviour [97]
Modifications in pain behaviour [98]

4.3.5 Effects on Other Systems

4.3.5.1 Effects in Metabolism

Estrogens have well-known effects on both peripheral and central energy homeostasis. During development, they regulate adipocyte number, whereas in adulthood they inhibit lipogenesis and adipose deposition exerting an anti-obesogenic action. Both estrogen receptors (alpha and beta) mediate estrogens' action in the adipose tissue, ERα being the principal modulator [99]. BPA, as a weak estrogen, is expected to mimic some of estrogens' actions on energy expenditure and adiposity.

BPA administered at high doses (4 or 5 mg/day) for 15 days in ovariectomised adult female rats had similar effects with estrogens on the reduction of body weight [100]. Another study conducted in adult mice [101] has also reported that BPA imitates the effects of 17beta-estradiol on blood glucose homeostasis through both genomic and non-genomic pathways, depending on the dose used. A single low dose (10 μg/kg) of either estradiol or BPA induced a rapid decrease in glucose levels with concomitant increase of plasma insulin. Longer exposures to estradiol or BPA at doses as low as 10 μg/kg/day induced an increase of insulin in pancreatic beta

cells. Upon 4 days of treatment with either hormone, the mice developed chronic hyperinsulinemia, with altered glucose and insulin tolerance. These findings support an enhancing role of BPA, upon adult exposure, in the development of insulin resistance and consequently of type 2 diabetes, hypertension and dyslipidemia.

Fetal or perinatal BPA exposure has recently been proposed to be a potentially risk factor for the development of obesity and related disorders in adulthood [102]. BPA exposure during this period advances puberty onset [103] and increases body weight gain, adipose tissue mass and cholesterol levels later in life of the exposed mice [35, 104]. However, more recent data do not support a programming effect of low BPA exposures in obesity-associated metabolic disturbances. Ryan et al. [105] reported that perinatal exposure to an ecologically relevant dose of BPA indeed resulted in heavier offspring at 4 weeks of age, compared to the controls, but these differences were no longer apparent when the mice reached adulthood, even when tested on a high-fat diet. These data suggest that perinatal exposure to low BPA doses leads to a faster rate of growth early in development, rather than in an obese, diabetic phenotype in adulthood.

4.3.5.2 Epigenetic Changes

Alterations in the pattern of DNA methylation at CpG-rich promoter sequences of a gene comprise a common epigenetic modification, which can activate (hypomethylation) or silence (hypermethylation) gene transcription. Search for methylation changes in the prostate gland of adult male rats, neonatally exposed to low BPA levels, revealed an altered methylation pattern in several genes involved in cell signalling [49]. One of these genes, the phosphodiesterase type 4 variant 4 gene, coding for an enzyme involved in cyclic AMP degradation, was highly hypomethylated and thus continuously expressed, compared to the controls. Importantly, overexpression of this gene was also detected in prostate cancer cells of neonatally BPA-exposed rats that were as adults treated with gonadal hormones. These findings showed for the first time that perinatal BPA exposure to human relevant doses can lead to epigenetic alterations in genes directly associated with preneoplastic prostatic lesions. In another study in mice, BPA exposure during early life also reduced DNA methylation in the reporter genes, implying a potential of BPA to cause epigenetic alterations in the genome [106]. In this study, the dose of BPA used was high (50 mg/kg of diet) that however is an order of magnitude lower than the dietary non-toxic threshold for rodents [107]. The above observations confer another mechanism of BPA action that directly affects fetal epigenome.

4.4 Concluding Remarks

Animal models provide a powerful tool for elucidating the in vivo effects of BPA exposure. There is evidence that low, human relevant, exposure of rodents during development can adversely modify several physiological functions. These include brain neurotransmission and plasticity, behaviour and neuroendocrine responses as

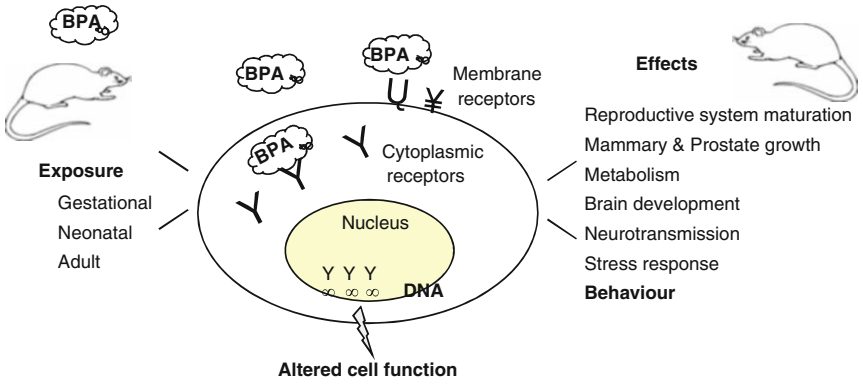

Fig. 4.1 Exposure of rodents to environmental relevant BPA doses during development or in adulthood can affect a number of systems in later life, including the reproductive and the central nervous system. At the cellular level, BPA binds to cytoplasmic receptors for gonadal steroids (ERα, ERβ, AR) and mimics or antagonises their actions. BPA can also bind to membrane receptors of steroid hormones or neurotransmitters, further modifying cell function

Table 4.2 Representative low BPA effects in laboratory rats or mice and their possible relevance for human health

Effect in rodents	'Translation' in human health
Developmental exposure	
Abnormal development of brain cortex	Cognitive and neuropsychiatric disorders
Spontaneous motor hyperactivity, related to midbrain dopamine dysfunction	Attention deficit hyperactivity disorder (ADHD)
Increased scores in behavioural tests of 'depression'	Depression
Heightened plasma corticosterone and altered levels of glucocorticoid receptors in the hippocampus	Altered stress response
Enhanced sensitivity of mammary gland to estrogens, hyperplasia	Enhanced sensitivity to mammary hyperplasia
Increased prostate size and incidence for neoplasms	Enhanced risk for prostate neoplasia
Adult exposure	
Decreased sperm production	Infertility
Impaired maternal behaviour	Postpartum emotional dysfunction
Senescence-like disruption of synaptic function in females	Brain aging
Enhancement of insulin resistance	Diabetes type II

well as normal growth of reproductive accessory organs (Fig. 4.1). At low levels, BPA can also exert epigenetic modifications in the whole genome. Several of the observed adversities are reminiscent of human pathologies (Table 4.2) and have sensitised both the public and the scientific communities. Apparently, there is need to revise the risk of such low exposures. However, further research should be conducted before we can safely extrapolate the knowledge from animal data to humans. First of all, the concentrations and kinetics of BPA in different tissues must be precisely determined, both in neonates and older animals, for oral and nonoral

exposures. This will help to delineate the discrepancies of varying effects from the use of rodent species with different genetic backgrounds and sensitivity. Inclusion of appropriate positive controls, taking into consideration all mechanisms of BPA actions, will also facilitate conclusions on BPA properties. Finally, concerted actions for the determination of active BPA levels in human fluids and whenever possible in fetal tissues will allow more reliable comparisons between rodents and humans.

References

1. Ben-Jonathan N, Steinmetz R (1998) Xenoestrogens: the emerging story of bisphenol A. Trends Endocrinol Metab 9:124–128
2. Richter CA, Birnbaum LS, Farabollini F, Newbold RR, Rubin BS, Talsness CE, Vandenbergh JG, Walser-Kuntz DR, vom Saal FS (2007) In vivo effects of bisphenol A in laboratory rodent studies. Reprod Toxicol 24:199–224
3. Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM (2009) Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. Endocr Rev 30:75–95
4. Vom Saal FS, Akingbemi BT, Belcher SM, Birnbaum LS, Crain DA, Eriksen M, Farabollini F, Guillette LJ Jr, Hauser R, Heindel JJ, Ho SM, Hunt PA, Iguchi T, Jobling S, Kanno J, Keri RA, Knudsen KE, Laufer H, LeBlanc GA, Marcus M, McLachlan JA, Myers JP, Nadal A, Newbold RR, Olea N, Prins GS, Richter CA, Rubin BS, Sonnenschein C, Soto AM, Talsness CE, Vandenbergh JG, Vandenberg LN, Walser-Kuntz DR, Watson CS, Welshons WV, Wetherill Y, Zoeller RT (2007) Chapel Hill bisphenol A expert panel consensus statement: integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure. Reprod Toxicol 24:131–138
5. Tyl RW, Myers CB, Marr MC, Sloan CS, Castillo NP, Veselica MM, Seely JC, Dimond SS, Van Miller JP, Shiotsuka RN, Beyer D, Hentges SG, Waechter JM Jr (2008) Two-generation reproductive toxicity study of dietary bisphenol A in CD-1 (Swiss) mice. Toxicol Sci 104: 362–384
6. Tyl RW, Myers CB, Marr MC, Thomas BF, Keimowitz AR, Brine DR, Veselica MM, Fail PA, Chang TY, Seely JC, Joiner RL, Butala JH, Dimond SS, Cagen SZ, Shiotsuka RN, Stropp GD, Waechter JM (2002) Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicol Sci 68:121–146
7. NTP-CERHR monograph on the potential human reproductive and developmental effects of bisphenol A (2008) http://cerhr.niehs.nih.gov/chemicals/bisphenol
8. Myers JP, vom Saal FS, Akingbemi BT, Arizono K, Belcher S, Colborn T, Chahoud I, Crain DA, Farabollini F, Guillette LJ Jr, Hassold T, Ho SM, Hunt PA, Iguchi T, Jobling S, Kanno J, Laufer H, Marcus M, McLachlan JA, Nadal A, Oehlmann J, Olea N, Palanza P, Parmigiani S, Rubin BS, Schoenfelder G, Sonnenschein C, Soto AM, Talsness CE, Taylor JA, Vandenberg LN, Vandenbergh JG, Vogel S, Watson CS, Welshons WV, Zoeller RT (2009) Why public health agencies cannot depend on good laboratory practices as a criterion for selecting data: the case of bisphenol A. Environ Health Perspect 117:309–315
9. Tyl RW (2009) Basic exploratory research versus guideline-compliant studies used for hazard evaluation and risk assessment: bisphenol A as a case study. Environ Health Perspect 117:1644–1651
10. Al-Hiyasat AS, Darmani H, Elbetieha AM (2002) Effects of bisphenol A on adult male mouse fertility. Eur J Oral Sci 110:163–167

11. Al-Hiyasat AS, Darmani H, Elbetieha AM (2004) Leached components from dental composites and their effects on fertility of female mice. Eur J Oral Sci 112:267–272
12. Pottenger LH, Domoradzki JY, Markham DA, Hansen SC, Cagen SZ, Waechter JM Jr (2000) The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. Toxicol Sci 54:3–18
13. Moors S, Diel P, Degen GH (2006) Toxicokinetics of bisphenol A in pregnant DA/Han rats after single i.v. application. Arch Toxicol 80:647–655
14. Doerge DR, Twaddle NC, Vanlandingham M, Fisher JW (2010) Pharmacokinetics of bisphenol A in neonatal and adult Sprague-Dawley rats. Toxicol Appl Pharmacol 247:158–165
15. Schönfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I (2002) Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environ Health Perspect 110:A703–A707
16. Takeuchi T, Tsutsumi O, Nakamura N, Ikezuki Y, Takai Y, Yano T, Taketani Y (2004) Gender difference in serum bisphenol A levels may be caused by liver UDP-glucuronosyltransferase activity in rats. Biochem Biophys Res Commun 325:549–554
17. Völkel W, Colnot T, Csanády GA, Filser JG, Dekant W (2002) Metabolism and kinetics of bisphenol a in humans at low doses following oral administration. Chem Res Toxicol 15: 1281–1287
18. EFSA (2008) Toxicokinetics of bisphenol A: scientific opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food (AFC). EFSA J 759:1–10
19. Ginsberg G, Rice DC (2009) Does rapid metabolism ensure negligible risk from bisphenol A? Environ Health Perspect 117:1639–1643
20. Takahashi O, Oishi S (2000) Disposition of orally administered 2,2-Bis (4-hydroxyphenyl) propane (Bisphenol A) in pregnant rats and the placental transfer to fetuses. Environ Health Perspect 108:931–935
21. Watson CS, Bulayeva NN, Wozniak AL, Alyea RA (2007) Xenoestrogens are potent activators of nongenomic estrogenic responses. Steroids 72:124–134
22. Honma S, Suzuki A, Buchanan DL, Katsu Y, Watanabe H, Iguchi T (2002) Low dose effect of in utero exposure to bisphenol A and diethylstilbestrol on female mouse reproduction. Reprod Toxicol 16:117–122
23. Rubin BS, Murray MK, Damassa DA, King JC, Soto AM (2001) Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. Environ Health Perspect 109(7):675–680
24. Vandenberg LN, Maffini MV, Schaeberle CM, Ucci AA, Sonnenschein C, Rubin BS, Soto AM (2008) Perinatal exposure to the xenoestrogen bisphenol-A induces mammary intraductal hyperplasias in adult CD-1 mice. Reprod Toxicol 26:210–219
25. Long X, Steinmetz R, Ben-Jonathan N, Caperell-Grant A, Young PC, Nephew KP, Bigsby RM (2000) Strain differences in vaginal responses to the xenoestrogen bisphenol A. Environ Health Perspect 108:243–247
26. Diel P, Schmidt S, Vollmer G, Janning P, Upmeier A, Michna H, Bolt HM, Degen GH (2004) Comparative responses of three rat strains (DA/Han, Sprague-Dawley and Wistar) to treatment with environmental estrogens. Arch Toxicol 78:183–193
27. Lee HJ, Chattopadhyay S, Gong EY, Ahn RS, Lee K (2003) Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. Toxicol Sci 75:40–46
28. Zoeller RT, Bansal R, Parris C (2005) Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist in vitro, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. Endocrinology 146:607–612
29. Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS (2003) Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environ Health Perspect 111:994–1006
30. Talsness CE, Andrade AJ, Kuriyama SN, Taylor JA, vom Saal FS (2009) Components of plastic: experimental studies in animals and relevance for human health. Philos Trans R Soc Lond B Biol Sci 364:2079–2096

31. Imanishi S, Manabe N, Nishizawa H, Morita M, Sugimoto M, Iwahori M, Miyamoto H (2003) Effects of oral exposure of bisphenol A on mRNA expression of nuclear receptors in murine placentae assessed by DNA microarray. J Reprod Dev 49:329–336
32. Ryan BC, Hotchkiss AK, Crofton KM, Gray LE Jr (2010) In utero and lactational exposure to bisphenol A, in contrast to ethinyl estradiol, does not alter sexually dimorphic behavior, puberty, fertility, and anatomy of female LE rats. Toxicol Sci 114:133–148
33. Markey CM, Coombs MA, Sonnenschein C, Soto AM (2003) Mammalian development in a changing environment: exposure to endocrine disruptors reveals the developmental plasticity of steroid-hormone target organs. Evol Dev 5:67–75
34. Susiarjo M, Hassold TJ, Freeman E, Hunt PA (2007) Bisphenol A exposure in utero disrupts early oogenesis in the mouse. PLoS Genet 3:e5
35. Newbold RR, Jefferson WN, Padilla-Banks E (2007) Long-term adverse effects of neonatal exposure to bisphenol A on the murine female reproductive tract. Reprod Toxicol 24: 253–258
36. Nikaido Y, Yoshizawa K, Danbara N, Tsujita-Kyutoku M, Yuri T, Uehara N, Tsubura A (2004) Effects of maternal xenoestrogen exposure on development of the reproductive tract and mammary gland in female CD-1 mouse offspring. Reprod Toxicol 18:803–811
37. Hunt PA, Koehler KE, Susiarjo M, Hodges CA, Ilagan A, Voigt RC, Thomas S, Thomas BF, Hassold TJ (2003) Bisphenol A exposure causes meiotic aneuploidy in the female mouse. Curr Biol 13:546–553
38. Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR, Hardy MP (2004) Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology 145:592–603
39. Kawai K, Nozaki T, Nishikata H, Aou S, Takii M, Kubo C (2003) Aggressive behavior and serum testosterone concentration during the maturation process of male mice: the effects of fetal exposure to bisphenol A. Environ Health Perspect 111:175–178
40. Della Seta D, Minder I, Belloni V, Aloisi AM, Dessì-Fulgheri F, Farabollini F (2006) Pubertal exposure to estrogenic chemicals affects behavior in juvenile and adult male rats. Horm Behav 50:301–307
41. Kato H, Furuhashi T, Tanaka M, Katsu Y, Watanabe H, Ohta Y, Iguchi T (2006) Effects of bisphenol A given neonatally on reproductive functions of male rats. Reprod Toxicol 22:20–29
42. Ema M, Fujii S, Furukawa M, Kiguchi M, Ikka T, Harazono A (2001) Rat two-generation reproductive toxicity study of bisphenol A. Reprod Toxicol 15:505–523
43. Howdeshell KL, Furr J, Lambright CR, Wilson VS, Ryan BC, Gray LE Jr (2008) Gestational and lactational exposure to ethinyl estradiol, but not bisphenol A, decreases androgen-dependent reproductive organ weights and epididymal sperm abundance in the male long evans hooded rat. Toxicol Sci 102:371–382
44. Vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S, Welshons WV (1998) A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. Toxicol Ind Health 14:239–260
45. Markey CM, Luque EH, Munoz De Toro M, Sonnenschein C, Soto AM (2001) In utero exposure to bisphenol A alters the development and tissue organization of the mouse mammary gland. Biol Reprod 65:1215–1223
46. Wadia PR, Vandenberg LN, Schaeberle CM, Rubin BS, Sonnenschein C, Soto AM (2007) Perinatal bisphenol A exposure increases estrogen sensitivity of the mammary gland in diverse mouse strains. Environ Health Perspect 115:592–598
47. Lapensee EW, Tuttle TR, Fox SR, Ben-Jonathan N (2009) Bisphenol A at low nanomolar doses confers chemoresistance in estrogen receptor-alpha-positive and -negative breast cancer cells. Environ Health Perspect 117:175–180
48. Ramos JG, Varayoud J, Kass L, Rodríguez H, Costabel L, Muñoz-De-Toro M, Luque EH (2003) Bisphenol A induces both transient and permanent histofunctional alterations of the

hypothalamic-pituitary-gonadal axis in prenatally exposed male rats. Endocrinology 144: 3206–3215
49. Ho SM, Tang WY, Belmonte de Frausto J, Prins GS (2006) Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. Cancer Res 66:5624–5632
50. Wetherill YB, Petre CE, Monk KR, Puga A, Knudsen KE (2002) The xenoestrogen bisphenol A induces inappropriate androgen receptor activation and mitogenesis in prostatic adenocarcinoma cells. Mol Cancer Ther 1:515–524
51. Matthews J, Gustafsson JA (2003) Estrogen signaling: a subtle balance between ER alpha and ER beta. Mol Interv 3:281–292
52. Welshons WV, Nagel SC, vom Saal FS (2006) Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology 147:S56–S69
53. Uchida K, Suzuki A, Kobayashi Y, Buchanan D, Sato T, Watanabe H, Katsu Y, Suzuki J, Asaoka K, Mori C, Arizono K, Iguchi T (2002) Bisphenol-A administration during pregnancy results in fetal exposure in mice and monkeys. J Health Sci 48:579–582
54. Miksys SL, Tyndale RF (2002) Drug-metabolizing cytochrome P450s in the brain. J Psychiatry Neurosci 27:406–415
55. Pang T, Atefy R, Sheen V (2008) Malformations of cortical development. Neurologist 14:181–191
56. Nakamura K, Itoh K, Yaoi T, Fujiwara Y, Sugimoto T, Fushiki S (2006) Murine neocortical histogenesis is perturbed by prenatal exposure to low doses of Bisphenol A. J Neurosci Res 84:1197–1205
57. Nakamura K, Itoh K, Sugimoto T, Fushiki S (2007) Prenatal exposure to bisphenol A affects adult murine neocortical structure. Neurosci Lett 420:100–105
58. Garcia-Falgueras A, Pinos H, Collado P, Pasaro E, Fernandez R, Jordan CL, Segovia S, Guillamon A (2005) The role of the androgen receptor in CNS masculinisation. Brain Res 1035:13–23
59. Kubo K, Arai O, Omura M, Watanabe R, Ogata R, Aou S (2003) Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. Neurosci Res 45:345–356
60. Simerly RB (1989) Hormonal control of the development and regulation of tyrosine hydroxylase expression within a sexually dimorphic population of dopaminergic cells in the hypothalamus. Mol Brain Res 6:297–310
61. Rubin BS, Lenkowski JR, Schaeberle CM, Vandenberg LN, Ronsheim PM, Soto AM (2006) Evidence of altered brain sexual differentiation in mice exposed perinatally to low, environmentally relevant levels of bisphenol A. Endocrinology 147:3681–3691
62. Kubo K, Arai O, Ogata R, Omura M, Hori T, Aou S (2001) Exposure to bisphenol A during the fetal and suckling periods disrupts sexual differentiation of the locus coeruleus and of behavior in the rat. Neurosci Lett 304:73–76
63. Nagao T, Saito Y, Usumi K, Kuwagata M, Imai K (1999) Reproductive function in rats exposed neonatally to bisphenol A and estradiol benzoate. Reprod Toxicol 13:303–311
64. Ishido M, Masuo Y, Kunimoto M, Oka S, Morita M (2004) Bisphenol A causes hyperactivity in the rat concomitantly with impairment of tyrosine hydroxylase immunoreactivity. J Neurosci Res 76:423–433
65. Kawato S (2004) Endocrine disrupters as disrupters of brain function: a neurosteroid viewpoint. Environ Sci 11:1–14
66. MacLusky NJ, Hajszan T, Leranth C (2005) The environmental estrogen bisphenol A inhibits estradiol-induced hippocampal synaptogenesis. Environ Health Perspect 113:675–679
67. Zhou R, Zhang Z, Zhu Y, Chen L, Sokabe M, Chen L (2009) Deficits in development of synaptic plasticity in rat dorsal striatum following prenatal and neonatal exposure to low-dose bisphenol A. Neuroscience 159:161–171
68. Masuo Y, Ishido M, Morita M, Oka S (2004) Effects of neonatal treatment with 6-hydroxydopamine and endocrine disruptors on motor activity and gene expression in rats. Neural Plast 11:59–76

69. Van der Kooij MA, Glennon JC (2007) Animal models concerning the role of dopamine in attention-deficit hyperactivity disorder. Neurosci Biobehav Rev 31:597–618
70. Matsuda S, Saika S, Amano K, Shimizu E, Sajiki J (2010) Changes in brain monoamine levels in neonatal rats exposed to bisphenol A at low doses. Chemosphere 78:894–906
71. Martini M, Miceli D, Gotti S, Viglietti-Panzica C, Fissore E, Palanza P, Panzica G (2010) Effects of perinatal administration of bisphenol A on the neuronal nitric oxide synthase expressing system in the hypothalamus and limbic system of CD1 mice. J Neuroendocrinol 22:1004–1012
72. Ceccarelli I, Della Seta D, Fiorenzani P, Farabollini F, Aloisi AM (2007) Estrogenic chemicals at puberty change ERalpha in the hypothalamus of male and female rats. Neurotoxicol Teratol 29:108–115
73. Kawai K, Murakami S, Senba E, Yamanaka T, Fujiwara Y, Arimura C, Nozaki T, Takii M, Kubo C (2007) Changes in estrogen receptors alpha and beta expression in the brain of mice exposed prenatally to bisphenol A. Regul Toxicol Pharmacol 47:166–170
74. Nishizawa H, Manabe N, Morita M, Sugimoto M, Imanishi S, Miyamoto H (2003) Effects of in utero exposure to bisphenol A on expression of RARalpha and RXRalpha mRNAs in murine embryos. J Reprod Dev 49:539–545
75. Charlier TD (2009) Importance of steroid receptor coactivators in the modulation of steroid action on brain and behaviour. Psychoneuroendocrinology 34(Suppl 1):S20–S29
76. Xu X, Liu Y, Sadamatsu M, Tsutsumi S, Akaike M, Ushijima H, Kato N (2007) Perinatal bisphenol A affects the behavior and SRC-1 expression of male pups but does not influence on the thyroid hormone receptors and its responsive gene. Neurosci Res 58:149–155
77. Ulrich-Lai YM, Herman JP (2009) Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci 10:397–409
78. De Kloet ER, Karst H, Joëls M (2008) Corticosteroid hormones in the central stress response: quick-and – slow. Front Neuroendocrinol 29:268–272
79. Handa RJ, Burgess LH, Kerr JE, O'Keefe JA (1994) Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. Horm Behav 28:464–476
80. McEwen BS, Alves SE (1999) Estrogen actions in the central nervous system. Endocr Rev 20:279–307
81. Ahima RS, Garcia MM, Harlan RE (1992) Intracellular localization of corticosteroid receptors in brain: potential interactions with signal transduction pathways. Proc Soc Exp Biol Med 201:244–253
82. Patchev VK, Hayashi S, Orikasa C, Almeida OF (1999) Ontogeny of gender-specific responsiveness to stress and glucocorticoids in the rat and its determination by the neonatal gonadal steroid environment. Stress 3:41–54
83. Poimenova A, Markaki E, Rahiotis C, Kitraki E (2010) Corticosterone-regulated actions in the rat brain are affected by perinatal exposure to low dose of bisphenol A. Neuroscience 167:741–749
84. Sencar-Cupović I, Milković S (1976) The development of sex differences in the adrenal morphology and responsiveness in stress of rats from birth to the end of life. Mech Ageing Dev 5:1–9
85. Burgess LH, Handa RJ (1992) Chronic estrogen -induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. Endocrinology 131:1261–1269
86. Carey MP, Deterd CH, de Koning J, Helmerhorst F, de Kloet ER (1995) The influence of ovarian steroids on hypothalamic-pituitary-adrenal regulation in the female rat. J Endocrinol 144:311–321
87. Adriani W, Seta DD, Dessì-Fulgheri F, Farabollini F, Laviola G (2003) Altered profiles of spontaneous novelty seeking, impulsive behavior, and response to D-amphetamine in rats perinatally exposed to bisphenol A. Environ Health Perspect 111:395–401
88. Farabollini F, Porrini S, Dessì-Fulgherit F (1999) Perinatal exposure to the estrogenic pollutant bisphenol A affects behavior in male and female rats. Pharmacol Biochem Behav 64: 687–694

89. Fujimoto T, Kubo K, Aou S (2006) Prenatal exposure to bisphenol A impairs sexual differentiation of exploratory behavior and increases depression-like behavior in rats. Brain Res 1068:49–55
90. Gioiosa L, Fissore E, Ghirardelli G, Parmigiani S, Palanza P (2007) Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. Horm Behav 52:307–316
91. Laviola G, Gioiosa L, Adriani W, Palanza P (2005) D-amphetamine-related reinforcing effects are reduced in mice exposed prenatally to estrogenic endocrine disruptors. Brain Res Bull 65:235–240
92. Dessì-Fulgheri F, Porrini S, Farabollini F (2002) Effects of perinatal exposure to bisphenol A on play behavior of female and male juvenile rats. Environ Health Perspect 110(Suppl 3): 403–407
93. Porrini S, Belloni V, Della Seta D, Farabollini F, Giannelli G, Dessì-Fulgheri F (2005) Early exposure to a low dose of bisphenol A affects socio-sexual behavior of juvenile female rats. Brain Res Bull 65:261–266
94. Tian YH, Baek JH, Lee SY, Jang CG (2010) Prenatal and postnatal exposure to bisphenol a induces anxiolytic behaviors and cognitive deficits in mice. Synapse 64:432–439
95. Ryan BC, Vandenbergh JG (2006) Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice. Horm Behav 50:85–93
96. Farabollini F, Porrini S, Della Seta D, Bianchi F, Dessì-Fulgheri F (2002) Effects of perinatal exposure to bisphenol A on sociosexual behavior of female and male rats. Environ Health Perspect 110(Suppl 3):409–414
97. Della Seta D, Minder I, Dessì-Fulgheri F, Farabollini F (2005) Bisphenol-A exposure during pregnancy and lactation affects maternal behavior in rats. Brain Res Bull 65:255–260
98. Aloisi AM, Della Seta D, Rendo C, Ceccarelli I, Scaramuzzino A, Farabollini F (2002) Exposure to the estrogenic pollutant bisphenol A affects pain behavior induced by subcutaneous formalin injection in male and female rats. Brain Res 937:1–7
99. Cooke PS, Naaz A (2004) Role of estrogens in adipocyte development and function. Exp Biol Med (Maywood) 229:1127–1135
100. Nunez AA, Kannan K, Giesy JP, Fang J, Clemens LG (2001) Effects of bisphenol A on energy balance and accumulation in brown adipose tissue in rats. Chemosphere 42:917–922
101. Alonso-Magdalena P, Morimoto S, Ripoll C, Fuentes E, Nadal A (2006) The estrogenic effect of bisphenol A disrupts pancreatic beta-cell function in vivo and induces insulin resistance. Environ Health Perspect 114:106–112
102. Heindel JJ, vom Saal FS (2009) Role of nutrition and environmental endocrine disrupting chemicals during the perinatal period on the aetiology of obesity. Mol Cell Endocrinol 304:90–96
103. Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenbergh JG, vom Saal FS (1999) Exposure to bisphenol A advances puberty. Nature 401:763–764
104. Miyawaki J, Sakayama K, Kato H, Yamamoto H, Masuno H (2007) Perinatal and postnatal exposure to bisphenol a increases adipose tissue mass and serum cholesterol level in mice. J Atheroscler Thromb 14:245–252
105. Ryan KK, Haller AM, Sorrell JE, Woods SC, Jandacek RJ, Seeley RJ (2010) Perinatal exposure to bisphenol-a and the development of metabolic syndrome in CD-1 mice. Endocrinology 151:2603–2612
106. Dolinoy DC, Huang D, Jirtle RL (2007) Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. Proc Natl Acad Sci U S A 104:13056–13061
107. Takahashi O, Oishi S (2003) Testicular toxicity of dietarily or parenterally administered bisphenol A in rats and mice. Food Chem Toxicol 41:1035–1044

Part III
Bisphenol-A in Dental Polymers

Chapter 5
BPA and Dental Materials

Jill Lewis

5.1 Introduction and Historical Perspective

BPA has been a controversial component of dental materials since the first reports of its potential toxicities in the mid-1990s [1]. Concerns for the estrogenic and other toxicities of BPA from all environmental sources have escalated over the past decade or so to the point that federal regulatory agencies have begun to address the issue. An early survey was conducted by the Centers for Disease Control and Prevention (CDC) in 2003–2004 (the National Health and Nutrition Examination Survey or NHANES) [2] that found detectable levels of BPA in 93 % of the Americans tested. Because of that survey, the National Toxicology Program (NTP) component of the National Institute of Environmental Health Sciences (NIEHS) focused efforts to evaluate research on the potential adverse effects of BPA [3]. Their findings are expressed on a 5-point scale of concern for adverse effects that includes (from lowest concern level to highest) negligible, minimal, some, concern, and serious. They reported negligible or minimal concern for almost all categories. However, they expressed "some concern for adverse effects" for the effect of BPA on brain, behavior, and prostate gland of fetuses, infants, and children. The US Food and Drug Administration (FDA) issued a statement in 2010 stating that it shares the perspective of the NTP findings concerning the potential effects of BPA [4]. It followed up in July 2012 with a new Food Additive Regulation prohibiting the use of BPA in the production of baby bottles and sippy cups [5].

Thus, what has emerged so far from these federal reviews and surveys is that BPA is extremely ubiquitous in our environment and the most at-risk populations are the very young.

J. Lewis
College of Dental Medicine, Western University of Health Sciences,
Pomona, CA, USA
e-mail: lewisj@westernu.edu

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5.2 What Is the Current "Safe" Level of Exposure?

The Environmental Protection Agency (EPA) and European Food Safety Authority (EFSA) both have set the acceptable BPA exposure limits at <50 μg/kg body weight/day. This level was based on animal studies performed over 20 years ago that showed rather global adverse effects (decreased offspring, low birth weight, delayed puberty) using high doses of BPA treatment (50–500 μg/kg/day). Since then, more recent studies have shown that BPA most likely does not have a monotonic dose response but rather exhibits a biphasic dose response curve suggesting that adverse effects may occur at lower levels than previously thought or, at the very least, responses are unpredictable at low doses [6]. In fact, studies using doses in the 10 μg/kg/day range are reportedly causing changes in urinary and prostate development and early-onset puberty [7]. Therefore, the current exposure guidelines are somewhat controversial.

5.3 Where Can We Get BPA Exposure from Dental Materials?

Patient exposure to BPA from dental materials is presumed to be primarily through ingestion of released components into the gastrointestinal tract following placement of dental sealants or composite restorations. Although BPA is not itself a component of composite resins, BPA is the starting material for production of common composite monomer, BPA glycidyl dimethacrylate (Bis-GMA), and may persist at trace levels as a contaminant in those preparations. In addition, BPA is released via hydrolysis of another common monomer, BPA dimethacrylate (Bis-DMA), by salivary esterases [7, 8]. Bis-GMA is not susceptible to action of salivary esterases and therefore is unlikely to release BPA into the oral cavity via this route. In addition, exposure to oxygen on the surface of dental sealants inhibits polymerization of monomers and may account for most of the 20–45 % of unreacted monomer that can leach into the saliva. Several studies have shown that BPA can be detected in patient saliva for a short time at detectable levels [9–12]. Salivary BPA levels consistently returned to baseline within a few hours following placement of dental sealants, indicating that BPA exposure from dental materials is primarily an acute event. Some of these studies also monitored BPA levels in serum and urine following sealant placement. While BPA was not detected in serum, elevated BPA levels in urine following sealant placement persisted for up to 5 days [9–12]. Longer-term studies are not available and would be valuable to help determine the contribution of dental materials to chronic BPA exposure. Release of degradation products could occur as composite fillings wear, become more porous, and release unpolymerized monomers that may have been trapped when the restoration was placed and cured. As detection methods have become more sensitive, these studies now are more feasible.

Another largely overlooked potential source for BPA exposure is via uptake of aerosolized or volatile components by the lungs. Dental professionals are at highest risk for occupational exposure by this route, and little research has been done in this arena. However, a 2009 study in Germany reported that low levels of volatile methacrylates have been detected in operatories using solid phase microextraction (SPME) to collect air samples during filling treatment [13]. These investigators found levels of MMA, HEMA, EGDMA, and TEG-DMA in their samplings suggesting that this exposure route deserves further attention.

5.4 Is BPA from Dental Material Sources Significant When Compared to Overall Exposure Levels?

Unfortunately, even after years of research and dozens of studies, controversy over exposure levels and potential adverse effects of BPA from dental materials remains. Since BPA is so pervasive in our environment, attempts to tease out the contributions to overall dose from any single source are exceedingly difficult. In addition, there is no accepted standardization for methodologies that study this problem. As a result, large variabilities in detection limits of various techniques and even variation in the reported units of the results make direct comparisons of studies difficult. Controversies over the studies chosen to set regulatory guidelines also exist [14]. Some investigators have done extensive reviews of the literature to try to standardize the results for comparison as well as recommend guidelines for standardization in future studies [15]. Others have tried to provide some insight into the relative contribution of overall BPA dose from dental materials [16]. These studies sometimes are complicated by the lack of information provided by the manufacturer, whose formulations often are proprietary. However, even with all of these caveats in data interpretation, the contribution to overall dose provided by dental materials often is significant, especially in children, and therefore should not be ignored completely.

5.5 What Effects Have Been Reported from Dental Material-Derived BPA?

The toxic and estrogenic effects of BPA have been studied for many years and summarized elsewhere in this text. More recent papers focus on epigenetic changes, developmental issues, and brain/psychosocial issues. Concerns for BPA toxicities prompted the NIEHS to devote $30 million in funds from the American Reinvestment and Recovery Act toward the study of BPA. Many of these studies that focused on dental materials examined the effects of BPA exposure on children and development. Based on other in vitro and in vivo studies, metabolic homeostasis and

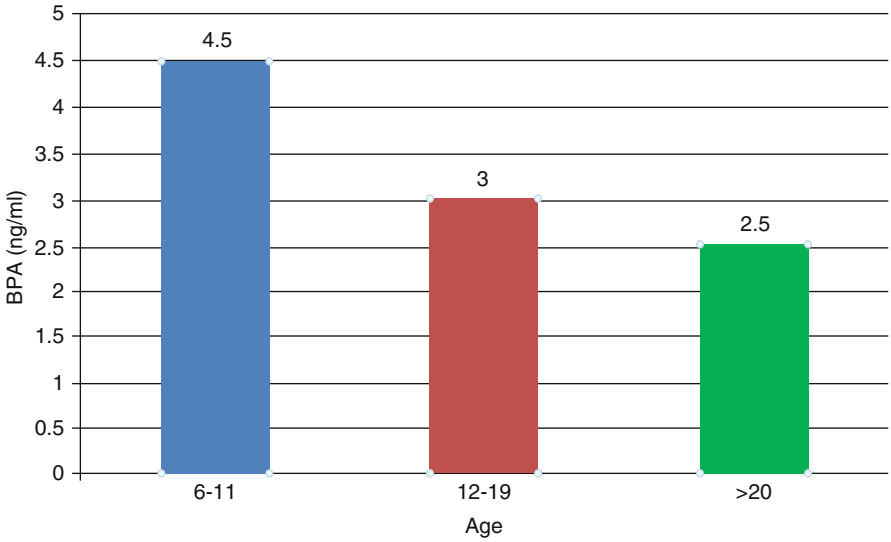

Fig. 5.1 Results of NHANES evaluation of BPA levels in urine

neuropsychological development were two areas of particular interest. Several studies using secondary analysis from the New England Children's Amalgam Trial conducted from 1997 to 2006 recently have been published. This database includes information on 534 children aged 6–10 with ≥2 posterior tooth caries who were randomized into amalgam or composite treatment groups. One such study addressed potential effects on physical growth and found no significant differences in growth rate, percent body fat, or BMI between the two groups. However, they did report some findings that suggest differences in the age of menarche in adolescent girls from the composite group that may warrant further investigation [17]. Other recent studies from this group have focused on neuropsychological development. Results from these studies have been equivocal, sometimes showing an association of composite resin with lower psychosocial scores and others showing trends toward lower executive functioning scores that did not reach statistical significance [18, 19]. This area of research likely will continue to be a topic of focus in the future.

5.6 What Is the Current Impact of BPA on the Dental Profession?

One of the most troubling findings of the NHANES survey was the clear and significant relationship between BPA levels and age. Children (6–11 years of age) had the highest levels of BPA, followed by adolescents, with adults (age >20 years) exhibiting the lowest levels of BPA (Fig. 5.1). No children under age 6 were included in the survey. Developmental effects of BPA exposure are likely to be most

deleterious in the prenatal and perinatal periods. Therefore, in terms of dental patients, pediatric patients and expectant/lactating mothers are most at risk. At the same time, efforts to increase the numbers of children receiving dental sealants have been successful, and the efficacy of dental sealants for reducing the incidence of pit and fissure caries and improving oral health in children is undeniable [20].

Recent articles have reviewed research reports on salivary BPA levels and provide a good perspective on the contribution of BPA exposure from dental materials. The findings of studies using current Bis-GMA-based composites and sealants showed that the acute BPA exposure levels associated with sealant placement were a minimum of 50,000–100,000 times lower than the daily recommended exposure limit for adults set by current EPA and NTP guidelines [21, 22]. Thus, exposure levels from dental materials alone are considered to be safe.

So what should be done? As with any biocompatibility issue, the decision to use these materials is a decision of risk to benefit ratio. The oral health benefits from these materials are well established [23–25]. Because the data on BPA levels and safety remains complex, it seems prudent to limit the exposure contribution from dental materials to these most vulnerable populations as much as possible. Some commonsense guidelines have been suggested in dealing with these issues [26]: Limit elective placement of dental sealant and composite resin restorations in pregnant women. When possible, choose materials that are BPA-free. In pregnant women and children, use precautionary application techniques when restorations or sealants are placed to limit BPA exposure. These techniques are aimed at removing the unpolymerized material on the surface and have been shown to return BPA levels to baseline, greatly reducing the acute, short-term BPA exposure associated with sealant placement [27, 28].

Because of the high profile of BPA safety in the press, many patients may express concerns about the use of these dental materials. The ADA recently published some guidelines to help the dental practitioner address these concerns [21].

BPA and Dental Materials: Addressing Patient Concerns

Here are some key points that can help you answer patient questions about BPA:

- According to manufacturers, BPA is not an added ingredient in dental composites or sealants currently on the market.
- The main ingredient in most commonly used composites and sealants is Bis-GMA, which has been shown to be stable within the mouth and does not decompose to BPA over time.
- Trace amounts of BPA present in raw Bis-GMA are a residue of its manufacturing process.
- Some products contain added Bis-DMA as a Bis-GMA viscosity modifier. Bis-DMA is known to decompose to BPA in the presence of salivary esterases (enzymes). However, many current dental resins severely limit or eliminate all Bis-DMA from their formulations.

- Although trace levels of BPA can be detected in dental products containing Bis-GMA, the potential exposure level is at least 100,000 times lower than current exposure limits.
- BPA exposure from dental materials likely lasts only a few hours after placement of a composite or sealant. Therefore, any BPA exposure is brief and transient.
- The preponderance of scientific data over the past 15 years indicates that the amount of BPA exposure from dental restoratives does not present a health hazard.

Compiled from Ref. [21], used with permission (pending)

5.7 What Can We Do in the Future?

BPA research would benefit from more standardized methodologies to study and report the effects of these compounds in the literature to better facilitate comparisons of the results. In addition, with newer and more sensitive techniques available, studies on longer-term/chronic release of these compounds from dental materials should be possible. Further assessment of volatile release of these compounds during dental procedures should be performed to better delineate potential risks to dental practitioners and staff.

Regulations concerning required product information could be modified to insure that the dental practitioner can make a better informed choice on which materials to use to help limit BPA exposure as much as possible. More extensive studies should be performed on other compounds released from these composite resin systems to assess their estrogenic/toxic effects. Manufacturers should continue to develop new BPA-free materials to offer as options. Of course, these new materials will need biocompatibility testing to insure that new risks are not being introduced.

References

1. Olea N, Pulgar R, Perez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A et al (1996) Estrogenicity of resin based composites and sealants used in dentistry. Environ Health Perspect 104: 298–305
2. Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL (2008) Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003–2004. Environ Health Perspect 116:39–44
3. National Toxicology Program (2008) NTP-CERHR monograph on the potential human reproductive and developmental effects of bisphenol A. NTP CERHR MON (22):v, vii–ix, 1–64 passim
4. US Food and Drug Administration (2010) Update on bisphenol A for use in food contact applications. www.fda.gov/NewsEvents/PublicHealthFocus/ucm197739. Accessed 20 Feb 2013

5. https://www.federalregister.gov/articles/2012/07/17/2012-17366/indirect-food-additives-polymers. Accessed 10 Mar 2013
6. Weiss B (2011) Endocrine disrupters as a threat to neurological function. J Neurol Sci 205:11–21
7. Arenholt-Bindslev D, Breinholt V, Preiss A, Schmalz G (1999) Time-related bisphenol-A content and estrogenic activity in saliva samples collected in relation to placement of fissure sealants. Clin Oral Investig 3(3):120–125
8. Schmalz G, Preiss A, Arenholt-Bindslev D (1999) Bisphenol-A content of resin monomers and related degradation products. Clin Oral Investig 3(3):114–119
9. Kingman A, Hyman J, Masten SA, Jayaram B, Smith C, Eichmiller F, Arnold MC, Wong PA, Schaeffer JM, Solanki S, Dunn WJ (2012) Bisphenol A and other compounds in human saliva and urine associated with the placement of composite resins. J Am Dent Assoc 143(12): 1292–1302
10. Fung EY, Ewoldsen NO, St Germain HA Jr et al (2000) Pharmacokinetics of bisphenol A released from a dental sealant. J Am Dent Assoc 131(1):51–58
11. Joskow R, Barr DB, Barr JR, Calafat AM, Needham LL, Rubin C (2006) Exposure to bisphenol A from bis-glycidyl dimethacrylate-based dental sealants. J Am Dent Assoc 137(3): 353–362
12. Sasaki N, Okuda K, Kato T et al (2005) Salivary bisphenol-A levels detected by ELISA after restoration with composite resin. J Mater Sci Mater Med 16(4):297–300
13. Marquardt W, Seiss M, Hickel R, Reichl FX (2009) Volatile methacrylates in dental practices. J Adhes Dent 11:101–107
14. Myers JP, vom Saal FS, Akingbemi BT, Arizono K, Belcher S, Colborn T, Chahoud I et al (2009) Why public health agencies cannot depend on good laboratory practices as a criterion for selecting data: the case of bisphenol A. Environ Health Perspect 117:309–315. Commentary
15. Van Landuyt KL, Nawrot T, Geebelen B, De Munck J, Snauwaert J, Yoshihara K, Scheers H, Godderis L, Hoet P, Van Meerbeek B (2011) How much do resin-based dental materials release? A meta-analytical approach. Dent Mater 27:723–747
16. von Goetz N, Wormuth M, Scheringer M, Hungerbühler K (2010) Bisphenol A: how the most relevant exposure sources contribute to total consumer exposure. Risk Anal 30(3):473–487
17. Maserejian NN, Hauser R, Tavares M, Trachtenberg FL, Shrader P, McKinlay S (2012) Dental composites and amalgam and physical development in children. J Dent Res 91(11): 1019–1025
18. Maserejian NM, Trachtenberg FL, Hauser R, McKinlay S, Shrader P, Bellinger DC (2012) Dental composite restorations and neuropsychological development in children: treatment level analysis from a randomized clinical trial. Neurotoxicology 33:1291–1297
19. Maserejian NN, Trachtenberg FL, Hauser R, McKinlay S, Shrader P, Tavares M, Bellinger DC (2012) Dental composite restorations and psychosocial function in children. Pediatrics 130:e328; originally published online July 16, 2012
20. Ahovuo-Saloranta A, Hiiri A, Nordblad A, Makela M, Worthington HV (2008) Pit and fissure sealants for preventing dental decay in the permanent teeth of children and adolescents. Cochrane Database Syst Rev 8(4), CD001830
21. Gruninger SE, Tiba A, Koziol N (2013) Update: bisphenol A in dental materials. ADA Prof Prod Rev 8(1):2–5
22. Rathee M, Malik P, Singh J (2012) Bisphenol A in dental sealants and its estrogen like effect. Indian J Endocrinol Metab 16(3):339–342
23. Truman BI, Gooch BF, Sulemana I et al (2002) Reviews of evidence on interventions to prevent dental caries, oral and pharyngeal cancers, and sports-related craniofacial injuries. Am J Prev Med 23(1 suppl):21–54
24. Beauchamp J, Caufield PW, Crall JJ, American Dental Association Council on Scientific Affairs et al (2008) Evidence-based clinical recommendations for the use of pit-and fissure sealants: a report of the American Dental Association Council on Scientific Affairs. J Am Dent Assoc 139(3):257–268

25. Griffin SO, Oong E, Kohn W et al (2008) The effectiveness of sealants in managing caries lesions. J Dent Res 87(2):169–174
26. Fleisch AF, Sheffield PE, Chinn C, Edelstein BL, Landrigan PJ (2010) Bisphenol A and related compounds in dental materials. Pediatrics 126(4):760–768
27. Rueggeberg FA, Dlugokinski M, Ergle JW (1999) Minimizing patients' exposure to uncured components in a dental sealant. J Am Dent Assoc 130(12):1751–1757
28. Komurcuoglu E, Olmez S, Vural N (2005) Evaluation of residual monomer elimination methods in three different fissure sealants in vitro. J Oral Rehabil 32(2):116–121

Chapter 6
Bisphenol A and Orthodontic Materials

Dimitrios Kloukos and Theodore Eliades

6.1 Introduction

Orthodontic polymers, and their applications, have been instrumental in introducing aesthetics, innovation, and practicality into the orthodontic specialty. Such materials constitute a large class of components including plastic elements and auxiliaries such as adhesives, polycarbonate brackets, and aligners.

The composition and configuration of these materials vary notably. Some of them are based on bisphenol A (BPA), which is used as a precursor of bisphenol A glycidyl dimethacrylate (Bis-GMA) or BPA dimethacrylate (Bis-DMA) during the production of many composite resins. The BPA structure assembles a bulk, stiff chain that offers low susceptibility to biodegradation as well as great rigidity and strength [1]. Although BPA is not used by itself as a raw material in composite resins, it is likely to be present as an impurity from the synthesis process [2, 3].

Since the 1960s, when the use of bisphenol A glycidyl dimethacrylate (Bis-GMA) began to flourish in dentistry, many studies have assessed the effects of dental composites on pulpal impairment [4] and their cytotoxic properties [5–7]. Nevertheless, the systemic health consequences of these chemicals, or their monomers, have not been thoroughly evaluated [8, 9].

Even though the patient may come in contact with significant amounts of unpolymerized monomers during the placement of composites, the release of uncured monomers after polymerization has been assumed to cause most of the unwanted effects [10]. In particular, BPA release from dental resins has attracted recent attention in the literature because of numerous experiments presenting adverse effects of BPA [2, 3, 11]. BPA has shown potential estrogenicity in a significant number of

D. Kloukos
Department of Orthodontics, University of Bern, Bern, Switzerland

T. Eliades (✉)
Department of Orthodontics and Paediatric Dentistry, Center of Dental Medicine, University of Zurich, Zurich, Switzerland
e-mail: theodore.eliades@zzm.uzh.ch

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studies [12] and is described as an endocrine disruptor chemical (EDC), owing to its ability to bind and activate the human estrogen receptor, however with a capacity of 1,000–5,000 times less than the endogenous 17-b estradiol [13].

Moreover, BPA can interact with other endocrine receptors, as thyroid hormone receptors and peroxisome proliferator-activated receptor gamma [14]. BPA was classified as a reproductive toxic substance of category 3, a significant risk factor for human fertility [15]. The concern is not isolated only at the molecular level. A recently published review indicated that exposure to dental composite resins based on BPA derivatives may even impact psychosocial health in children. Increased levels and duration of exposure (5 years) to composite indicated higher levels of anxiety, depression, social stress, and interpersonal-relation problems in children [16].

The European Food Safety Authority published an initial risk assessment on BPA in 2006, based on a tolerable daily intake (TDI) of 50 μg/kg body weight/day [17]. Several scientists arguably disputed the use of TDI for risk assessments on EDCs, suggesting that the effects of EDCs are observed at very low doses, non-monotonic dose–response curves, as well as on effects occurring from very specific windows of exposure [18].

The uncertainty in the dental literature was initially provoked by a study published by Olea et al. [19] who reported elevated salivary levels of BPA in patients with dental sealants. Since then, the extensive implementation of new polymers has triggered the investigation of their long-term effects at subtoxic levels. The investigation of the biological properties of materials has deviated from various routine cytotoxicity assays, for example, DNA synthesis or MTT proliferation assay [20].

The orthodontic concerns originate from the fact that monomers equivalent to those used for dental sealants are also used for the construction of orthodontic polymeric adhesives, plastic polycarbonate brackets, and other polycarbonate-made appliances that might also be sources of BPA. However, the actual effects induced by the possible release of BPA are difficult to be assessed because the mode of application of the materials, the growth stage and age of the individual, and potential other environmental factors might alter the extrapolation of results.

The purpose of this chapter is to briefly summarize the limited evidence available on the topic, which is associated with (a) polymeric orthodontic adhesive resins, (b) plastic polycarbonate brackets, and (c) polymeric aligners and their relationship to the possibility of bisphenol A (BPA) release and the subsequent phenomena of estrogenicity. A recently published systematic review was utilized as basis [21] for providing the evidence discussed in this chapter.

6.2 Orthodontic Adhesives

Bonding of brackets to enamel has been an enduring critical issue in orthodontics research.

Biomechanical principles necessitated a relatively inelastic interface that would transfer a load applied to the bracket directly to the tooth or to its root. Furthermore, the engagement of an archwire to the bracket should not exceed the bond strength between bracket and tooth [22]. Based on these requirements a considerable volume

of research was undertaken, aimed to find new materials and new perspectives in the province of orthodontic adhesives.

Orthodontic adhesive exposure to the oral environment involves three patterns:

(a) The bracket peripheral margins

The average thickness of these margins is quantified as between 150 and 250 μm [23]. The effect of aging and leaching of the material throughout these margins and under oral conditions might not be that potent.

(b) Bonded fixed lingual retainers

Fixed retainers have been used in orthodontics for many years. In both arches, mandibular and maxillary, they are routinely used for a prolonged period of time or even permanently. The use of these bonded retainers has been proven and well documented to be efficient in preventing relapse of the orthodontic treatment in most patients [24]. Two main types of fixed retainers are generally used: large-diameter wires, usually made of stainless steel, bonded only to the lingual surfaces of the canines, or small-diameter wires bonded to the lingual surfaces of all six anterior teeth.

For bonding both retainer types, specific orthodontic adhesives, mainly light-cured, are used. The adhesive in this case is used in a mode that involves full exposure of its surface to the oral environment. An extremely large surface-to-volume ratio of the applied adhesive is the main reason that increases its reactivity with the surrounding oral environment and facilitates aging and degradation, with volatile BPA release [25].

(c) Removal of the brackets and cleaning up of the enamel surface

This procedure follows the completion of orthodontic treatment [26]. This standard technique involves grinding and removal of the adhesive layer that existed between the bracket and the tooth with rotating instruments at low or high speed. This process discharges three main fragments in the aerosol that is created: polymer matrix pieces, filler degradation by-products, and particles descending from the wear of the bur [27].

The potentially hazardous nature of this aerosol is double. Potential concerns deal with the respiratory health of the patient and the treatment-providing team, since the produced dust is capable of reaching the alveoli of the lungs [27–29]. If we also take into consideration that the medical team is exposed on a long-term basis to this condition, we can easily assume the importance of these concerns.

Secondly, the particles attained from the presence of a double benzoyl ring in the released Bis-GMA monomers lead, as proclaimed, to the formation and release of bisphenol A (BPA) and hence to potentially disruptive hormonal action [30–33].

6.3 Orthodontic Adhesives: In Vitro BPA Release

Published studies are contradictory with respect to the qualitative and quantitative parameters of elution and BPA release from adhesives, probably because of the varying methodologies that have been employed. Eliades et al. were the first to

investigate the release of bisphenol A from orthodontic adhesives after their artificial accelerated aging with an in vitro study [34]. The results showed no indication of BPA identified for either type of adhesive across all time intervals used in the study, i.e., 1 day and 1, 3, and 5 weeks. Nevertheless the authors concluded that although the lack of BPA release was demonstrated in a particularly severe environment and under artificial accelerated aging conditions, these results should not be unquestionably extrapolated to real-life clinical conditions. The given reasons were three: Initially, the analysis of the adhesive extracts should be handled with caution, as far as it concerns the estrogenicity of polymers, because of the documented reactivity of BPA at very low levels [35]. In addition, the detection threshold level of the analytical apparatus used could be well above the potential BPA levels in the analyzed samples. Finally, intraoral aging, which is rather inconsistent with the extraoral reproductive aging, involves complex mechanical and chemical aging with the action of human enzymes, such as esterases, that induce degradation [36].

Similar protocol and techniques for assessing BPA release with the previous research were also used in a recent in vitro study of Sunitha et al. [37]. The scope of this study was to assess the BPA released from an orthodontic adhesive by varying the light cure tip distance and correlate it with the degree of conversion (DC). The degree of conversion of a resin composite material is the range of transformation of carbon double bonds (C═C) that exist in the monomer into carbon single bonds (C–C) to form polymers during the polymerization process. This has been found to significantly affect the physical [38, 39], mechanical [40–42], and biological [43] properties of dental composites.

The outcomes of the study displayed that increase in light cure tip distance from the adhesive caused a decrease in the degree of conversion of the substance which, in turn, led to a greater BPA release.

The release of bisphenol A from an orthodontic adhesive used to bond lingual fixed retainers on the surface of teeth was also studied recently from Eliades et al. [25]. Eighteen recently extracted teeth, divided into three groups of six teeth each, were used for this study. A light-cured adhesive was bonded to a twist flex wire adjusted to the lingual surface of the teeth. Then the arches were immersed in double-distilled water for 10, 20, and 30 days. Thereafter, the concentration of BPA in the three eluents was investigated with gas chromatography–mass spectroscopy. The results certified measurable amounts of BPA that were identified for all groups, with the highest found in the immersion media of the 30 days groups: 2.9 mg/L. The control group, which consisted of teeth maintained in immersion media, showed BPA in the mean of 0.16 mg/L.

6.4 Orthodontic Adhesives: In Vitro Estrogenicity

The actual contribution of the above amounts of BPA to adolescents and adults remains indefinite, and it is not likely that it would have a direct effect, considering the age of the average orthodontic patient in the retention phase of the treatment,

which may be well above 14 years of age. At such developmental stages, the action of BPA might not have the distinct effects reported for utero or early stages of life.

On the other hand, infants and children, examined on a pound-for-pound basis, have higher relative intakes of many widely detected environmental chemicals because they eat, drink, and breathe more than adults [44].

A recent statement of the US National Toxicology Program concluded that, along with high doses, BPA may show a diversity of effects at much lower ones [12]. A close example is that of phthalate esters, for instance, octaphenol, a substance added to plastics to make them more flexible, durable, and transparent. These plasticizers are capable of altering the uptake of dopamine by hypothalamic cells, at levels as low as 10 parts per trillion [45].

Therefore, there is unfortunately a large window of uncertainty on BPA potential estrogenicity, even if a precise and reliable quantitative estimation is attained. Moreover, there are about 20 different formations of bisphenol, and some of them share estrogenic action with BPA, such as Bis-DMA [30]. Therefore, the direct analysis of the estrogenic action of, artificial or not, aged adhesive eluents may be the method of choice for the inquiry about the potential estrogenic action of orthodontic polymers.

Appraisal of estrogenicity of orthodontic adhesive resins with in vitro studies has started to blossom mainly in the last 10 years. Eliades et al. assessed the estrogenic action of a chemically cured and a light-cured orthodontic adhesive resin [46]. The adhesives were bonded to 40 stainless steel brackets divided into two equal groups. The clinical handling of materials was reliably simulated. In total, three representative series of samples were prepared for each adhesive and bracket group. After immersion of the specimens in normal saline, samples of eluent were discharged from each group at 1 day and 1 week following incubation. The probable estrogenicity was measured by the effect of the eluents on the proliferation of cells. Estrogen-responsive MCF-7 breast cancer cells and estrogen-insensitive MB-231 human breast adenocarcinoma cells were used as active group and as control, respectively. The data from both cell lines indicated that no estrogenic activity was detected in the eluents from the resins tested.

Gioka et al. considered that whereas bulk, unimpaired orthodontic adhesive samples, used for the previous research, had not demonstrated estrogenic action, the biological features of their small-scale particles had not been assessed. One of the purposes of her study was to evaluate the estrogenicity of orthodontic adhesive particulates assembled by simulated debonding [26]. A chemically cured and a light-cured adhesive were included in the study. Specimens were prepared by simulating clinical bonding procedures. The adhesives prepared with this method were grounded in glass chambers with a high-speed handpiece. The collected amounts of the ground adhesives were immersed in saline for 1 month at 37 °C, replicating body temperature. Estrogenicity was assessed with estrogen-responsive cell line derived from human breast adenocarcinoma (MCF-7). Estradiol and bisphenol A as positive and saline as negative controls were also used. The proliferation rate of MCF-7 cells was clearly elevated, 160 and 128 %, compared to control for both chemically cured and light-cured adhesives, respectively. Both adhesives

demonstrated therefore an estrogenic behavior. The possibility of irrelevant effects to estrogenicity interfering with proliferation was excluded as the estrogen-insensitive cell line MB-231 did not show any discrepancy in the experimental groups.

6.5 Orthodontic Adhesives: In Vivo BPA Release and Estrogenicity

The estrogenicity in eluents of tested adhesives with in vitro studies is usually measured by an established assay, for example, as seen before through the estimation of the proliferation of the estrogen-responsive cell line. These cells are known to express estrogen receptor-α (ERα), which is of paramount importance for the proliferative effect of estrogens. The typical method for measuring estrogenic action in vivo is the increase of mitotic indices of rodent epithelia [47]. This strategy may have, however, limited relevance to humans. That is because estrogenicity is diminished from rat hepatic microsomes in contrast with human liver [48]. Receptors for estrogens have been additionally identified in human gingival tissues, supplying evidence that this tissue can be a target organ for human sex hormones [49]. There are also indications of a sex hormone influence on the oral human epithelium reacting to chemical challenge [50]. It has been reported that the oral mucosa of premenopausal woman was appreciably more sensitive to sodium lauryl sulfate found in toothpastes than that of postmenopausal woman.

Up-to-date information about in vivo assessment of BPA released from orthodontic adhesives in humans has to do mainly with a recent study of Kang et al. [51]. This study assessed the changes in bisphenol A level in the saliva and urine before and after placing a lingual bonded retainer on the lower dentition of 22 volunteers. The samples were obtained immediately before placement of the retainer and 30 min, 1 day, 1 week, and 1 month after placement. The only significant level of BPA was detected in the saliva collected immediately after lingual retainer placement. Age and gender of the volunteers did not seem to affect the BPA level in the saliva or urine. The salivary BPA level (mean 5.04 ng/mL, levels ranging from 0.85 to 20.88 ng/mL) observed in the immediately collected sample was, as implied by the authors, far lower than the reference daily intake dose. Nevertheless, they concluded that, since some evidence of “low-dose effect” exists, clinicians should reduce the uncured layer of the material, using pumice surface prophylaxis of the adhesive.

The US human exposure limit and European Food Safety Authority have set the tolerable daily intake level of BPA to 50 μg/kg/day [17, 52]. The BPA released level from the lingual bonded retainer in this study was far below these doses. However and as already mentioned before, there is some controversy regarding the safe level of BPA exposure. Vom Saal and Hughes [53] proposed the need for a new risk assessment for BPA. They based this proposal on more than 100 in vivo and in vitro study results indicating that a BPA level far below 50 μg can cause modifications in the biological activities of cultured cells.

Finally, it should be also outlined that there are plenty reports of allergic dermatitis in dental personnel [54–58], which can reasonably be attributed to released monomers

from dental composite resins and, in our case, orthodontic adhesives. A smaller number of case reports of allergic responses in patients, which appear to be linked with the monomers, also exist. The last of these reports [59] described two cases of allergic contact dermatitis to bisphenol A glycidyl dimethacrylate (Bis-GMA) during the application of orthodontic fixed appliances. The authors concluded that these cases highlighted the importance for clinicians of two matters. Firstly, the importance of documenting which bonding agent the clinicians use rather than just recording "bonding upper and lower" and secondly, the conflict to the popular belief that dental adhesives are not eventually all the same, i.e., some have Bis-GMA, others do not.

6.6 Polycarbonate Brackets: In Vitro BPA Release

One of the first to describe BPA release from orthodontic polycarbonate brackets was Suzuki et al. [60]. The materials used in this in vitro experiment were, among others, four different types of polycarbonate orthodontic brackets. Analysis of total and released amounts of BPA resulted in the conclusion that during the synthesis of polycarbonates, nonreacted BPA probably remains inside the materials and is released when they are immersed in water or organic solvents. As for polycarbonates, the thermal conditions during the inclusion of their fillers and fabrication of restorations lead to polymer decomposition and BPA production.

This study was followed by another one from Watanabe et al. [61], who investigated the change in the bisphenol A content in a polycarbonate orthodontic bracket and its leaching characteristics during incubation in water. Polycarbonate brackets were placed in water at 37 and 60 °C. The BPA content in the bracket and the amount of BPA released into the water were analyzed at different time intervals. The BPA content increased in the water with time and was 3.8-fold after 12 months at 37 °C and 12.4-fold after 14 weeks at 60 °C compared with the virgin value. The rate of BPA release also increased with time.

The same team validated the previous findings with their second study [62]. The purpose was to investigate long-term degradation of polycarbonates and the formation of BPA in vivo and in vitro.

The degradation of polycarbonate brackets placed in the oral cavity for up to 40 months was examined regarding surface morphology, BPA content, molecular weight, and glass filler content.

The release of BPA from polycarbonate used in orthodontic brackets, temporary crowns, and denture base resins was examined by immersing them in water at 37 °C for up to 34 months. This study was principally conducted in vitro, but an in vivo attitude was also implied from the brackets retrieved from three patients' oral cavities. The results showed linear relationship for the cumulated amount of BPA eluted into water against time for bracket, denture plate, and temporary crown. BPA eluate increased linearly with time during 12–34 months. The elution of BPA was faster for the polycarbonate bracket. The formation and the release of larger amount of BPA found in the bracket were correlated to larger amount of water absorption in the bracket (2.69 %) compared to that in denture plate and temporary crown (0.07 %).

6.7 Polycarbonate Brackets: In Vivo BPA Release and Estrogenicity

The in vivo BPA release and in vivo estrogenicity from orthodontic polycarbonates are only suggested as logical consequence from the two in vitro studies of Watanabe et al. [61, 62] that were mentioned previously. Specifically, the first one specified the BPA content in the polycarbonate brackets retrieved from patients and attempted to clarify whether the BPA content might change in the oral cavity. It was found that the BPA content in five samples was 56–102 μg/g after 5–15 months. The BPA content was not necessarily correlated with the time the brackets stayed in the oral cavity.

The findings suggested that polycarbonate would degrade in the oral cavity to produce BPA. Based on the in vitro findings, the amount of BPA released in the oral cavity during 5–15 months could be estimated to be a maximum of 3.8 μg/g. This estimation was found to be reasonable because the BPA contents in vivo (56–102 μg/g) were lower than that in vitro (132 μg/g), and the BPA release should be proportional to the BPA content.

The second study suggested that BPA was released from the bracket in the oral cavity more than expected from the in vitro data. However, it was difficult to estimate the amount of BPA released.

The in vitro data obtained in water at 37 °C were as follows: the BPA content in the bracket and the BPA release were 132 and 3.8 μg/g after 12 months and 472 and 37.4 μg/g after 34 months, respectively. Therefore, it was expected that the BPA content would be 132–472 μg/g during 12–34 months. However, the BPA content in vivo was 39–125 μg/g during 18–30 months. Therefore, these results suggested that more amount of BPA was released in the oral cavity compared to that expected from the in vitro data.

Nevertheless, the researchers declared that while in vitro specimens were placed under a static condition in water, the brackets in vivo were, as well understood, exposed to complicated and dynamic conditions. While in oral cavity, toothbrushing, mechanical stresses, thermal alterations, and intake of heterogeneous foods and drinks may all have influenced the degradation of polycarbonates and the release of BPA from the brackets.

Therefore, BPA content released in the oral cavity will not always be correlated to the degradation of polycarbonates, since BPA content is the result of the balance of BPA formed and BPA released in the oral cavity, even if molecular weight decrease is correlated with the degradation of polycarbonate molecules.

6.8 Aligners

The development of clear polymeric aligners as a potential substitutional option instead of conventional brackets and archwires is already a fact in modern orthodontics [63, 64]. Patients are typically required to wear the set of aligners for a

minimum of 2 weeks, for 22 h per day, to achieve progressive tooth movement [64]. Although some controversy exists over the efficiency and limitations of this method, polymeric aligners have become an integral part of the daily orthodontic practice.

The fundamental constituent polymeric module of Invisalign aligners is polyurethane. Polyurethane is not an inanimate or inactive material and is affected by moisture, heat alterations, and sustained contact with enzymes that usually exist in the oral cavity [65, 66]. Eliades et al. assessed the cytotoxicity and estrogenicity of Invisalign appliances (Align Technology, Santa Clara, Calif) [67]. The results failed to demonstrate measurable biologic effects from aligners. Two reasons were thought from the authors to might have contributed to this effect: the short time frame of the study model, although it was longer than in actual clinical conditions, and the stability of the aligners as a material, which are basically, as described before, polyurethane-derived products [68].

6.9 Conclusions

The variety of setups did not allow quantitative synthesis of individual study findings. However, the release of BPA is a well-demonstrated phenomenon in oral conditions, which requires special clinical handling and further research. Despite the lack of consistency in methodological approaches, the qualitative analysis of the studies revealed that:

1. High level of BPA was detected in the saliva collected immediately after lingual bonded retainer placement.
2. Increase in light cure tip distance from the adhesive caused a decrease in the DC of the substance which, in turn, led to a greater BPA release.
3. Direct exposure of the adhesive to the oral fluids appears to play an important role in BPA release. Thus, adhesives used to bond lingual retainers leached more components in contrast to adhesives used to bond brackets (exposure through peripheral margins of the bracket).
4. Polycarbonate was found to show evidence of degradation in both in vitro and in vivo conditions and, under specific conditions, released BPA.

Clinical Recommendations

1. It is recommended to keep the light cure tip as close to the adhesive as clinically possible.
2. The use of pumice prophylaxis after bonding may reduce the potential for BPA release.
3. The use of indirect irradiation (around the bracket edges) instead of direct irradiation (through the bracket) is recommended.
4. Mouth rinsing during the first hour after bracket or retainer bonding may prevent the exposure of patients to the potential hazard of leaching monomers.

Future Research Recommendations

1. Large-scale in vivo studies, focusing on the effects of BPA released in saliva or blood of patients after placement of brackets or lingual retainers on developmental and reproductive toxicity.

Recommendations for Standardization Across Studies

1. It is recommended to express the quantitative data of release in standardized units. When the release is expressed per surface area or volume, the data can be linked to teeth or oral conditions.
2. It can be that a compound is released, even if it is not detected, if the concentration is below the detection threshold; therefore, limits of detection of eluates should be always mentioned.
3. The use of polymer-based materials, such as plastic instruments, plastic containers, or disposable gloves, is discouraged, as they may leach components themselves and they can cause contamination, leading to false-positive results.
4. When human saliva is used as incubation medium, it should originate from volunteers without resin restorations and with baseline check for BPA.
5. If long-term release is to be assessed, refreshing the elution medium at predetermined time periods is recommended. This way the solution is not saturated by leached compounds.
6. A constant temperature of 37 °C is preferable.

References

1. Artham T, Doble M (2008) Biodegradation of aliphatic and aromatic polycarbonates. Macromol Biosci 8:14–24
2. Fleisch AF, Sheffield PE, Chinn C, Edelstein BL, Landrigan PJ (2010) Bisphenol A and related compounds in dental materials. Pediatrics 126:760–768
3. Van Landuyt KL, Nawrot T, Geebelen B, De Munck J, Snauwaert J, Yoshihara K et al (2011) How much do resin- based dental materials release? A meta-analytical approach. Dent Mater 27:723–747
4. Ranks CT, Craig RG, Diehi ML, Pashley DH (1988) Cytotoxicity in dental composites and other materials in a new in vitro device. J Oral Pathol 17:396–403
5. Hensten-Pettersen A, Helgeland K (1981) Sensitivity of different human cell lines in the biologic evaluation of dental resin-based restorative materials. Scand J Dent Res 89:102–107
6. Terakado M, Yamazaki M, Tsujimoto Y, Kawashima T, Nagashima K, Ogawa J, Fujita Y, Sugiya H, Sakai T, Furuyama S (1984) Lipid peroxidation as a possible cause of benzoyl peroxide toxicity in rabbit dental pulp a microsomal lipid peroxidation in vitro. J Dent Res 63:901–905
7. Hanks CT, Strawn SE, Wataha JC, Craig RG (1991) Cytotoxic effects of resin components on cultured mammalian fibroblasts. J Dent Res 70:1450–1455
8. Bourne LB, Milner FJM, Alberman KB (1959) Health problems of epoxy resins and amine curing agents. Br J Ind Med 16:81–97
9. Morrissey RE, George JD, Price CJ, Tyl RW, Marr MC, Kimmel CA (1987) The developmental toxicity of bisphenol A in rats and in mice. Fundam Appl Toxicol 8:571–582
10. Mohsen NM, Craig RG, Hanks CT (1998) Cytotoxicity of urethane dimethacrylate composites before and after aging and leaching. J Biomed Mater Res 39:252–260

11. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV (2007) Human exposure to bisphenol A (BPA). Reprod Toxicol 24:139–177
12. Chapin RE, Adams J, Boekelheide K, Gray LE Jr, Hayward SW, Lees PS et al (2008) NTP-CERHR Expert Panel report on the reproductive and developmental toxicity of bisphenol A. Birth Defects Res B 83:157–395
13. Roy JR, Chakraborty S, Chakraborty TR (2009) Estrogen-like endocrine disrupting chemicals affecting puberty in humans – a review. Med Sci Monit 15:137–145
14. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM et al (2009) Endocrine-disrupting chemicals: an endocrine society scientific statement. Endocr Rev 30:293–342
15. Institute of National Health and Medical Research (INSERM) (2010) Bisphenol A: effects on reproduction. Preliminary report. INSERM, Paris
16. Maserejian NN, Trachtenberg FL, Hauser R, McKinlay S, Shrader P, Tavares M, Bellinger DC (2012) Dental composite restorations and psychosocial function in children. Pediatrics 130:328–338
17. European Food Safety Authority (EFSA) (2006) Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food on a request from the commission related to 2,2-bis(4-hydroxyphenyl) propane (Bisphenol A). Question number EFSA-Q-2005-100. Eur Food Saf Auth J 428:1–75
18. French Agency for Food, Environmental and Occupational Health & Safety (ANSES) (2010) Opinion of 29 January 2010 of the French Agency for Food Safety on the critical analysis of the results of a toxicity study on the development of the nervous system as well as other recently published data on the toxic effects of bisphenol A. ANSES, Paris
19. Olea N, Pulgar R, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V et al (1996) Estrogenicity of resin-based composites and sealant used in dentistry. Environ Health Perspect 104:298–305
20. Eliades T (2007) Orthodontic materials research and applications: Part 2. Current status and projected future developments in materials and biocompatibility. Am J Orthod Dentofacial Orthop 131:253–262
21. Kloukos D, Pandis N, Eliades T (2013) Bisphenol-A and residual monomer leaching from orthodontic adhesive resins and polycarbonate brackets: a systematic review. Am J Orthod Dentofacial Orthop 143(4):S104-12.e1-2
22. Brantley WA, Eliades T (2001) Orthodontic materials: scientific and clinical aspects, 1st edn. Thieme, Stuttgart
23. Eliades T, Viazis AD, Eliades G (1991) Bonding of ceramic brackets to enamel: morphologic and structural considerations. Am J Orthod Dentofacial Orthop 99:369–375
24. Renkema AM, Al-Assad S, Bronkhorst EM, Weindel S, Katsaros C, Lisson JA (2008) Effectiveness of lingual retainers bonded to the canines in preventing mandibular incisor relapse. Am J Orthod Dentofacial Orthop 134:1–8
25. Eliades T, Voutsa D, Sifakakis I, Makou M, Katsaros C (2011) Release of bisphenol-A from a light-cured adhesive bonded to lingual fixed retainers. Am J Orthod Dentofacial Orthop 139:192–195
26. Gioka C, Eliades T, Zinelis S, Pratsinis H, Athanasiou AE, Eliades G et al (2009) Characterization and in vitro estrogenicity of orthodontic adhesive particulates produced by simulated debonding. Dent Mater 25:376–382
27. Almeida MA (2006) Analysis of aerodynamically respirable dust generated from quartz containing orthodontic and dental composites utilizing air rotary abrasion. MS thesis, Oregon Health & Science University, Portland
28. Ireland AJ, Moreno T, Price R (2003) Airborne particles produced during enamel cleanup after removal of orthodontic appliances. Am J Orthod Dentofacial Orthop 124:683–686
29. Day CJ, Price R, Sandy JR, Ireland AJ (2008) Inhalation of aerosols produced during the removal of fixed orthodontic appliances: a comparison of 4 enamel cleanup methods. Am J Orthod Dentofacial Orthop 133:11–17
30. Tarumi H, Imazato S, Narimatsu M, Matsuo M, Ebisu S (2000) Estrogenicity of fissure sealants and adhesive resins determined by reporter gene assay. J Dent Res 79:1838–1843

31. Schmalz G, Preiss A, Arenholt-Bindslev D (1999) Bisphenol-A content of resin monomers and related degradation products. Clin Oral Investig 3:114–119
32. Pulgar R, Olea-Serrano MF, Novillo-Fertrell A, Rivas A, Pazos P, Pedraza V et al (2000) Determination of bisphenol A and related aromatic compounds released from bis-GMA-based composites and sealants by high performance liquid chromatography. Environ Health Perspect 108:21–27
33. Atkinson JC, Diamond F, Eichmiller F, Selwitz R, Jones G (2002) Stability of bisphenol A, triethylene-glycol dimethacrylate, and bisphenol A dimethacrylate in whole saliva. Dent Mater 18:128–135
34. Eliades T, Hiskia A, Eliades G, Athanasiou AE (2007) Assessment of bisphenol-A release from orthodontic adhesives. Am J Orthod Dentofacial Orthop 131:72–75
35. Timms BG, Howdeshell KL, Barton L, Bradley S, Richter CA, vom Saal FS (2005) Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra. Proc Natl Acad Sci U S A 102:7014–7019
36. Lee YK, Lim BS, Powers JM (2004) Color changes of dental resin composites by a salivary enzyme. J Biomed Mater Res B Appl Biomater 70:66–72
37. Sunitha C, Kailasam V, Padmanabhan S, Chitharanjan AB (2011) Bisphenol A release from an orthodontic adhesive and its correlation with the degree of conversion on varying light-curing tip distances. Am J Orthod Dentofacial Orthop 140:239–244
38. Eliades GC, Vougiouklakis GJ, Caputo AA (1987) Degree of double bond conversion in light-cured composites. Dent Mater 3:19–25
39. Ferracane JL, Greener EH (1986) The effect of resin formulation on the degree of conversion and mechanical properties of dental restorative resins. J Biomed Mater Res 20:121–131
40. Ruyter IE (1985) Monomer systems and polymerization. In: Vanherle G, Smith DC (eds) Posterior composite resin dental restorative materials. Szulc Publishing, Amsterdam, pp 109–135
41. Ferracane JL, Moser JB, Greener EH (1985) Ultraviolet light- induced yellowing of dental restorative resins. J Prosthet Dent 54:483–487
42. Söderholm K-J, Zigan M, Ragan M, Fischlschweiger W, Bergman M (1984) Hydrolytic degradation of dental composites. J Dent Res 63:1248–1254
43. Rathbun MA, Craig RG, Hanks CT, Filisko FE (1991) Cytotoxicity of a Bis-GMA dental composite before and after leaching in organic solvents. J Biomed Mater Res 25:443–457
44. Miyamoto K, Kotake M (2006) Estimation of daily bisphenol A intake of Japanese individuals with emphasis on uncertainty and variability. Environ Sci 13:15–29
45. Christian M, Gillies G (1999) Developing hypothalamic dopaminergic neurones as potential targets for environmental estrogens. J Endocrinol 160:R1–R6
46. Eliades T, Gioni V, Kletsas D, Athanasiou A, Eliades G (2007) Oestrogenicity of orthodontic adhesive resins. Eur J Orthod 29:404–407
47. Al-Hiyasat AS, Darmani H, Elbetieha AM (2004) Leached components from dental composites and their effects on fertility of female mice. Eur J Oral Sci 112:267–272
48. Elsby R, Maggs JL, Ashby J, Park BK (2001) Comparison of the modulatory effects of human and rat liver microsomal metabolism on the estrogenicity of bisphenol A: implications for extrapolation to humans. J Pharmacol Exp Ther 297:103–113
49. Vittek J, Hernandez MR, Wenk EJ, Rappaport SC, Southren AL (1982) Specific estrogen receptors in human gingiva. J Clin Endocrinol Metab 54:608–612
50. Herlofson BB, Barkvoll P (1996) Oral mucosal desquamation of pre- and post-menopausal women. A comparison of response to sodium lauryl sulphate in toothpastes. J Clin Periodontol 23:567–571
51. Kang YG, Kim JY, Kim J, Won PJ, Nam JH (2011) Release of bisphenol A from resin composite used to bond orthodontic lingual retainers. Am J Orthod Dentofacial Orthop 140:779–789
52. Environment Protection Agency (EPA) (1988) Oral RfD assessment: bisphenol A. Integrated Risk Information System

53. vom Saal FS, Hughes C (2005) An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. Environ Health Perspect 113:926–933
54. Kanerva L, Estlander T, Jolanki R (1989) Allergic contact dermatitis from dental composite resins due to aromatic epoxy acrylates and aliphatic acrylates. Contact Dermatitis 20:201–211
55. Kanerva L, Turjanmaa K, Estlander T, Jolanki R (1991) Occupational allergic contact dermatitis caused by 2- hydroxyethyl methacrylate (2-HEMA) in a new dentin adhesive. Am J Cont Dermatol 2:24–30
56. Kanerva L, Henriks-Eckerman M-L, Estlander T, Jolanki R, Tarvainen K (1994) Occupational allergic contact dermatitis and composition of acrylates in dental bonding systems. J Eur Acad Dermatol Venereol 3:157–169
57. Munksgaard EC (1999) Toxicology versus allergy in restorative dentistry. Adv Dent Res 6:17–21
58. Slavin RG, Ducomb DF (1999) Allergic contact dermatitis. Hosp Pract 30:39–51
59. Connolly M, Shaw L, Hutchinson I, Ireland AJ, Dunnill M, Sansom J (2006) Allergic contact dermatitis from bisphenol-A- glycidyldimethacrylate during application of orthodontic fixed appliance. Contact Dermatitis 55:367–368
60. Suzuki K, Ishikawa K, Sugiyama K, Furuta H, Nishimura F (2000) Content and release of bisphenol A from polycarbonate dental products. Dent Mater J 19:389–395
61. Watanabe M, Hase T, Imai Y (2001) Change in the bisphenol A content in a polycarbonate orthodontic bracket and its leaching characteristics in water. Dent Mater J 20:353–358
62. Watanabe M (2004) Degradation and formation of bisphenol A in polycarbonate used in dentistry. J Med Dent Sci 51:1–6
63. Align Technology, Inc. (2002) The Invisalign reference guide. Santa Clara, p 8
64. Align Technology, Inc. (2003) Material safety data sheet. MSDS Aligner EX203040. Customer support, 11 Aug 2003
65. Eliades T, Eliades G, Watts DC (1999) Structural conformation of in vitro and in vivo aged orthodontic elastomeric modules. Eur J Orthod 21:649–658
66. Huget EF, Patrick KS, Nunez LJ (1990) Observations on the elastic behavior of a synthetic orthodontic elastomer. J Dent Res 69:496–501
67. Eliades T, Pratsinis H, Athanasiou AE, Eliades G, Kletsas D (2009) Cytotoxicity and estrogenicity of Invisalign appliances. Am J Orthod Dentofacial Orthop 136:100–103
68. Eliades T, Eliades G, Silikas N, Watts DC (2005) In vitro degradation of polyurethane orthodontic elastomeric chains. J Oral Rehabil 32:72–77

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