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## Estrogenic activity of biological samples as a biomarker

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1 **ESTROGENIC ACTIVITY OF BIOLOGICAL SAMPLES AS A BIOMARKER**

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26 **Abbreviations**

27 BPA - bisphenol A

28 DCC - dextran-coated charcoal

29 E2 - 17 $\beta$ -estradiol

30 EDCs - endocrine disrupting chemicals

31 EEQ - 17 $\beta$ -estradiol equivalent quantity

32 ER - Estrogen receptor

33 FSH - Follicle-stimulating hormone

34 GH - Growth hormone

35 ISO - International Organization for Standardization

36 HELN - human uterine cervix carcinoma cells (HeLa) stably transfected with ERE- $\beta$ Glob-Luc-SVNeo and pSG5ER $\alpha$ puro or pSG5ER $\beta$ puro plasmids (HELN $\alpha$  or HELN $\beta$ , respectively).

38 HPLC - high-performance liquid chromatography

39 LH - Luteinizing hormone

40 MELN - breast cancer cells (MCF-7) stably transfected with ERE- $\beta$ Glob-Luc-SVNeo plasmid.

41 OECD - Organisation for Economic Co-operation and Development

42 PCBs - polychlorinated biphenyls

43 PFAA - perfluorinated alkyl acids

44 POPs - persistent organic pollutants

45 PP - precocious puberty

46 TEXB - total estrogenic burden

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51 **ABSTRACT**

52 Biological assays can evaluate the cumulative effect of a mixture, considering  
53 synergistic/antagonistic interactions and effects of unknown/unconsidered compounds.

54 Therefore, their application could increase in the next years also to analyse biological samples.

55 The aim of this review is to discuss the methodological approach and the application of  
56 estrogenic activity assays in human biological samples. 75 research articles were analysed and  
57 divided according to whether they used these assays: i) to quantify the level of estrogens and/or  
58 as a biomarker of estrogenic status ii) as a biomarker of exposure to endocrine disrupting  
59 chemicals (EDCs).

60 For the first purpose, some authors extracted biological samples while others tested them  
61 directly without any treatment. The study of these methodologies outlined that the methodology  
62 applied influenced the specificity of analysis. The estrogenic activity biomarker was used to  
63 analyse physiological variations of estrogens, pediatric diseases, hormone-dependent diseases  
64 and estrogen suppression/enhancement after pharmaceutical treatments.

65 For the second purpose, some authors extracted samples while others tested them directly, some  
66 authors divided endogenous estrogens from xenoestrogens while others tested samples without  
67 separation. The analysis of these methodologies outlined some limitations related to the  
68 efficiency of extraction and the incorrect separation of some compounds. The studies which  
69 applied this EDC biomarker showed that it was correlated with some EDCs, it varied according  
70 to the exposure of the population and it allowed the identification of some relationships  
71 between EDC exposure and breast cancer, type 1 diabetes and adverse health effects on  
72 children.

73 In conclusion, the estrogenic activity of biological samples can be a useful tool: to quantify low  
74 levels of 17 $\beta$ -estradiol, to assess the combined effect of endogenous estrogens and  
75 xenoestrogens, to estimate the estrogenic status providing considerable insight into

76 physiological or pathological conditions, to evaluate EDC presence implementing the existing  
77 knowledge about EDC exposure and adverse health effects.

78

79 **Keywords:** estrogenic activity, biological samples, EDC, exposure biomarker, hormone-  
80 dependent diseases, adverse health effects.

81

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## 112 1. Introduction

113 The endocrine system is based on hormones, which are molecules produced by endocrine  
114 glands, organs and tissues and released into the blood. Once they reach cells and tissues,  
115 hormones can induce several effects through hormone receptors. Hormones are involved in  
116 complex signalling pathways, which regulate numerous development stages of human and  
117 animal life: foetal development, childhood and puberty. The endocrine system also controls  
118 several functions in adulthood such as reproduction, metabolism and thermal regulation, and it  
119 interacts with other systems such as nervous and immune systems (Bergman et al. 2013,  
120 Demeneix and Slama 2019). Therefore, a correct functioning of the endocrine system is crucial  
121 in order to ensure the correct regulation of numerous physiological processes and its  
122 dysfunction or perturbation can lead to several adverse health effects such as malformations,  
123 metabolic disorders, reduced fertility and cancer (Bergman et al. 2013, Kabir et al. 2015,  
124 Pamplona-Silva et al. 2018).

125 Even if the endocrine system includes several different hormones, the scientific community  
126 focused in particular on estrogens because of their peculiar characteristics. Estrogens exert a  
127 crucial role in human organisms since they regulate menstrual/estrous reproductive cycles and  
128 they are also involved in more complex mechanisms such as the physiology of reproductive

129 organs and tissues (e.g., breast, ovary and endometrium), lipid metabolism, protein synthesis  
130 and diseases (e.g., cancer and neurodegenerative/cardiovascular diseases) (Kiyama and Wada-  
131 Kiyama 2015). Moreover, estrogens play a critical role in the physiology and pathology of the  
132 immune system, and therefore they can influence the onset and the progression of some  
133 autoimmune diseases (Benagiano et al. 2019, Merrheim et al. 2020).

134 The most important endogenous estrogens are 17 $\beta$ -estradiol (E2), estrone and estriol (Kiyama  
135 and Wada-Kiyama 2015) and their actions occur at very low concentrations (Pamplona-Silva  
136 et al. 2018).

137 Due to their key role in the organism, the evaluation of estrogen levels can be helpful to  
138 understand and treat a wide range of physiological or clinical conditions in children as well as  
139 adults (Rosner et al. 2013). The least invasive methods to estimate estrogen levels are indirect  
140 methods which consist in tracking physiologic changes. These methods include monitoring  
141 basal body temperature, using tests of urine to detect ovulation, examining vaginal discharge  
142 and measuring other body changes, such as uterine length at pelvic sonography to evaluate  
143 female pubertal status (Bellem et al. 2011, Paris et al. 2002). However, since they have a low  
144 specificity and sensitivity, indirect methods are generally coupled with direct methods such as  
145 immunoassays and spectrometry analysis which can quantify hormone levels in biological  
146 samples (e.g. saliva, blood, urine) (Bellem et al. 2011). Immunoassays measure hormones  
147 using the binding between antigen and antibody, which is amplified using different markers:  
148 radioisotope, enzyme, fluorescent or chemiluminescent labels (radioimmunoassay, enzyme  
149 immunoassay, fluorescence immunoassay, chemiluminescence immunoassay). Immunoassays  
150 are generally considered to be quite specific, but their sensitivity is often insufficient to detect  
151 low hormone levels and it seems that they tend to overestimate hormonal levels (Santen et al.  
152 2008). Moreover they are unable to measure different estrogens simultaneously (Bellem et al.  
153 2011) and they do not reflect the hormonal activity in the samples since they only quantify the

154 concentration of compounds that are structurally recognized by the antibody, thus neglecting  
155 the overall activity induced by compounds with the same action mechanism (Widschwendter  
156 et al. 2009). Mass spectrometry identifies and quantifies each chemical through its mass-to-  
157 charge ratio after ionization (electron spray or electron impact ionization). Before the mass  
158 spectrometry analysis, the sample is generally prepared using a separation technique (gas  
159 chromatography or liquid chromatography). The tandem mass spectrometry, which is coupled  
160 with spectral analysis in multiple rounds, is accepted as the golden standard for hormone assays  
161 but it shows some limitations such as the expensive equipment needed and the technical  
162 complexity of the analysis, which involves several steps and thus requires a long time (Bellem  
163 et al. 2011). Immunoassays and mass spectrometry have been widely used for their specificity  
164 since they quantify the concentrations of specific hormones. However, their high specificity  
165 may oversimplify the physiological situation. The physiological hormonal activity is mainly  
166 based on the effect of a specific hormone, but it can also be induced by other hormones,  
167 hormone metabolites and growth factors. Also, it can be altered by exogenous substances.  
168 Therefore, in recent years bioassays have been used for hormonal activity quantification by an  
169 increasing number of authors. Bioassays are based on biological reactions that depend on the  
170 presence or absence of the hormone, but also on the presence of other substances, which can  
171 induce the same effect. For example, bioassays for estrogenic activity can detect the total  
172 estrogenic effect in human biological samples: they measure the activity of E2 but are also able  
173 to detect the activity induced by other estrogens such as estrone and estriol (Paris et al. 2002).  
174 In addition to estrogens, a great number of exogenous compounds can exert and modulate the  
175 estrogenic activity which can be measured by means of bioassays. These estrogenic compounds  
176 are part of a group of substances called endocrine disrupting chemicals (EDCs). The World  
177 Health Organization defined EDCs as “exogenous substances or mixtures that alter function(s)  
178 of the endocrine system and consequently cause adverse health effects in an intact organism,



179 or its progeny, or (sub) populations” (Damstra et al. 2002). EDCs are a highly heterogeneous  
180 group of natural (i.e., steroids and phytoestrogen) and synthesized chemicals (i.e., synthetic  
181 chemicals, plastics, plasticizers, pesticides, pharmaceutical agents) (Diamanti-Kandarakis et  
182 al. 2009, Kabir et al. 2015) which can interfere with the endocrine system in different ways:  
183 first, they can act directly by binding to hormone receptors. In particular, agonist EDCs can  
184 imitate hormones, thus producing over or under responses, while antagonist EDCs can block  
185 the response. Second, EDCs can indirectly interact with receptors as they can interfere with the  
186 synthesis, transport, metabolism and excretion of hormones (Hampl et al. 2016, Kabir et al.  
187 2015). These contaminants are ubiquitous in the environment and human exposure to them  
188 occurs in different ways, such as by inhalation (mainly in the working environment), ingestion  
189 of contaminated food and/or water and dermal contact through personal care products (Kabir  
190 et al. 2015). Since low doses of EDCs could be enough to induce effects - and exposure during  
191 specific lifetime periods could induce permanent adverse effects - EDCs may represent an  
192 alarming health and environmental problem.

193 In this context, the assessment of EDC exposure appears crucial in order to monitor populations  
194 at higher risk of exposure and to understand the link between exposure and adverse health  
195 effects. In biological samples concentrations of EDCs (e.g., bisphenol A (BPA), phthalates,  
196 parabens, polychlorinated biphenyls (PCBs), perfluorinated compounds, polybrominated  
197 diphenyl ethers) and metabolites of EDCs (e.g., metabolites of pyrethroids, insecticides,  
198 pesticides, phthalates) have been used as conventional biomarkers of exposure (Calsolaro et al.  
199 2017, Dziewirska et al. 2018, Hampl et al. 2016, Karwacka et al. 2019). However, since 1995  
200 (Sonnenschein et al. 1995) some authors proposed novel biomarkers of exposure to EDCs,  
201 which focus on detecting the biological effect of chemical compounds or metabolites, rather  
202 than detecting the presence of the EDC itself. In particular, the estrogenic activity of biological  
203 samples, measured with estrogenic activity assays, has been proposed and used as a novel

204 biomarker of exposure to EDCs. The main advantage of estrogenic activity assays is that they  
205 can assess the total effect induced by multiple, exogenous chemicals with estrogenic activity  
206 (xenoestrogens). Indeed, the chemical quantification of xenoestrogens provides an estimate of  
207 the real exposure. However, the cumulative estrogenic activity of a mixture is not the sum of  
208 the individual estrogenic activity of each xenoestrogen, since synergistic/antagonistic  
209 interactions must be taken into account. Moreover, the chemical analysis of xenoestrogens only  
210 takes into account specific known xenoestrogens and can not quantify the effect of unknown  
211 or unevaluated compounds (Bicchi et al. 2009, Escher et al. 2018, Jarošová et al. 2014, Kase  
212 et al. 2018, Könemann et al. 2018).

213 Consequently, the use of estrogenic activity assays in human biological samples may have two  
214 main purposes, namely i) the assessment of total estrogenic effect to allow for the management  
215 of a wide range of physiological or clinical conditions in children as well as in adults, and ii)  
216 the improvement of the assessment of EDC exposure to allow for a better management of this  
217 important health and environmental issue.

218 For the time being the development and application of estrogenic activity assays in human  
219 biological samples do not seem to be widespread, compared to other experimental tools.  
220 Nevertheless, their use could increase exponentially in the next years due to their versatility  
221 and holistic approaches. It is thus much needed to gain additional knowledge of their  
222 methodological approaches and applications relevant to them.

223 The aim of the present review is to discuss the methodological approaches and the applications  
224 of estrogenic activity assays in human biological samples. A bibliographic research was  
225 performed: 75 research articles were analysed in order to summarize the methods used to treat  
226 biological samples and the results obtained.

227 The examined articles were divided into two categories, according to the use of estrogenic  
228 activity: i) to quantify the level of estrogens and/or as a biomarker of estrogenic status, ii) as a  
229 biomarker of exposure to EDCs.

230 To quantify the level of estrogens and/or as a biomarker of estrogenic status, the estrogenic  
231 activity assays were applied in 38 of the analysed articles (table 1). Some authors mainly  
232 considered estrogenic activity assays as tools for the evaluation of estrogen levels, specifically  
233 for the evaluation of E2 levels (table 1, n° ref. 1- 8, 10-13, 18, 29, 32, 38). Other authors instead  
234 used these assays for their ability to measure the total estrogenic effect defined as “evaluation  
235 of estrogenic bioactivity” (table 1, n° ref. 9, 17). In this review, the application of estrogenic  
236 activity assays was intended as a tool to detect the total estrogenic effect, meaning that it was  
237 intended as a biomarker of “estrogenic status”, except for the articles published by Klein et al.  
238 where it was intended as a tool for estrogen quantification. In these 38 studies, serum and  
239 plasma were used as biological samples and they were analysed using gene reporter assays  
240 (using mammalian and yeast cells) and proliferation assays (E-screen assays).

241 As biomarkers of exposure to EDCs, estrogenic activity assays were used in 37 of the analysed  
242 articles (table 2). In these studies, the biomarker was defined as “assessment of total estrogenic  
243 burden (TEXB)” (table 2, n° ref. 3), “total estrogenic xenobiotic burden” (table 2, n° ref. 4),  
244 “total effective xenoestrogen burden” (table 2, n° ref. 7, 15, 16, 21, 22, 35) or “xenoestrogenic  
245 activity” (table 2, n° ref. 11, 14, 32, 37). In this article the abbreviation “EDC biomarker” will  
246 be used. In these 37 studies, serum, adipose tissue, placenta and milk were used as biological  
247 samples and they were analysed using ligand-binding assays, gene reporter assays (using  
248 mammalian and yeast cells) and proliferation assays (E-screen assays).

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## 2. Assays for estrogenic activity evaluation

Assays for estrogenic activity evaluation have been described in recent review articles (Mueller 2004, Kiyama and Wada-Kiyama 2015, Wagner et al. 2017, Wangmo et al. 2018). For the assessment of the estrogenic activity in biological samples, three kinds of assays have been applied: ligand-binding assays, gene reporter assays and proliferation assays (E-screen assays). In the following sections the characteristics of these assays are briefly described considering their strengths/weaknesses (table 3) and their detection limits (table 4) in the assessment of estrogenic activity in biological samples. The assays validated by the Organisation for Economic Co-operation and Development (OECD) as tools to test potential EDC substances or by the International Organization for Standardization (ISO) as tools to assess water/waste water estrogenic activity are also listed.

### 2.1. Ligand-binding assays

Ligand-binding assays detect receptor–ligand interactions. These assays quantify the ability of a test chemical or a mixture to compete with E2 in binding to ER, and the result is often expressed as the concentration showing 50% displacement of E2 from receptor. The result can also be expressed as relative binding affinity with respect to E2 (Kiyama and Wada-Kiyama, 2015). Ligand-binding assays were initially performed using radioactive ligands, while, more recently, assays using non-radioactive ligands have been developed. Ligand-binding assays are able to detect the binding to ER of both agonist and antagonist substances without distinguishing between the two effects (Seifert et al. 1999). This characteristic is an advantage. Indeed, while in other estrogenic activity assays agonist and antagonist effects could compensate each other resulting in a null activity, in ligand-binding assays this compensatory effect can not occur. However, the same characteristic can be a disadvantage, since these assays are unable to assess the total estrogenic effect induced by the interaction of agonist and antagonist substances (Mueller 2004).

277 To assess the estrogenic activity in biological samples, a ligand-binding assay has been applied:  
278 the enzyme linked receptor assay (Sapbamrer et al. 2010), a competitive enzyme immunoassay  
279 which uses non-radioactive ligands. Moreover, the OECD has validated two ligand-binding  
280 assays to screen and test potential EDC substances (OECD 2018), namely the Freyberger-  
281 Wilson ER binding assay and the Chemical Evaluation and Research Institute (CERI) ER  
282 binding assay (OECD n° 493 2015).

## 283 **2.2. Gene reporter assays**

284 Gene reporter assays consist of the transfection of a reporter construct into mammalian or yeast  
285 cells. ER are transcription factors that induce the transcription of target genes after binding to  
286 specific DNA sequences in their promoter; the reporter construct is made of these DNA  
287 sequences linked to the gene of a measurable protein (for example the enzyme luciferase)  
288 (Sonneveld et al. 2005). Gene reporter assays are able to quantify the total estrogenic effect  
289 and are characterized by short incubation periods. However, the results of these assays can be  
290 ambiguous when substances with overactivation effects are included in the analysed sample.

291 Gene reporter assays can be divided into two main categories. Some reporter gene assays are  
292 performed on cells that already express ERs, while others are performed on cells that do not  
293 express endogenous ERs. In the first category of gene reporter assays, cells are transfected with  
294 an estrogen-inducible reporter gene. These cells can be transiently or stably transfected,  
295 however, since stably transfected cells remain stable and ready for use, they are generally  
296 preferred (Soto et al. 2006, Wangmo et al. 2018). In the second category of gene reporter  
297 assays, cells are transfected with both an estrogen-inducible reporter gene and an ER  
298 expression construct. Since these cells do not express other ERs, the advantage of these assays  
299 is that they allow the evaluation of estrogenic activity in the samples distinguishing between  
300 ER $\alpha$  and ER $\beta$  estrogenic activity.

301 Numerous gene reporter assays have been applied for the assessment of estrogenic activity in  
302 biological samples. Moreover, two gene reported assays have been validated by the OECD to  
303 screen and test potential EDC substances (OECD 2018): the first uses ER $\alpha$ -HeLa-9903 cells  
304 and the second uses VM7Luc4E2 cells (OECD n° 455 2016). Both assays use human cells  
305 stably transfected with ER $\alpha$ , but the VM7Luc4E2 cells also express a minor amount of  
306 endogenous ER $\beta$ . As stated by the OECD, VM7Luc4E2 cells were originally designated as the  
307 BG1Luc cells, however, in-depth analyses revealed that these cells were not the BG1 cells, but  
308 instead a variant of the MCF-7 cells (OECD 2018). Recently, a gene reporter assay using stably  
309 transfected human cells has also been described in the ISO 19040-3 (ISO 2018) for the  
310 assessment of estrogenic activity in water and waste water. As for gene reported assays based  
311 on yeasts, two assays, based on *Saccharomyces cerevisiae* or *Arxula adenivorans*, have been  
312 described in the ISO 19040-1 (2018) and in the ISO 19040-2 (2018) as methods for the  
313 assessment of estrogenic activity in water and waste water (Hettwer et al. 2018).

### 314 **2.3. Proliferation assays (E-screen assays)**

315 E-screen assays are proliferation tests. They measure the proliferative effect induced by  
316 estrogens or estrogenic substances on estrogen-responsive cells. Proliferation is determined  
317 through different procedures, including DNA staining and metabolic activity quantification.  
318 The first E-screen assay was developed by Soto et al. (1997) using human breast cancer cells  
319 (MCF-7), whose proliferation is mainly induced by the activation of ER $\alpha$  (Wagner et al. 2017).  
320 Later on E-screen assays were also applied using MCF-7 sublines, such as the MCF-7 BUS, in  
321 order to obtain a higher proliferative response (Martinez et al. 2016, Villalobos et al. 1995).  
322 Many E-screen assays have been applied for the assessment of estrogenic activity in biological  
323 samples, while they have not been reported by the OECD nor by the ISO (OECD 2018) to test  
324 potential EDC substances or water/waste water estrogenic activity.

325

### 3. Estrogenic activity as estrogen quantification and biomarker of estrogenic

#### status: methodologies

The research group of Klein was the first to developed an estrogenic activity assay in biological samples (table 1, n° ref. 1). They developed an estrogenic activity assay as estrogen quantification using yeasts (i.e. a strain of *Saccaromyces cerevisiae*) in which the detection limit was 0.02 pg/mL, in order to overcome the lack of sensitivity of available assays for E2. Klein et al. (1994) treated serum samples performing a liquid-liquid extraction with ether and demonstrated that this assay was highly specific for E2 with low cross-reactivity for estrogen metabolites and other hormones, suggesting that this high specificity could be due to many factors, including the extraction of the samples.

The protocol of Klein et al. was mainly focused on measuring E2 and it was applied in many studies by the same research group (table 1, n°ref. 2-8, 10-13, 18, 22, 29, 32, 38).

Other authors applied extraction to biological samples before the analysis: in the studies of Pedersen et al. (2010) and Chamas et al. (2017) samples were extracted using a liquid-liquid extraction with methyl tert-butyl ether and diethyl ether respectively, while in the study of Kanaya et al. (2015) samples were extracted using a solid-phase extraction.

In 2002, the research group of Paris et al. developed an estrogenic activity assay for the determination of serum estrogenic activity using human uterine cervix carcinoma cells (HeLa) stably transfected with ERE- $\beta$ Glob-Luc-SVNeo and pSG5ER $\alpha$ puro or pSG5ER $\beta$ puro plasmids (HELN $\alpha$  or HELN $\beta$ , respectively). In contrast to Klein et al. (1994), Paris et al. (2002) tested serum samples without extraction and without any other sample treatment to keep their conditions closer to those of their physiological status, since their aim was to develop a protocol for the assessment of the total estrogenic activity rather than for E2 quantification. Their protocol, based on HeLa cells, was subsequently applied by several authors (table 1, n° ref. 15, 17, 23, 24, 28) and it was also used by Séronie-Vivien et al. (2004) as a model to develop

351 another serum estrogenic activity assay based on breast cancer cells (MCF-7) stably transfected  
352 with ERE- $\beta$ Glob-Luc-SVNeo plasmid (MELN).

353 Other 11 articles reported in this review treated biological samples without any extraction (table  
354 1, n° ref. 16, 19, 20, 25, 26, 27, 30, 31, 34, 35, 37). In almost all protocols, samples were added  
355 to culture medium without any treatment. In four studies, however, samples were filtered (pore  
356 size 0.22  $\mu$ m) before being added to culture medium (table 1, n° ref. 20, 25, 30, 32) and an  
357 aromatase inhibitor was added with a view to preventing the conversion of testosterone to E2  
358 by aromatase in the cells (table 1, n° ref. 20). Biological samples were tested in one or in  
359 different concentrations ranging from 1 to 20% in culture medium.

360 The analysis of samples without extraction could be influenced by the complexity of  
361 serum/plasma samples, therefore some authors proposed different methodological approaches  
362 in order to consider the variability of these samples.

363 The variability caused by serum components was considered by many authors in the  
364 construction of the standard curve. In five studies, the standard curve was produced for each  
365 sample and consisted of stripped serum from the same patient, to which incremental amounts  
366 of E2 were added (table 1, n° ref. 9, 15, 17, 23, 24, 28). Séronie-Vivien et al. (2004) considered  
367 this approach as essential when the assay was performed to quantify E2 in serum, because it  
368 was useful to remove the interference due to serum compounds different from E2, which  
369 modulate the estrogenic activity and differ from patient to patient. However, for the detection  
370 of estrogenic activity unrelated to E2 serum concentration, Séronie-Vivien et al. (2004) stated  
371 that a single standard curve could be performed using charcoal-stripped serum from a healthy  
372 volunteer in order to take into account a “normal overall estrogenic (transcriptional) activity of  
373 human serum”.

374 Similarly to Séronie-Vivien et al. (2004), other authors used a single standard curve for all  
375 samples (table 1, n° ref. 19, 20, 25, 26, 27, 30, 31). The standard curve was generally



376 constructed by adding E2 to medium with charcoal-stripped serum/plasma at the same  
377 concentration as the sample's one. For example, in the study of Lim et al. (2014a), samples  
378 were tested at a concentration of 10% in medium and the standard curve was constructed by  
379 adding E2 to 10% charcoal-stripped commercial human serum in medium.

380 A similar approach was adopted by Sonneveld et al. (2005) in order to test different  
381 concentrations of serum samples (0-10%) avoiding the variability caused by serum  
382 components. For this purpose, the authors maintained the final serum concentration at 10% by  
383 supplementing lower percentages of the tested sera with charcoal-stripped bovine serum.

384 Data were usually expressed as E2 equivalent quantity (EEQ), which is the total concentration  
385 of estrogenic active compounds normalised to the E2. However, Martínez et al. (2016)  
386 presented the results as estrogenic activity in comparison with a standard serum pool. In this  
387 study, each serum sample was tested with 0.5% serum pool, which was used as a reference  
388 standard to normalize the results. Other studies did not specify any peculiar approach to  
389 evaluate the results (table 1, n° ref. 35, 37).

390 Like many hormones, estrogenic activity has a circadian rhythm, with nocturnal and early  
391 morning rises (Janfaza et al. 2006, Li et al. 2009). For this reason, some authors pointed out  
392 that they collected fasting blood samples during specific hours of the day, especially in the  
393 morning (table 1, n° ref. 3, 4, 5, 6, 7, 8, 10, 12, 13, 18, 21, 32, 34, 35, 38).

394

#### 395 **4. Estrogenic activity as estrogen quantification and biomarker of estrogenic** 396 **status: applications**

397 Estrogenic activity as estrogen quantification and biomarker of estrogenic status was applied:  
398 i) to detect physiological variations of estrogens, ii) to study pediatric diseases, iii) to analyse  
399 hormone-dependent diseases in women, iv) to evaluate estrogen suppression or enhancement  
400 after pharmaceutical treatments.

#### 4.1. Physiological variations of estrogens

401 Estrogenic activity assays were used to detect low concentrations of estrogens in children and  
402 in post-menopausal women, since the sensitivity of other assays for E2 quantification was too  
403 low.

404 Klein et al. (1994) measured physiological estrogen levels using an estrogenic activity assay in  
405 prepubertal children, and found that the estrogen levels in prepubertal girls were higher than in  
406 prepubertal boys, suggesting that these hormones may contribute to higher rates of skeletal  
407 maturation, earlier puberty and earlier interruption of growth in girls compared to boys. The  
408 same results were obtained in the study of Paris et al. (2002) using a different assay for the  
409 evaluation of serum estrogenic activity. The role of estrogen levels in skeletal growth was  
410 confirmed in another study (Klein et al. 1996): estrogen levels measured in healthy growing  
411 boys were low throughout childhood, increased before puberty and rose steadily during  
412 adolescence. Moreover, there was a relationship between estrogen levels and testosterone  
413 concentrations and between estrogen levels and the time of peak growth velocity.

414 The physiological variations of estrogens were further evaluated by Janfaza et al. (2006) in 800  
415 healthy children from birth to puberty. In the same study, the estrogen levels were measured  
416 every hour for 24 hours in 55 children. Estrogen levels: (i) increased with age and pubertal  
417 stage in both genders, (ii) showed a circadian rhythm with a nocturnal rise and (iii) were always  
418 higher in girls than in boys.

419 The estrogenic activity/E2 level of prepubertal children was also evaluated to investigate the  
420 relationship between estrogenic activity and adiposity. The first study on this topic was  
421 performed by Klein et al. (1998b), whose results revealed that obese and nonobese children in  
422 prepubertal or early pubertal stage showed similar circadian rhythms and similar E2 levels. E2  
423 levels were not correlated with fat mass, body mass index, or arm fat, suggesting that E2 levels  
424 are not directly correlated with markers of adiposity. Similar results were obtained by Larmore  
425

426 et al. (2002), who did not find a significant correlation between estrogen levels and body mass  
427 index or weight in their analysis of prepubertal and pubertal girls (obese and nonobese).  
428 Furthermore, the study of Mesa Valencia et al. (2019) did not show any association between  
429 estrogenic activity and markers of adiposity and metabolic and hormonal factors in prepubertal  
430 girls, suggesting that estrogenic activity is not influenced by adiposity.

431 Estrogenic activity assays were also applied to detect low concentrations of  
432 estrogens/estrogenic compounds in postmenopausal women. Wang et al. (2005) applied a gene  
433 reporter assay using HeLa cells in samples of postmenopausal women, suggesting that such  
434 assays could be useful not only to determine the concentration of E2, but also to assess the total  
435 estrogenic activity.

436 Indeed, the authors claimed that the assay was not completely specific for E2 but seemed to  
437 measure other biologically active estrogens as well. This hypothesis was supported by a  
438 subsequent study (Wang et al. 2013), in which another estrogenic activity assay was applied  
439 (E-screen assay). In this study, the serum estrogenic activity of postmenopausal women was  
440 strongly associated with serum E2 levels, but it was also positively or negatively associated  
441 with other serum molecules and women characteristics. These results suggested that the  
442 estrogenic activity is influenced not only by endogenous estrogen levels but also by other  
443 factors.

444 Finally, the estrogenic activity was evaluated in mother-child pairs. The study of Pedersen et  
445 al. (2010) evaluated the estrogenic activity of both mother's and child's plasma (cord blood).  
446 The results showed that the estrogenic activity of cord blood plasma was higher than the one  
447 of mother plasma, and that the two parameters were loosely correlated. The authors also  
448 suggested that the high estrogenic activity of cord blood probably reflected the elevated  
449 estrogen production in the placenta and was not due to the presence of other estrogenic  
450 compounds such as EDCs.

451 **4.2. Estrogenic activity and pediatric diseases**

452 Estrogenic activity assays were applied in order to analyse biological samples collected from  
453 children and adolescents with pediatric diseases characterized by hormonal dysregulation:  
454 precocious puberty, Turner's syndrome, type 1 diabetes and sex differentiation disorders.

455 **4.2.1. Female precocious puberty**

456 Precocious puberty (PP) is defined as the appearance of secondary sex characteristics in girls  
457 aged under 8 years. PP is clinically distinguished between incomplete PP (premature thelarche,  
458 premature pubarche, isolated menarche) and true PP (complete or central PP) (Sultan et al.  
459 2018). Estrogenic activity assays have been used on both forms of PP by multiple studies.  
460 Three studies focused on incomplete PP and specifically on premature thelarche, which refers  
461 to the isolated breast development in girls aged under 8 without the development of any other  
462 sexual characteristics. The first study (Klein et al. 1999) performed on young girls (less than 3  
463 years of age) found that E2 levels in girls with premature thelarche were significantly higher  
464 than in normal prepubertal girls. In the second study (Paris et al. 2013) information on parental  
465 environmental/occupational exposure to EDCs during prenatal/postnatal patient life were  
466 collected through surveys, in addition to estrogenic activity evaluation. The estrogenic activity  
467 of girls with premature thelarche and whose parents had been exposed to EDCs was  
468 significantly higher compared to the one of healthy girls. Moreover, it was significantly higher  
469 compared to the estrogenic activity of girls with premature thelarche whose parents had not  
470 been exposed to EDCs. This suggested that some patients may be affected by this condition  
471 due to prenatal/postnatal EDC exposure. The third study (Pereira et al. 2015) investigated the  
472 relationship between estrogen levels and risk of premature thelarche, assessing the estrogen  
473 levels of prepubertal girls aged 7, who were monitored during the following years for the onset  
474 of thelarche. The results showed that girls with estrogen levels over 5 pg/ml at 7 years had an  
475 increased risk of presenting earlier thelarche onset.

476 E2 levels were evaluated using estrogenic activity assays also in girls affected by true PP. In  
477 the study of Larmore et al. (2002), average E2 levels were higher in pubertal girls than in  
478 prepubertal girls and in girls with PP. However, only E2 levels of normal pubertal and normal  
479 prepubertal girls were statistically different. A high estrogenic activity was also detected in a  
480 baby girl (4 months old) affected by precocious puberty (Gaspari et al. 2011b). Since high  
481 concentrations of pesticides were detected in the plasma of both the patient and her parents, as  
482 well as in the soil of their farm, the authors hypothesized a correlation between her precocious  
483 puberty and exposure to pesticides.

#### 484 **4.2.2. Other female diseases**

485 Wilson et al. (2003) compared the serum E2 levels in prepubertal girls affected by Turner's  
486 syndrome with healthy prepubertal girls, and found that girls with Turner's syndrome had  
487 significantly lower E2 levels. Martínez et al. (2016) compared the serum estrogenic activity of  
488 post-menarcheal girls with type 1 diabetes to the estrogenic activity of normal post-menarcheal  
489 girls, finding a statistically significant difference in the estrogenic activity of girls with type 1  
490 diabetes compared to the control group. In these two studies, the application of estrogenic  
491 activity assay allowed to demonstrate that the lack of normal ovarian function in girls with  
492 Turner's syndrome is evident even before puberty, and that type 1 diabetes can also affect  
493 estrogen metabolism.

#### 494 **4.2.3. Sex differentiation disorders in males**

495 The study of Paris et al. (2006) found that the serum estrogenic activity of three children with  
496 male pseudo-hermaphroditism was higher than in controls. This result, coupled with the  
497 mother's exposure to environmental EDCs during pregnancy, suggested that ambiguous  
498 genitalia could be related to foetal exposure to EDCs.

499 Similarly, the study of Gaspari et al. (2011a) found that the serum estrogenic activity of young  
500 males with sex differentiation disorder was significantly higher in 11 males who had been

501 exposed to EDCs during the foetal period compared to 17 cases who had not been exposed and  
502 compared to controls. Also, the estrogenic activity of patients who had not been exposed to  
503 EDCs was not statistically different compared to controls, suggesting the possible relationship  
504 between EDC exposure and sex differentiation disorder of some males.

### 505 **4.3. Estrogenic activity and hormone-dependent diseases in women**

506 Many studies investigated the relationship between estrogenic activity and breast cancer.  
507 Séronie-Vivien et al. (2004) found that in controls the estrogenic activity was significantly  
508 correlated with serum E2 concentration, while in advanced breast cancer patients it was less  
509 correlated. In this study, the estrogenic activity was higher in controls than in patients, probably  
510 because many pre-menopausal women were included in the control group.  
511 Another study performed a similar comparison using a higher number of subjects  
512 (Widschwendter et al. 2009). In contrast with the previous study, the estrogenic activity,  
513 measured as estrogen receptor- $\alpha$  and estrogen receptor- $\beta$  (ER $\alpha$  and ER $\beta$ ) transcriptional  
514 activity, was higher in postmenopausal women with breast cancer compared to postmenopausal  
515 controls, although no difference was observed in serum E2 levels between the two groups.  
516 Moreover, this study suggested that estrogenic activity assays might predict ER-positive breast  
517 cancer at the time of the diagnosis, since women with estrogenic activity (ER $\alpha$  and ER $\beta$ ) in the  
518 highest quintile among controls had 7.57- and 10.14-fold risk of general and ER-positive breast  
519 cancer respectively.

520 Recently, Fourkala et al. (2012) performed a case-control study nested in a cohort study  
521 demonstrating that ER $\alpha$  transcriptional activity of serum collected more than two years before  
522 diagnosis was independently associated with breast cancer risk, while ER $\alpha$  activity, collected  
523 less than two years before diagnosis, and ER $\beta$  activity were not. The association between ER $\alpha$   
524 transcriptional activity of serum, collected before diagnosis, and breast cancer risk was also  
525 confirmed by the analysis of Asian women in the study of Lim et al. (2014a). Women in the

526 highest quartile for ER $\alpha$  activity had an odds ratio of 2.39 compared with those in the lowest  
527 quartile. As reported also by Fourkala et al. (2012), cases and controls did not differ for ER $\beta$   
528 transcriptional activity.

529 Considering the results of these studies, estrogenic activity assays seem to be a useful tool to  
530 assess the risk assessment of breast cancer, since in most of the studies the serum estrogenic  
531 transcriptional activity was associated with breast cancer risk.

532 Fejerman et al. (2016) measured the estrogenic activity of Latin-American women in order to  
533 understand whether estrogenic activity may be related to higher breast cancer risk in US-born  
534 Latinas compared to foreign-born. Using linear regression models, the authors found a positive  
535 association between estrogenic activity and years of US residence (considering foreign-born  
536 Latinas only), suggesting that the breast cancer risk observed in Latin-American women might  
537 be related to the estrogenic activity.

538 Starting from this pilot study, Sanchez et al. (2019) assessed the association between estrogenic  
539 activity, demographic factors and breast cancer risk factors in American women of different  
540 racial/ethnic groups: Non-Latina Black, Non-Latina White and Latina women. Non-Latina  
541 Black women showed the highest estrogenic activity, followed by Non-Latina White women,  
542 while Latina women showed the lowest estrogenic activity. The multivariable analysis (which  
543 included several independent variables) showed that the difference between Non-Latina White  
544 and Latina women was statistically significant, as opposed to the difference between Non-  
545 Latina Black and Non-Latina White women. The results of these two studies combined  
546 suggested that the use of estrogenic activity assays might also provide explanation regarding  
547 different breast cancer risk in different racial/ethnic groups living in the US.

548 The estrogenic activity of serum was also evaluated in relation to another hormone-dependent  
549 disease in adults. Lim et al. (2012) considered the possible association between estrogenic  
550 activity (measured as ER $\alpha$  transcriptional activity) and hip fracture risk in postmenopausal

551 Asian women. A high serum estrogenic activity, evaluated before hip fracture, was associated  
552 with a reduced hip fracture risk. This reduction was still evident after adjustment for other  
553 known risk factors of hip fracture were made, including age and body mass index. The results  
554 suggested that a high estrogenic activity might prevent this hormone-dependent injury.  
555 Finally, the same authors evaluated the influence of serum estrogenic activity (measured as  
556 ER $\alpha$  and ER $\beta$  transcriptional activity) on lung cancer survival in postmenopausal Asian women  
557 (Lim et al. 2014b). Using regression analysis, they found that high ER $\beta$  transcriptional activity  
558 in sera was associated with a lower probability of lung cancer survival, while on the contrary  
559 estrogen levels and ER $\alpha$  transcriptional activity were not associated with the probability of  
560 survival. These results suggested that some serum activators of ER $\beta$  may lower the probability  
561 of lung cancer survival and that the evaluation of ER $\beta$  estrogenic activity in sera might serve  
562 as a prognostic marker to predict lung cancer survival.

#### 563 **4.4. Estrogen suppression/enhancement after pharmaceutical treatments**

##### 564 **4.4.1. Females**

565 The suppression of E2 levels after pharmaceutical treatments was tested in girls affected by  
566 PP. In the study of Klein et al. (1998a), girls affected by PP were treated with different doses  
567 of deslorelin for 9 months and serum E2 levels were evaluated using estrogenic activity assay.  
568 The results showed that the suppression of E2 was dependent on the dose of deslorelin.  
569 However, E2 concentrations were always above the E2 levels of normal prepubertal girls  
570 regardless of the dose.

571 Other authors evaluated E2 levels using estrogenic activity assays after pharmaceutical  
572 treatments in unhealthy girls. Taboada et al. (2011) assessed pharmacokinetics and  
573 pharmacodynamics of the same form of E2 administered orally and transdermally to girls with  
574 Turner's syndrome. Girls were treated for two weeks with either a high or a low dose of E2  
575 administered orally or transdermally. This preliminary short-term study demonstrated that the



576 high dose of transdermal administration managed to restore the physiological levels of E2 and  
577 estrone, while oral administrations increased estrone concentration compared to both  
578 transdermal administration and controls regardless of the dose. High transdermal E2  
579 administration proved to be the most effective way to make the estrogenic activity of the  
580 patients the most similar to the estrogenic activity of controls.

581 This preliminary short-term study was carried forward by the subsequent study of Torres-  
582 Santiago et al. (2013). The authors assessed the metabolic effects and body composition  
583 changes in girls with Turner's syndrome treated with the same form of E2 given orally or  
584 transdermally for a year. E2 doses were titrated in order to achieve E2 levels within the normal  
585 range of menstruating adolescents. The two treatments induced similar effects on numerous  
586 monitored parameters: follicle-stimulating hormone (FSH) and luteinizing hormone (LH)  
587 concentrations, body composition (weight, body mass index, percentage of fat mass, fat-free  
588 mass, abdominal fat), lipid concentrations and oxidation, resting energy expenditure rates and  
589 bone mineral density. However, since the estrogenic activity of serum samples, estrone and  
590 estrone sulfate concentrations were higher in girls treated orally with E2 than in girls treated  
591 with transdermal E2 administration, the authors concluded that transdermal administration  
592 might be more effective than oral administration in inducing a more physiological estrogenic  
593 status in girls with Turner's syndrome.

594 Estrogenic activity assays were also used to test women after pharmaceutical treatments. The  
595 first study to focus on women was the one of Klein et al. (1995), in which the E2 levels were  
596 measured using a bioassay in women affected by breast cancer and treated with different doses  
597 of an aromatase inhibitor for twelve weeks (100 µg/day - 5.0 mg/day of letrozole). The result  
598 of the study demonstrated that all doses of the drug induced an equivalent suppression of E2  
599 levels.

600 The second study on women tested the suppression and recovery of E2 after injection of a  
601 potent gonadotropin-releasing hormone receptor agonist (leuprolide acetate) in healthy  
602 volunteers (Larmore et al. 2000). Measured with an estrogenic activity assay, E2 levels were  
603 significantly suppressed by week 3 and further suppressed by week 4 after one injection of  
604 leuprolide acetate. E2 remained below postmenopausal levels for 5-8 weeks after one injection  
605 and for 6 weeks after a second injection (4 weeks apart).

606 Finally, Santen et al. (2002) investigated the estrogenic activity of serum collected from  
607 postmenopausal women affected by urogenital atrophy and treated with vaginal E2, in order to  
608 determine the lowest dosage needed to reverse signs and symptoms of urogenital atrophy  
609 without substantially increasing serum E2 levels. As measured by bioassay, a dose equal to 10  
610  $\mu\text{g}$  of vaginal E2 relieves the symptoms of urogenital atrophy and induces objective vaginal  
611 changes without increasing serum E2 levels.

#### 612 **4.4.2. Males**

613 E2 suppression can be induced in boys with severe growth retardation in order to delay puberty  
614 and then the epiphyseal fusion, thus maximizing height potential. For this purpose, Mauras et  
615 al. (2000) analysed the performance of anastrozole, a nonsteroidal aromatase inhibitor that  
616 blocks the conversion of  $\Delta 4$ -androstenedione to estrone and of testosterone to E2. The drug  
617 was tested in healthy young boys and the results showed that E2 concentrations were  
618 dramatically reduced after anastrozole administration. The drug seemed to be well tolerated  
619 and safe, since the treatment did not affect body composition, protein kinetics/substrate  
620 oxidation rates, muscle strength, and bone calcium metabolism. Based on these results, the  
621 authors stated that anastrozole appears to be suitable to treat boys with growth retardation.

622 This hypothesis was further supported by the same authors in a subsequent study (Mauras et  
623 al. 2004). In this study, growth hormone (GH) deficient boys were treated for 12 months with  
624 GH (control group) or with GH and anastrozole in order to suppress estrogen production and

625 delay epiphyseal fusion. After the treatment, E2 concentrations decreased in boys treated with  
626 anastrozole and increased in the control group. Testosterone levels were higher in boys treated  
627 with anastrozole compared to control group. Although both treatments did not increase  
628 predicted adult height, anastrozole treatment dramatically decreased estrogen concentrations  
629 without effects on body composition, plasma lipids, and bone metabolism.

630 Estrogenic activity assays were also used to assess the effect of the decoction of a Chinese  
631 medicinal plant (*Epimedium pubescens*), compared to the effect of a standard estrogenic  
632 prodrug (estradiol valerate) generally used to counteract menopausal symptoms (Li et al. 2009).  
633 The study was performed on male subjects in order to reduce the interference due to  
634 endogenous estrogens, and the estrogenic activity was evaluated in serum using different assays  
635 (ER $\alpha$  and ER $\beta$  gene reporter assays and E-screen assay). Ingestion of the standard drug induced  
636 a significant increase of serum estrogenic activity using all assays, while the plant decoction  
637 only induced a small but significant increase of ER $\alpha$  transcriptional activity, suggesting that  
638 the administration of this traditional decoction may not be suitable to counteract menopausal  
639 symptoms.

640

#### 641 **5. Estrogenic activity as a EDC biomarker: methodologies**

642 Human biological samples can contain endogenous steroidal estrogens, but also EDCs with  
643 estrogenic activity called xenoestrogens, which can be introduced in the human body through  
644 inhalation, ingestion of contaminated food/water, and dermal contact. Since the evaluation of  
645 estrogenic activity through assays measures the effect induced by all estrogenic compounds,  
646 the use of these methods alone is unable to determine whether a phenomenon is induced by  
647 endogenous estrogens or xenoestrogens. Therefore, in order to use estrogenic activity assays  
648 as a EDC biomarker, in most studies the biological samples were treated in order to remove  
649 endogenous estrogens.

650 The studies of Sonnenschein et al. (1995) and Soto et al. (1997) are to our knowledge the first  
651 ones that applied estrogenic activity assays as a biomarker. The protocol follows multiple  
652 stages: (i) serum sample extraction, (ii) acid cleanup and (iii) separation of xenoestrogens from  
653 endogenous estrogens by high-performance liquid chromatography (HPLC) in silica column  
654 using the method proposed by Mendina and Sherman (1986). The separation is based on the  
655 elution time from the HPLC column. Since xenoestrogens (estrogenic pesticides, PCBs,  
656 hydroxylated PCBs, phenolic antioxidants and plasticizers) and endogenous estrogens have  
657 different elution times, xenoestrogens can be collected during the first 10 minutes of elution.  
658 However, some xenoestrogens, such as phytoestrogens, diethylstilbestrol and mycoestrogens  
659 cannot be extracted through this technique, as their retention time is longer than 12 minutes  
660 (Soto et al. 1997).

661 The protocol proposed by Sonnenschein et al. (1995) and optimized by Soto et al. (1997) was  
662 further refined and modified by Rasmussen et al. (2003) by the introduction of solid-phase  
663 extraction and a modified HPLC gradient, with elution of xenoestrogens within 5.5 minutes.  
664 Rasmussen et al. (2003), who analysed serum samples from pregnant women, stated that two  
665 subfractions of the extract collected during the first 5.5 minutes could contain pregnancy-  
666 related hormones (5 $\alpha$ - dihydroprogesterone and fatty acid esters of estrone or E2). These  
667 subfractions (1.8 – 2.3 min and 3.8 – 4.3 min) were thus removed for the sake of analysis. In a  
668 subsequent study, Andersen et al. (2007) collected the xenoestrogens during the first 8 minutes  
669 in order to include more hydrophilic pesticides. However, they removed an additional  
670 subfraction (6.2–7.1 min) to avoid pregnenolone. The protocol, improved by Rasmussen et al.  
671 (2003), was applied in eight other studies (table 2, n° ref. 10, 11, 12, 13, 14, 19, 20, 27).

672 Starting from this protocol, which was focused mainly on the extraction of persistent EDCs,  
673 Bjerregaard-Olesen et al. (2015) developed an extraction method for serum samples which was  
674 optimized for perfluorinated alkyl acids (PFAA). As the previous one, this method is composed

675 by solid-phase extraction (water phase), liquid/liquid extraction (tetrahydrofuran/n-hexane)  
676 and HPLC fractionation (extract collected between 22.01 and 26.00 min). However, since after  
677 the HPLC fractionation estriol and estetrol are still present, this method also contains a weak  
678 anion exchange extraction in order to remove these endogenous hormones from the final  
679 extract. This protocol optimized for PFAA was subsequently applied in three studies (table 2,  
680 n° ref. 33, 36, 37).

681 Recently, a third protocol with HPLC fractionation was developed in order to analyse human  
682 serum samples (Pastor-Barriuso et al. 2016). In this protocol, the HPLC fractionation is  
683 preceded by liquid-liquid and solid-phase extractions.

684 In 1997, the Spanish research group of Rivas and Olea (1997) published another methodology  
685 to assess the estrogenic activity as a EDC exposure biomarker using adipose tissue samples  
686 instead of serum samples. The authors proposed a protocol in which the adipose tissue was  
687 dissolved in hexane and eluted with hexane in a glass column filled with Alumina Merck 90;  
688 the eluate obtained was then concentrated and injected in HPLC for separation of  
689 xenoestrogens from endogenous estrogens. The HPLC fractionation was performed using the  
690 method previously proposed for serum samples (Sonnenschein et al. 1995). Three fractions  
691 from HPLC were collected:  $\alpha$ -fraction, during the first 11 minutes, containing xenoestrogens;  
692 x-fraction from 11 to 13 minutes;  $\beta$ -fraction from 13 to 25 minutes containing endogenous  
693 hormones. However, since additives and monomers from plastics (such as bisphenols) are  
694 collected alongside endogenous hormones, their effect can not be evaluated analysing the  
695 fraction of xenoestrogens.

696 This HPLC separation protocol was applied by some authors on human adipose tissues (table  
697 2, n° ref. 4, 7, 8, 15, 16, 21, 26, 35), while others applied it on human placentas (table 2, n° ref.  
698 16, 17, 22, 23, 25, 30, 31).

699 In contrast to previously reported studies, which used extraction and HPLC fractionation, two  
700 articles (Natarajan et al. 2002, Sapbamer et al. 2010) separated endogenous estrogens by  
701 xenoestrogens with two alternative methods. The former used polyclonal antibodies to  
702 immunoprecipitate the endogenous estrogens (E2) and then separate them from the  
703 xenoestrogens. In the latter dextran-coated charcoal (DCC) was used to remove all gonadal  
704 hormones. The DCC is made from acid washed charcoal powder and dextran and it is generally  
705 used to reduce the levels of estrogens in foetal bovine serum, producing the dextran coated-  
706 charcoal stripped serum. However, it is important to bear in mind that, as reported by the  
707 manufacturer's instructions (Sigma Product Information), although this treatment may reduce  
708 the levels of estrogens in the sample it might not completely eliminate estrogens or any other  
709 steroids from the serum.

710 Other studies did not apply any separation technique but extracted the biological sample and  
711 tested the whole extract. In the study of Plíšková et al. (2005), half of a crude extract was used  
712 for the determination of overall estrogenic activity, while the other half was placed on a sulfuric  
713 acid-activated silica column, eluted with n-hexane: diethyl ether mixture, evaporated, and  
714 redissolved in DMSO, in order to elute only persistent compounds (including PCBs,  
715 polychlorinated dibenzo-p-dioxins and dibenzofurans).

716 In the studies of Arrebola et al. (2012, 2013), an evaluation without separation was performed  
717 in order to assess the combined effect of endogenous estrogens and xenoestrogens, since the  
718 estrogenic activity of the whole extract can be considered as a measure of the effect of complex  
719 interactions among all estrogenic compounds (i.e. xenoestrogens and endogenous hormones).  
720 However, the estrogenic effect of endogenous hormones may be underestimated using this  
721 approach. Indeed, as specified by the same research group (Fernández et al. 2007a), this  
722 protocol was developed to efficiently extract lipophilic xenoestrogens and, as such, it may not  
723 be so effective to extract endogenous hormones. Nonylphenol, octylphenol and BPA, the most

724 polar xenoestrogens, may also be inefficiently extracted by increasing the under-estimation of  
725 the interaction among estrogenic compounds within the sample (Fernández et al. 2007a).  
726 Finally, some studies did not apply any separation techniques and did not extract biological  
727 samples but tested them without any treatment as a EDC biomarker (Brouwers et al. 2011,  
728 Kanno et al. 2007).

729

## 730 **6. Estrogenic activity as a EDC biomarker: applications**

731 The evaluation of estrogenic activity as a EDC biomarker might be a useful tool to understand  
732 the relationship between emission, exposure, biological effects and health risks associated with  
733 EDCs.

734 This evaluation can be used for EDC exposure assessment (Andersen et al. 2007) and it can be  
735 considered as a biomarker of biological effective dose (Sonnenschein et al. 1995, Soto et al.  
736 1997). However, some authors seem to apply the biomarker also as a biomarker of effect  
737 (Bonefeld-Jørgensen 2014), as it can measure the effects induced on the organism by multiple  
738 chemicals considering all the possible interactions among them.

### 739 **6.1. EDC biomarker and exposure to pesticides, polychlorinated biphenyls and** 740 **perfluorinated alkylacids**

741 Rivas et al. (2001) evaluated the estrogenic activity of adipose tissue extracts containing  
742 xenoestrogens (HPLC fractionation technique) collected in 400 women as a EDC biomarker.  
743 The concentrations of 16 organochlorine pesticides were also quantified in the extracts, but the  
744 concentration of each pesticide was not correlated with the EDC biomarker. The authors  
745 claimed that this finding could be due to the combined effect of pesticides and/or to the effect  
746 of substances that had not been measured. A subsequent study of Fernández et al. (2004)  
747 confirmed that the combined effect of compounds is difficult to predict considering the effect  
748 of each one: the authors found that the estrogenic activity of a serum extract (consisting of

749 different extract fractions obtained with HPLC fractionation technique) rarely corresponded to  
750 the sum of the estrogenic activity of each fractions.

751 In contrast with the results of Rivas et al. (2001), in the study of Andersen et al. (2007) the  
752 estrogenic activity of serum extracts containing xenoestrogens (HPLC fractionation technique)  
753 was significantly and positively associated with pesticide exposure evaluated through  
754 interviews both with pregnant and non-pregnant women working in Danish greenhouses. The  
755 results of this study also demonstrated that the EDC biomarker is representative of recent  
756 exposures. Indeed, among pregnant women, the positive association was only significant for  
757 those who had been working one week before the sampling, while no association was observed  
758 for women working in the previous period.

759 A negative association between PCB exposure and estrogenic activity was observed by  
760 Plíšková et al. (2005) who studied men living in a PCB polluted area and in a control  
761 background area (different districts in eastern Slovakia). Serum extracts containing persistent  
762 compounds (extraction technique optimized for persistent compounds) from people living in  
763 the background area showed higher estrogenic activity, while extracts from the polluted area  
764 showed an antiestrogenic activity. The association between PCB exposure and antiestrogenic  
765 activity was also confirmed by the comparison between the total estrogenic activity and PCB  
766 concentrations, since the estrogenic activity of the extracts containing endogenous estrogens  
767 and persistent compounds (crude extracts) was lower in the samples with high PCB levels.  
768 Since the levels of E2 decreased in the samples with high PCB levels, the authors stated that  
769 exposure to high PCB levels might also affect concentration of E2 in blood, causing the  
770 decrease of estrogenic activity and an overall antiestrogenic effect.

771 Kanno et al. (2007) studied the estrogenic activity of serum samples (without treatment)  
772 collected from patients who underwent hemodialysis and peritoneal dialysis compared to a  
773 control group. The patients' sera had higher levels of BPA compared to controls and they



774 induced a higher estrogenic activity compared to the activity expected for the measured BPA  
775 levels. The authors discussed the results, claiming that the high level of BPA was probably due  
776 to the release of BPA from the dialyzers to patient's serum and that the unexpectedly high  
777 estrogenic activity could be due to the release of other EDCs, such as phthalate diesters in  
778 patient's serum.

779 Brouwers et al. (2011) studied men with different EDC exposure levels and found that  
780 occupational exposure to pesticides, disinfectants and exhaust fumes were associated with  
781 increased plasma estrogenic activity (without treatment). Instead, body mass index, use of  
782 personal care products and proximity to city centre were not associated with the EDC  
783 biomarker.

784 However, the results of Kanno et al. (2007) and Brouwers et al. (2011) could have been  
785 influenced by a possible interference of xenobiotics with endogenous estrogen levels, since the  
786 authors used the estrogenic activity in total plasma/serum as a EDC biomarker without dividing  
787 endogenous estrogens from xenoestrogens.

788 Finally, Bjerregaard-Olesen et al. (2016) studied the relationship between exposure to PFAAs  
789 and estrogenic activity of serum extracts containing PFAAs (HPLC fractionation technique) in  
790 pregnant women. Their study found positive linear associations between the estrogenic activity  
791 and the PFAA serum levels but also inverse linear associations between extracts with an  
792 antiestrogenic activity (when tested with E2) and PFAA serum levels.

## 793 **6.2. EDC exposed populations**

794 The EDC biomarker was used to study Nordic populations, which are considered to be highly  
795 exposed to persistent organic pollutants (POPs) and EDCs, since numerous POPs (such as  
796 PCBs, dioxins and organochlorine pesticides) are also classified as EDCs. Due to their  
797 resistance to environmental degradation, POPs are transported to the Arctic by the atmosphere  
798 and ocean currents; here, due to their chemical characteristics, they are bioaccumulated in the

799 adipose tissue of animals and then of humans through their diet. Nordic populations are  
800 particularly exposed to POPs since their diet consists of a high intake of fish and marine animals  
801 (seals, whales, polar bears, seabirds) and includes the consumption of tissues and organs that  
802 contain animal fats where POPs are highly accumulated (liver, blubber, skin) (Bonefeld-  
803 Jørgensen et al. 2010).

804 Rasmussen et al. (2003) studied the estrogenic activity of serum extracts containing  
805 xenoestrogens (HPLC fractionation technique) collected from three groups of women: 30  
806 pregnant Danish women (slightly exposed), 60 non-pregnant Danish women (slightly exposed)  
807 and 211 pregnant Faroese women (highly exposed through diet), and found that the EDC  
808 biomarker was higher in Faroese women compared to Danish women.

809 Other authors studied the Inuit Greenlandic populations as exposed populations, and compared  
810 the Inuit men with European men from Sweden, Poland (Warsaw) and Ukraine (Kharkiv). The  
811 results of the comparison showed that Inuit serum extracts containing xenoestrogens (HPLC  
812 fractionation technique) induced no estrogenic activity but elicited an antagonistic effect when  
813 the extracts were tested in combination with E2 (71% of samples) (Bonefeld-Jørgensen et al.  
814 2006). Contrarily, some European extracts induced estrogenic activity and only a few extracts  
815 elicited an antagonistic effect when tested with E2 (7-30% of samples). The EDC biomarker  
816 was not strongly associated with serum concentration of POPs (Bonefeld-Jørgensen et al. 2006)  
817 and no consistent association was found between the EDC biomarker and the adult semen  
818 quality assessed as sperm concentration, motility and morphology (Toft et al. 2007). In addition  
819 to lower estrogenic activity, Inuits were found to have lower sperm DNA damage, while in  
820 Europeans the estrogenic activity was positively correlated with DNA damage, suggesting that  
821 altered estrogenic activity together with genetic and/or nutrient factors may protect Inuits'  
822 sperm DNA from damage (Long et al. 2007). Different correlations between sperm DNA

823 damage and the EDC biomarker were further showed when comparing Inuits and Europeans  
824 using another assay (sperm chromatine structure assay) (Krüger et al. 2008b).  
825 The EDC biomarker was also used to study differences among Inuit populations across  
826 Greenland. The study of Krüger et al. (2008a) measured the estrogenic activity of serum  
827 extracts (HPLC fractionation) in men and women from different Greenlandic districts (Nuuk,  
828 Sisimiut and Qaanaaq) to evaluate associations between the EDC biomarker, POPs  
829 concentrations (14 PCBs and 10 pesticides), and lifestyle characteristics. The EDC biomarker  
830 showed different levels depending on districts and genders. In accordance with previous  
831 studies, male and female serum extracts generally induced a decrease in estrogenic activity and  
832 showed an antagonistic effect when the extracts were tested in combination with E2. Overall,  
833 few correlations were observed between the EDC biomarker and concentrations of each PCB  
834 and pesticide. A similar experimental study was performed in 2012 by the same authors  
835 (Krüger et al. 2012) in other Greenlandic districts (Ittoqqortoormiit, Narsaq and Qeqertarsuaq).  
836 The levels of the EDC biomarker were different depending on the district. The Ittoqqortoormiit  
837 serum extracts contained high levels of POPs and elicited an antagonistic effect similar to the  
838 extracts tested in the previous study (Nuuk, Sisimiut and Qaanaaq). On the contrary, a higher  
839 agonistic effect was observed in Qeqertarsuaq and Narsaq extracts. As summarized by two  
840 reviews (Bonfeld-Jørgensen 2010, Bonfeld-Jørgensen et al. 2014), the results of studies on  
841 Greenlandic Inuit suggested that the EDC biomarker is negatively correlated with POPs and it  
842 can be used as a biomarker in order to detect POP exposure.

### 843 **6.3. EDC exposure and adult adverse health effects**

844 The EDC biomarker was also applied to study the association with type 2 diabetes and breast  
845 cancer.  
846 Arrebola et al. (2013) applied the estrogenic activity measured in adipose tissues (extraction  
847 technique) of adults from Southern Spain as pesticide and PCB exposure biomarker, in order

848 to evaluate the possible role of these substances for the onset of type 2 diabetes. In the study,  
849 the concentrations of pesticides and PCBs in adipose tissue and serum were associated with  
850 type 2 diabetes, while the EDC biomarker was not associated with the disease and did not  
851 influence the effect of the substances on the disease, indicating that estrogenic activity may not  
852 be a critical factor for the onset of diabetes.

853 Among the studies on breast cancer, Ibarluzea et al. (2004) evaluated the estrogenic activity of  
854 two types of adipose tissue extracts (containing xenoestrogens and containing endogenous  
855 estrogens- HPLC fractionation technique) and the concentration of 16 organochlorine  
856 pesticides in the adipose tissue in a case-control study, comparing women who had just been  
857 diagnosed with cancer and control women. No significant differences were observed in  
858 concentrations of pesticides and in the estrogenic activity of extracts containing xenoestrogens  
859 between the two groups. However, in women with a body mass index below the median  
860 (especially for the postmenopausal group), the high estrogenic activity of extracts containing  
861 xenoestrogens was associated with increased risk of breast cancer. The association was  
862 detected only by considering the activity of extracts containing xenoestrogens, while no  
863 association was found between breast cancer risk and the estrogenic activity of extracts  
864 containing endogenous estrogens (Fernández et al. 2007b). This first evidence of a significant  
865 association between the EDC biomarker and the risk of breast cancer was further investigated  
866 considering potential confounders and covariates (Fernández et al. 2007a). In patients, the  
867 estrogenic activity of adipose tissue extracts containing xenoestrogens (EDC biomarker)  
868 (HPLC fractionation technique) was associated with age, family history of breast cancer,  
869 lactation experience and smoking, while in controls it was only associated with age. Moreover,  
870 in patients the estrogenic activity of adipose tissue extracts containing endogenous estrogens  
871 (HPLC fractionation technique) was associated with age, educational level, age at menarche,  
872 menopausal status, marital status, lactation experience and smoking, while in controls it was

873 only associated with menopausal status. Therefore, the analysis of confounders and covariates  
874 showed that the EDC biomarker might also be sensitive to environmental, dietary, lifestyle,  
875 genetic and reproductive factors.

876 A similar case-control study (Pastor-Barriuso et al. 2016) investigated the relationship between  
877 the EDC biomarker and breast cancer risk using serum instead of adipose tissue samples. No  
878 significant difference was observed in concentrations of each organohalogenated compound  
879 (PCBs, hexachlorobenzene, p,p'-dichlorodiphenyldichloroethylene) between women with  
880 breast cancer and controls. In contrast with the previous study, the estrogenic activity of both  
881 types of extracts (containing xenoestrogens and containing endogenous estrogens -HPLC  
882 fractionation technique) was higher in cases than in controls and the estrogenic activity  
883 measured in serum extracts was not associated with potential confounders (e.g. age, body mass  
884 index) except for geographical region. Nevertheless, in accordance with the previous study,  
885 this study highlighted the importance of the EDC biomarker since it demonstrated a strong  
886 positive association between the estrogenic activity of extracts containing xenoestrogens and  
887 the risk of breast cancer. Moreover, it revealed an association with a sigmoidal trend between  
888 estrogenic activity of extracts containing endogenous estrogens and breast cancer risk.

889 A perspective study was conducted by the same research group in order to evaluate the effect  
890 of breast cancer treatment (chemotherapy and/or radiotherapy) and the influence of cancer  
891 progression on the EDC biomarker (Fernández et al. 2017). The authors assessed the estrogenic  
892 activity of adipose tissue in breast cancer patients at the diagnosis and during four other  
893 consecutive periods (<6, 6-12, 12-18, >18 months) and found that estrogenic activity of both  
894 types of extracts (containing xenoestrogens and containing endogenous estrogens -HPLC  
895 fractionation technique) increased during the treatment with a maximum peak reached at 6-12  
896 months, suggesting that cancer treatment might influence the levels of the EDC biomarker.

897 These studies demonstrated the association of the EDC biomarker with the risk and progression  
898 of breast cancer in Spanish women. Nevertheless, the same results were not obtained in a  
899 similar study performed on serum samples of Inuit women (Wielsøe et al. 2018). Indeed, in  
900 this case-control study, the authors evaluated the estrogenic activity of two types of serum  
901 extracts containing lipophilic xenoestrogens and containing PFAAs (HPLC fractionation  
902 technique); the results showed that the estrogenic activity of both extracts was not associated  
903 with breast cancer risk. This unexpected result can be due to the different assays used for the  
904 assessment of estrogenic activity. Indeed, this study applied a gene reporter assay, while the  
905 previous studies evaluated the estrogenic activity through the E-screen assay which is a  
906 proliferation assay. Moreover, other differences might be responsible for the discrepancy  
907 among the results, namely different characteristics among the studied populations, extraction  
908 methods and exposure levels.

#### 909 **6.4. Mother EDC exposure and child adverse health effects**

910 Since exposure to EDCs during pregnancy could represent a risk for children, some authors  
911 applied the EDC biomarker in order to detect a possible association between the estrogenic  
912 activity of placenta/serum extracts collected in mothers and adverse health effects in their  
913 children.

914 To our knowledge, Fernández et al. (2007c) is the first study in which the EDC biomarker was  
915 applied for this purpose. The aim of this study was to investigate the mother-child exposure to  
916 organochlorine chemicals and its association with the risk of male urogenital malformations.  
917 The estrogenic activity was evaluated on placenta samples collected from mothers whose  
918 children were affected by cryptorchidism and/or hypospadias (n=46) and from controls. The  
919 estrogenic activity of two types of extracts (containing endogenous estrogens and containing  
920 xenoestrogens- HPLC fractionation technique), was not associated with the concentration of  
921 16 organochlorine pesticides measured in the extracts. However, the estrogenic activity of

922 extracts containing xenoestrogens (EDC biomarker) showed a weak association with the risk  
923 of malformation, which was stronger when adjusted for maternal age and birth weight. The  
924 results of the study suggested that the EDC biomarker may be a risk factor for cryptorchidism  
925 (Fernández et al. 2007b).

926 Other studies evaluated the effects on children of exposure to xenoestrogens during pregnancy.  
927 In the study of Vilahur et al. (2013), the estrogenic activity of placenta extracts containing  
928 xenoestrogens (HPLC fractionation technique) was positively associated with increased birth  
929 weight and with a decrease in the risk of a rapid growth only in boys, although no association  
930 was found between the EDC biomarker and body mass index measured at 14 months of age.  
931 These results suggested that exposure to xenoestrogens during pregnancy may affect male child  
932 health in particular. A sex difference was also observed in two subsequent studies of the same  
933 authors (Vilahur et al. 2014a, Vilahur et al. 2014b). The first study showed an association  
934 between increasing levels of estrogenic activity in extracts containing xenoestrogens (HPLC  
935 fractionation technique) and lower AluYb8 DNA methylation, both measured in placentas of  
936 male children, while no significant effect was detected analysing placentas of female children.  
937 In the second study (Vilahur et al. 2014b), a lower average on motor development tests at 1-2  
938 years of age was observed in boys with mothers having high estrogenic activity of placentas  
939 (extracts containing xenoestrogens- HPLC fractionation technique), while no associations were  
940 observed in girls. The association was not found in children at 4-5 years and neither in the  
941 analysis of mental and cognitive tests in children at 1-2 years and 4-5 years.

942 Recently, Bjerregaard-Olesen et al. (2019) investigated the associations between exposure to  
943 xenoestrogens during pregnancy (evaluated with the EDC biomarker) and child parameters (i.e.  
944 birth weight, length and head circumference). Differently from the other studies, in this study  
945 the biomarker was evaluated not in placenta extracts but in serum extracts and an extraction  
946 optimized for PFAAs was used (HPLC fractionation technique and PFAA extraction). The

947 results showed that a higher estrogenic activity of serum extracts containing PFAAs was  
948 associated with lower birth weight and length of children, suggesting that PFAA exposure  
949 during pregnancy may affect child growth.

950 Finally, since EDC exposure during the first life period of children can occur through milk,  
951 Sapbamrer et al. (2010) studied the relationship between maternal estrogenic activity of serum  
952 and activity of breast milk (both treated in order to remove endogenous estrogens-DCC  
953 technique) and the correlation between estrogenic activity and lipid levels. The results showed  
954 a correlation between serum estrogenic activity and serum lipid levels, while no correlation  
955 was found in milk. Moreover, the study demonstrated that the estrogenic activity in breast milk  
956 was 8–13.5 times higher than in serum and that the two EDC biomarkers were not significantly  
957 correlated, suggesting that child exposure through milk can not be precisely estimated  
958 analysing maternal serum.

959

## 960 **7. Conclusion**

961 Estrogenic activity assays in human biological samples were applied for two main purposes:

- 962 i) as estrogen quantification and/or biomarker of estrogenic status;
- 963 ii) as a biomarker of exposure to EDCs.

964 Different methodologies were applied for the first purpose: some authors extracted biological  
965 samples, while others tested them directly without any treatment. Several methodologies were  
966 applied in order to obtain different levels of specificity: highly specific protocols with  
967 extraction allowed the quantification of low concentrations of E2, while less specific protocols  
968 without extraction, allowed the quantification of the total estrogenic activity.

969 Estrogenic activity as estrogen quantification and biomarker of estrogenic status was useful to  
970 detect low concentrations of estrogens/estrogenic compounds and allowed the detection of  
971 physiological variations of these compounds in prepubertal children and postmenopausal



972 women. This biomarker was also useful to study pediatric diseases characterized by hormonal  
973 dysregulation and women hormone-dependent diseases. Overall, in many studies a high  
974 estrogenic activity was associated with PP in females and sex differentiation disorders in males  
975 and in some studies the use of this biomarker allowed the identification of EDC exposure as a  
976 possible risk factor for these two diseases. Moreover, in girls the biomarker was also associated  
977 with Turner syndrome (one study) and type 1 diabetes (one study), while in women a high  
978 estrogenic activity was associated with higher breast cancer risk (three studies), lower hip  
979 fracture risk (one study) and lower lung cancer survival (one study). These relationships should  
980 be evaluated with caution since they were only demonstrated in a limited number of studies  
981 and through the analysis of small groups of subjects. Furthermore, the results may have been  
982 influenced by the type of estrogenic activity assay applied: some correlations were statistically  
983 significant mainly using a type of estrogenic activity assay (e.g. ER $\alpha$  transcriptional activity  
984 was correlated with breast cancer in all studies, while ER $\beta$  was correlated only in one out of  
985 three studies; ER $\beta$  transcriptional activity was correlated with lower probability of lung cancer  
986 survival, while ER $\alpha$  transcriptional activity was not). This biomarker was also important to  
987 monitor estrogen suppression/enhancement in females and males treated with different drugs:  
988 deslorelin, oral E2, transdermal E2, vaginal E2, letrozole, leuprolide acetate, anastrozole,  
989 decoction of a Chinese medicinal plant.

990 For the second purpose, in order to use estrogenic activity assays as a EDC biomarker different  
991 methodologies were applied on biological samples: some authors extracted biological samples,  
992 while others tested them directly without any treatment; some authors divided endogenous  
993 estrogens from xenoestrogens, while others tested samples without separation. The analysis of  
994 these methodologies outlined some limitations. Regardless of the technique used for extraction  
995 (solid phase extraction, liquid/liquid extraction or both) and separation (HPLC,  
996 immunoprecipitation, DCC), the extraction collects different compounds with different

997 efficiencies depending on the protocol. Thus it may underestimate the effect of some  
998 compounds, while the separation using HPLC is not always able to divide endogenous  
999 hormones from xenoestrogens correctly.

1000 Since the EDC biomarker might be a useful tool to understand the relationship between  
1001 exposure, biological effects and health risks associated with EDCs, it was used: (i) to study its  
1002 relationship with exposure to EDCs, (ii) to investigate its relationship with adverse health  
1003 effects, and (iii) to compare exposed populations with not exposed populations. The EDC  
1004 biomarker showed different associations with exposure to different EDCs. This result can be  
1005 explained considering that different EDCs may induce different estrogenic effects (agonistic  
1006 or antagonistic effects), and that the EDC biomarker quantifies the cumulative effect induced  
1007 by the combination of all these effects. Moreover, this result may also be due to different  
1008 methodologies applied in different studies (extraction/no extraction and separation/no  
1009 separation). Indeed, when samples were tested without separation the EDC biomarker also  
1010 quantified the interaction between EDCs and endogenous hormones, while, when samples were  
1011 tested after separation, this interaction was not measured. Conflicting results were also found  
1012 analysing exposed populations (i.e. antagonistic effect measured in most of the Inuit  
1013 populations, estrogenic activity measured in Faroese women), these results could be due to the  
1014 use of different estrogenic activity assays (i.e. gene reporter assay in studies on Inuits, E-screen  
1015 assay in the study on Faroese women). Finally, the EDC biomarker highlighted some possible  
1016 associations between EDC exposure and diseases (i.e. breast cancer in Spanish women, child  
1017 adverse health effects).

1018 In conclusion, the estrogenic activity on biological samples may have numerous applications  
1019 and, depending on the methodology used, it can provide different information:

- 1020 i) It can be applied as a new method to quantify low levels of E2 in biological samples  
1021 and for this purpose it should be applied extracting the biological sample;

1022 ii) It can be applied to evaluate the estrogenic status providing considerable insight into  
1023 both physiological and pathological human conditions and for this purpose it should be  
1024 applied without any treatment.

1025 iii) It can be applied to evaluate the presence of EDCs in biological samples and for this  
1026 purpose it should be applied using a separation technique, which allows the separation  
1027 of endogenous estrogens from xenoestrogens.

1028 iv) It can be applied to evaluate the presence of specific categories of EDCs (such as  
1029 PFAAs) in biological samples and for this purpose the analytical techniques should be  
1030 chosen according to the chemical characteristics of the EDCs investigated.

1031 v) It can be applied to assess the combined effect of endogenous estrogens and  
1032 xenoestrogens in biological samples and for this purpose it should be applied without  
1033 any separation technique.

1034 Therefore, the choice of methodology implies the evaluation of the research aim and the  
1035 definition of the estrogenic activity as a biomarker of estrogenic status or EDC exposure.

1036 Studies using this kind of biomarker are still limited in number; however, considering its  
1037 promising applications, future research is needed in this field in order to improve the  
1038 standardization of the different methods.

1039

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1048

### 1049 **9. Declaration of competing interest**

1050 The authors declare that they have no known competing financial interests or personal  
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1052

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1426 **11. Table captions**

1427 **Table 1.** Studies applying estrogenic activity assay as estrogen quantification and/or biomarker  
1428 of estrogenic status.

1429 Footnotes:

1430 AroER tri-screen cells = breast cancer MCF-7 variant without endogenous ER expression (C4-  
1431 12) transfected with these plasmids: pTomo-ER $\alpha$  vector or pTomo-ER $\beta$  vector, pCHGP-2,  
1432 pCMV-G, pCMV-rev, pGL4.26 [luc2/minP/Hygro] (ERE)<sub>3</sub>.

1433 *Arxula adenivorans* = strain G1212 transformed with YRC102-hER-DsRed2 plasmid.

1434 ER $\alpha$  CALUX cells = human osteoblastic osteosarcoma cells (U2-OS) transfected with 3x ERE-  
1435 TATA-Luc and pSG5-neo-hER $\alpha$  (Wong et al. 2007).

1436 HeLa transfected = human uterine cervix carcinoma cells (HeLa) stably transfected with  
1437 pERE4-Luc<sub>hygro</sub> and pEGFP-ER $\alpha$ <sub>neo</sub> or pEGFP-ER $\beta$ <sub>neo</sub> (HeLa ER $\alpha$  transfected or HeLa ER $\beta$   
1438 transfected, respectively).

1439 HELN = human uterine cervix carcinoma cells (HeLa) stably transfected with ERE- $\beta$ Glob-  
1440 Luc-SVNeo and pSG5ER $\alpha$ puro or pSG5ER $\beta$ puro plasmids (HELN $\alpha$  or HELN $\beta$ , respectively).

1441 MELN = breast cancer cells (MCF-7) stably transfected with ERE- $\beta$ Glob-Luc-SVNeo plasmid.

1442 *Saccharomyces cerevisiae* = strain BJ3505 (MAT $\alpha$ , ura3-52, tryp1 $\Delta$ 101, lys2-208) transformed  
1443 with YEPKB1 and YRPE2 plasmids.

1444 *Saccharomyces cerevisiae*<sub>1</sub> = triply deleted *pdr5 snq2 yor 1* strain transformed with hER $\alpha$ -  
1445 ERE-GFP or hER $\beta$ -ERE-GFP plasmids (Hasenbrink et al. 2006).

1446 T47D-Kbluc = human breast cancer cells (T-47D) transfected with pGL2.TATA.Inr.luc.neo  
1447 (Wilson et al. 2004).

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1451 **Table 2.** Studies applying estrogenic activity assay as a biomarker of exposure to EDCs.

1452 Footnotes:

1453 BG1Luc4E<sub>2</sub> = human ovarian carcinoma cells (BG1) stably transfected with pGudLuc7.0

1454 (Rogers and Denison 2000).

1455 ER $\alpha$  CALUX cells = human osteoblastic osteosarcoma cells (U2-OS) transfected with 3x ERE-

1456 TATA-Luc and pSG5-neo-hER $\alpha$  (Wong et al. 2007).

1457 MCF-7 transfected = breast cancer cells (MCF-7) transfected with ERE-tk109 luc, ERE2-

1458 tk109 luc, ERE-tk81 luc.

1459 MVLN = breast cancer cells (MCF-7) stably transfected with pVit-tk-Luc and pAG-60

1460 plasmids (Pons et al. 1990).

1461 *Saccharomyces cerevisiae*\_2 = yeast genome integrated with human ER and transformed with

1462 plasmid carrying the reporter gene *lac-Z* (plasmid code not specified) (Routledge and Sumpter

1463 1996).

1464 T47D.Luc = human breast cancer cells (T-47D) stably transfected with pEREtataLuc.

1465

1466 **Table 3.** Characteristics of assays for the assessment of estrogenic activity in human biological

1467 samples (Mueller 2004, Kiyama and Wada-Kiyama 2015, Seifert et al. 1999, Soto et al. 2006,

1468 Wangmo et al. 2018, Wagner et al. 2017). Abbreviations of mammalian and yeast cells reported

1469 in table 1 and table 2 (footnotes).

1470

1471 **Table 4.** Detection limits of estrogenic activity assays compared to detection limits of direct

1472 methods for estrogen quantification. Data are expressed as sensitivity for measuring

1473 estrogens/progestagens (direct methods) and as sensitivity for measuring E2 (estrogenic

1474 activity assays). Ns = not specified. Quantitation limits of direct methods for estrogen

1475 quantification are reported in table S.1 (Supplementary Material). Abbreviations of mammalian  
1476 and yeast cells reported in table 1 and table 2 (footnotes).