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Endocrine disrupters and female reproductive health

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There is growing evidence of the impact of estrogenic contaminants in the environment. Studies have shown that male fish in detergent-contaminated water express female characteristics, turtles are sex-reversed by polychlorinated biphenyls (PCBs), male frogs exposed to a common herbicide form multiple ovaries, pseudohermaphroditic offspring are produced by polar bears, and seals in contaminated water have an excess of uterine fibroids. Endocrine-disrupting chemicals (those found in the external environment that can mimic or inhibit endogenous hormones) mostly exhibit estrogenic effects, but a few are anti-estrogenic or anti-androgenic. Many of these compounds are industrial contaminants, such as pesticides and plasticizers, and others are natural phytoestrogens found in plants such as soy and in herbal supplements. Recent work shows that human development can also be feminized by exposure to estrogenic chemicals. Estrogen is the key hormone in the initiation (puberty) and the end (menopause) of reproductive life in women and thus of considerable importance in women's health. The same chemicals that affect wildlife may affect breast growth and lactation, and could have a role in uterine diseases such as fibroids and endometriosis. New studies

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provide a mechanism of action for estrogenic chemicals and other endocrine disrupters at the molecular level (called epigenetics) that may help explain the long-term effects of endocrine disruption.

Key words: early onset of puberty; epigenetics; endocrine disrupters; female reproductive health; feminization; uterus; uterine fibroids; endometriosis; estrogen; phytoestrogens.

THE PREVALENCE OF ENVIRONMENTAL ESTROGENS

Evidence from wildlife

The news today is full of stories about strange things happening to the reproductive systems of a wide variety of animals; and they all seem to be linked to hormones. For example, there are reports in the literature of fish in the Potomac River being feminized by water-borne pollutants [1]. Researchers demonstrated that the protein made in the liver that is deposited in the egg before it is laid is being made in great quantities by male fish. Egg yolk protein or vitellogenin is not usually made or secreted into the blood of male fish unless they are treated with estrogen; in this case, environmental estrogens are affecting the male fish.

Turtles can be sex-reversed, male to female, in the laboratory by painting their eggs with estrogen or, surprisingly, estrogenic polychlorinated biphenyls (PCBs) [2,3]. The effect of the PCBs is seen at relatively low levels. Some of these PCBs act like estrogen when tested in estrogen receptor binding assays or mouse uterotrophic assays. In the wild, data from Guillette and his colleagues [4] have shown that penis length and circulating levels of hormones in alligators can be compromised in contaminated lakes as compared to less contaminated sites.

Male frogs' eggs or embryos exposed in the lab to environmentally relevant levels of the widely used pesticide atrazine develop ovaries and are subfertile or infertile [5]. In fact, not only do the males—which normally only have testes—develop ovaries, they develop multiple ovaries, sometimes six or seven. And they are functioning gonads, making estrogen. Unlike the PCBs and DDT, atrazine is not an estrogen mimic even though it developmentally feminizes the frog. In fact, it is a kind of endocrine modulator that one might imagine in clinical practice. It actually is an aromatase *inducer*, enhancing the conversion of testosterone to estradiol [6], so even through the effect is indirect (production of more endogenous estrogen), the net effect is one of estrogenization.

Even mammals are affected. Polar bears in contaminated sites in the Arctic have had pseudohermaphroditic offspring [7], and a recent report described an association between estrogenic contaminants in the Baltic Sea environment and the increased prevalence of uterine fibroids in seals (yes, seals, like people, get uterine fibroids) [8]. Sheep that graze on subterranean clover ingest such high amounts of estrogenic materials from the plants (the so-called phytoestrogens) that they are essentially infertile [9,10]. More recently, Setchell et al. used knowledge of phytoestrogens to solve a reproductive mystery at the Cincinnati Zoo. Cheetahs were not breeding and had benign hepatomas, leading some to suspect that they were being exposed to high doses of estrogens (infertility and benign hepatomas being a symptom of intoxication or long-term use of high-dose oral contraceptives). There was no obvious source of estrogen in the cheetah's environment. Setchell and colleagues suspected soy-based materials in the diet as a source of estrogen. In fact that was the case. When the wild cats were given a diet without soy their symptoms resolved and several gave birth to kittens [11].

Finally, a fungus *Fusarium* sp., which infests corn and other grain in storage, is known to produce a potent estrogenic chemical called zearalenone [12]. Rather than a phytoestrogen, zearalenone is a mycotoxin estrogen. In much the same way as the Setchell group did with the cheetah, Mirocha and colleagues sought the material responsible for cessation of lactation and hyperestrogenization in female pigs that ate corn infested with Fusarium. The condition in pigs was called the *moldy corn syndrome* by farm veterinarians. Knowing that the symptoms were those of hyperestrogenization, they sought a fungal product with that activity and found it [13]. It is estimated that humans consume approximately 3 *(g*/person per day of zearalenone in North America.

Relevance to human health

What does all this have to do with the topic of female reproductive health? A lot! We know that one of the most prominent hormones in female reproduction is estrogen; the right amount at the right time and in the right place is the key to its proper function. The previous examples, chosen from among many others, demonstrate that our environment contains estrogenic chemicals, that they come in a variety of forms, including dietary, that they can have effects on virtually every vertebrate (fish, amphibians, reptiles, birds, mammals), and that the effects are similar across species. In other words, an estrogenic chemical by any other name is still an estrogen. Estradiol-17β is produced and is active in all the vertebrates. The estrogen receptors α and β are also extensively conserved across species [14]. That is to say, the effects of environmental estrogens in wildlife are relevant to medicine because they are working on cellular systems and physiological mechanisms that are very similar to our own.

In addition to the synthetic industrial pollutants that mimic estrogens, the dietary substances called phytoestrogens, and the fungal materials that give an estrogenic response, we humans of late have been adding our own natural and synthetic pharmaceutical estrogens to the environment. So when we ask the question: 'Are there estrogens in the environment?' we don't just mean contaminants that mimic estradiol or dietary chemicals that the body recognizes as estradiol, we mean estradiol. Or at least ethinyl estradiol, the major estrogen in oral contraceptives [15–17]. Pharmaceuticals including the constituent hormones of oral contraceptives have been found in virtually every stream and waterway tested in the USA [19]. In some cases the amounts of pharmaceutical estrogens are potentially quite large. For example, Joakim Larsson [20] estimated the amount of estradiol in a vaginal ring contraceptive device (now used in the US under the name NuvaRing) at disposal was 2.4 mg; that is enough estradiol in one disposed ring to reach biologically active levels in fish in 24,000,000 l of water. Thus, one of the take-home lessons for women's reproductive health will be the responsible disposal of these potent hormones.

THE BIOCHEMISTRY OF DISRUPTION

As is evident from the previously described examples, most of the environmental chemicals that mimic or block hormones, the endocrine disrupters, are estrogenic, and are from natural dietary sources or are man-made pollutants. Only a few have anti-androgenic or anti-estrogenic activity. Estrogenicity is measured via the ability of a compound to bind to the estrogen receptor and initiate a transcriptional response.

Due to the complexity of receptor interactions, one cannot simply look at the structure of a chemical and conclude that it will be estrogenic. It is worth noting that, in most cases, endocrine-disrupting chemicals are much less potent than the ovarian steroid estradiol-17β, ranging from one thirtieth to one ten thousandth as active. The phytoestrogens in soy have activity in the range of 130th–100th the activity of estradiol. Thus, it may be important to pay special attention to the diet of women with reproductive dysfunction both in terms of total soy intake (soy milk, tofu, supplements) as well as over-the-counter soy extracts and derivatives usually classified as isoflavones. We can put these relative potencies in perspective by considering that the other ovarian estrogens, estrone and estriol, are also in the range of one fortieth that of estradiol and yet are certainly biologically active.

RECOGNIZING ESTROGEN DISRUPTION

So the potential for estrogenization of humans is there. What would it look like? Our favorite example is called *The Mortician's Mystery*[21]. A 50-year-old man presents with enlarged breasts, low libido, low sperm count, etc. This suite of symptoms is associated with estrogen exposure in an adult male. The physician's differential diagnosis is that he has an estrogen-producing tumor. However, the clinical work-up finds very low testosterone levels, but no increase in estradiol, estrone or estriol. Upon interview, his physicians determine that he uses a cream in his work as an undertaker, but does not wear protective gloves. Organic extracts of the patient's serum and the embalming cream reveals an unknown compound with potent estrogenic activity. With proper care the symptoms reverse over 2 years. One of the lessons drawn by the authors was that "…some principles in our patient may be generalizable to groups. First, although he presented with striking clinical findings, it is possible that lesser degrees of exposure to estrogen in embalming compounds or other industrial exposures are more common and induce less profound disturbances of reproductive function, such as oligospermia in men or menstrual irregularities in women." (Emphasis is our own.)

One of the issues raised in the mortician's mystery is that the patient presented with *striking clinical findings*. As with many other approaches to environmental medicine, the causal chain is often hard to establish, and the effects may be subtle. Thus, the physician must rely on a thorough knowledge of biology to develop the insights necessary to bring sense to the clinical picture. One such recent example involved the adaptation of an almost classical technique for sex determination in laboratory rodents to the clinical setting. For over half a century, animal-room workers could predict successfully the sex of newborn mouse or rat pups by measuring the distance from their anus to their as yet undifferentiated genital swelling; this was known as the ano-genital distance (AGD). It is invariably larger in males than in females, so much so that skilled animal-room technicians don not actually have to measure except by eye. Chemicals such as the synthetic estrogen diethylstilbestrol (DES) or other environmental estrogens given to the pregnant rodent would cause a decrease in this measure, or feminization, if you will, of the AGD. Shanna et al. [22] wanted to know if they could use this measure to test whether exposure to known endocrine-disrupting chemicals—in this case phthalates—would result the AGD in newborn males being shortened toward the female distance in male infants. They were able to show a strong positive correlation between phthalate levels in the

mother's urine and decreasing or feminized AGD. The decrease in AGD was also associated with incomplete testicular descent. This represents an important paper in environmental medicine in general, and in endocrine disruption in particular. Phthalates are widespread environmental contaminants detected in 75% of urine samples from normal men in a CDC study [23].

ENDOCRINE DISRUPTION AND FEMALE REPRODUCTIVE HEALTH

Estrogens are the gatekeepers of female reproductive health. By their sudden increase they open the gate to enter reproductive life at puberty and with their decrease they close it at menopause. Thus, environmental chemicals that mimic or block estrogen could be expected to play a role in advancing puberty, or at least breast development, and possibly in delaying menopause. There is a great deal more information on the former than the latter. While most information on the effects of endocrine disruption on females comes from laboratory animal studies, there is a growing body of information concerning effects of exogenous or environmental estrogens and uterine function in women.

Puberty and breast development

The evidence for environmental factors effecting puberty or early breast development in girls is growing stronger and is, to a large extent, based on an increasing understanding of the physiology of puberty and a re-evaluation of the standard measurements of breast growth in girls.

An early sign of puberty is breast development, which in humans is triggered by an increase in the ratio of estrogen to androgen. There are many factors that can work together to induce the onset of breast development. One of these is metabolism of androgen. When an enzyme (CYP3A4) responsible for testosterone hydroxylation, an inactivating step in testosterone metabolism, is found as the homozygous alleles for a high-activity variant of the enzyme (CYP3A4*1B) there is an early onset of breast development [24]. Of the girls (aged 9.5 ± 0.3 years) found to be homozygous for CYP3A4*1B, 90% exhibited breast development (Tanner score 2B or higher), whereas only 40% of the girls homozygous for the low-activity variant (CYP3A4*1A) exhibited breast development at the same age. Removal of testosterone would increase the estrogen-to-androgen ratio, perhaps explaining why girls with increased testosterone hydroxylation would begin breast development earlier than girls expressing a lower-activity enzyme. Other means of increasing the estrogen-to-androgen ratio, such as increased estrogen due to over-expression of aromatase (the enzyme that converts T–E2), have been shown to cause premature breast development in girls and gynecomastia in boys [25,26]. Can environmental estrogens be raising the estrogen/androgen ratio? Given the findings of Hayes' group mentioned earlier, that the pesticide atrazine can increase aromatase in frogs to a functionally estrogenizing level, we must at least consider this possibility in humans.

There is also concern of possible increases in rates of precocious puberty in girls. For decades, the definition of precocious puberty has been based on norms established by Marshall and Tanner [27] using a British study population. The Herman–Giddens study [28] reassessed onset of puberty in US girls ($n = 17{,}077$)

and found that breast growth or thelarche (Tanner stage 2B) occurs much earlier (mean age 8.87 years and 9.96 years for African-American and Caucasian girls, respectively, compared to 11.2 years in the study of Marshall and Tanner) [27] than the previously established norms, and that menarche also occurs somewhat earlier (12.16 years and 12.88 years, compared to 13.5 years), although the difference is not as drastic for this measure. Moreover, the percentage of girls under the age of 8 years with one or more secondary sexual characteristics (27.2% of African–American girls and 6.7% of Caucasian girls) is much higher than previously reported [28]. Laboratory studies on rodents showing that pre-pubertal exposure to environmental estrogens can advance puberty in females [29] suggest that environmental estrogens may be causing this change. Whether these changes reflect a redefinition of normal or could be due to environmental influences is a matter of debate. Additionally, because the dramatic shifts are limited to breast development and/or pubic hair appearance (adrenarche), but not menarche, it is unclear whether these results reflect a true change in puberty onset or increased incidence of isolated premature thelarche and adrenarche. Pelvic ultrasonography indicates increases in mean uterine (>1.8 mL) and ovarian (>1.2 mL) volumes in cases of early central precocious puberty, but not in isolated premature thelarche [30].

Several case studies of premature thelarche after known exposure (oral or trans-dermal) to estrogens (reviewed by New [31]) indicate that these pre-pubertal tissues are especially sensitive to exogenous estrogens. A survey of parents at four US Army pediatric clinics ($n=521$) found that 64% of African Americans (but only 6.9% of Caucasians) use hair-care products containing hormones or placenta, and that half of those parents also use the products on their children [32]. This may account for some of the racial differences in the Herman–Giddens study. A case study by the author of the survey describes four African–American girls (aged 14–93 months) with premature thelarche and/or pubic hair that resolved after their mothers ceased using estrogen- or placenta-containing hair products on the girls, suggesting a transient effect of exogenous estrogen rather than a central neuroendocrine effect [33].

A more extreme example of early thelarche has been called the ‘Puerto Rico epidemic’ [34], in which toddlers were presenting at Tanner stage 2B and above at alarming rates. The incidence of premature thelarche was estimated at 6.2 per 1000 live births for children under 2 years old, and 1.62 per 1000 for girls between 2 and 8 years old. Many possible explanations have been proposed after analyses of hormones, diet, and environment. More recently, studies from an early puberty cohort in Puerto Rico showed a link between phthalic esters used as plasticizers and premature thelarche [35]. The primary endocrine-disrupting mechanism of phthalates is to act as anti-androgens [36]. As mentioned before, decreasing androgenic activity would increase the effective estrogen-to-androgen ratio, which may account for the connection between early thelarche and elevated plasma levels of phthalic esters in the Puerto Rican girls.

Breast disease

The effects of EDCs on breast cancer are covered in Chapter 9 of this volume. Here we will touch on the effects of EDCs and breast disease, specifically lactation suppression. It is well known that exogenous estrogens such as DES (diethylstilbestrol) will

effectively suppress lactation. Gladen and Rogan evaluated the role of estrogenic organochlorine compounds on lactation and found a striking adverse effect [37]. In fact, one may say that two areas that are likely affected by EDCs in women's reproductive health are thelarche and the control of lactation. Both of these are important events in the establishment of reproductive capacity.

Uterine disease

Uterine health and disease is another area where environmental endocrine-active compounds are likely to play a role. For example, it has been shown that non-human primates exposed to the widespread environmental contaminant TCDD (dioxin) have a high rate of endometriosis. The recent evaluation of the cohort of women exposed to massive doses of dioxin after a chemical accident in Seveso, Italy [38], does not support these earlier finings in non-human primates. Since dioxin is not an estrogenic contaminant its effect on the uterus may not be profound.

Another benign uterine disease, leiomyomas or fibroids, has both high prevalence (affecting from 25% of white women to 70% of black women) and morbidity (including infertility and abnormal bleeding) and is dependent on circulating estrogen. Onset of fibroids occurs after puberty, and these benign tumors regress after menopause. It has been shown that fibroids are hypersensitive to the effects of estrogen [39]. Because of its dependence on estrogen for growth, a role of environmental estrogens in fibroid disease should be considered. Our lab has shown, for example, that human uterine fibroid cells in culture proliferate to a greater degree in response to various estrogenic chemicals than do comparably cultured uterine myometrial cells (Simpson et al., unpublished observation).

A unifying theory for cellular programming of female reproductive tract response to estrogen by fetal exposures will be presented below.

FETAL DETERMINANTS OF ADULT FEMALE REPRODUCTIVE HEALTH AND DISEASE

One of the most important considerations for endocrine disruption and female reproductive health is the timing of exposure. A growing body of evidence and a developing medical hypothesis suggests that some adult diseases are programmed by the environment in utero. In the concept expressing a fetal basis for adult disease, Barker [40] has suggested that maternal malnutrition in pregnancy increases the risk of schizophrenia in the offspring.

The almost classical case for endocrine disruption in utero that leads to adult disease in the offspring is that of prenatal exposure to DES. Between 1958 and 1976 this medication was prescribed during pregnancy to prevent miscarriage. Some 4–6 million pregnancies were treated with DES in the US alone. In 1971 Herbst and colleagues described the occurrence of a rare gynecologic neoplasm in the female offspring of DES-treated pregnancies. Subsequent studies have confirmed the association between maternal treatment with the hormone and cervico-vaginal cancer in the daughters. This was the first demonstration of transplacental carcinogenesis in humans. Remarkably, the expression of disease, always after puberty, was seen from 14 years to 42 years of age. At the time of publication, the

DES phenomenon was termed a 'biological time bomb', set in utero to go off decades later. In addition to a small number of genital tract cancers, the daughters of DES-exposed mothers also had functional and anatomical abnormalities of the uterus and fallopian tubes. Fertility was also compromised (see Mittendorf and Herbst [41] for a summary of clinical outcomes).

Changes in uterine function as a result of prenatal DES continue to be described. Missmer et al. have explored the role of estrogenic chemicals in the fetal environment and the subsequent occurrence of endometriosis using the Nurses Health Study II cohort [42]. They found an 80% increased risk for laparoscopically determined endometriosis in women with a documented history of DES exposure in utero. Wise et al. [43] report an increased risk for paraovarian cysts but not ovarian cysts or uterine leiomyomas (fibroids) in a collaborative cohort study of women with known DES exposure. On the other hand, Baird and Newbold [39] report a 2.5-fold increased risk for uterine leiomyomas in a population of 819 black and 504 white women whose fibroid status was determined by ultrasound screening or surgical record review; their DES exposure was determined by interview. Collectively, one may conclude that prenatal exposure to exogenous estrogens alters the adult function of female reproductive tract.

It is hard to imagine a mechanism that could explain a fetal effect associated with estrogen that would persist into adult life and result in reproductive dysfunction. Results from animal studies are starting to provide such a mechanism, and it involves a process called epigenetics. Epigenetics describes persistent, heritable change in gene expression without change in the sequence of the DNA itself. The most common process for epigenetic change is through DNA methylation; increased DNA methylation is usually associated with a decrease in gene expression, while decreased methylation usually accompanies increased expression. Epigenetic change during development can be permanent and heritable; something as simple as feeding folic acid as a source of methyl groups for methylation to a pregnant mouse can alter the coat color of the offspring through methylation [44].

Our laboratory approached the mechanism of epigenetic change with environmental estrogens, first creating a mouse model for cervico-vaginal adenocarcinoma seen after prenatal exposure to DES in women [45]. This model also replicates the functional and anatomical changes seen in the uterus, fallopian tubes, paraovarian cysts and fertility seen in similarly exposed women [46]. We demonstrated the persistent over-expression of genes in the uterus of mice treated developmentally with DES [47]. We further showed that some of these genes were persistently hypomethylated in their promoter regions [48]. This provided a mechanism for heritable change in gene expression by environmental estrogens. Subsequently, other genes were altered and methylated [49,50]. Moreover, the cervico-vaginal cancers of women were shown by Boyd et al. [51] to display genetic instability consistent with epigenetic imprints in the absence of mutation in any expected oncogenes or tumor suppressor genes. Thus, a common element for developmental disease associated with estrogens or estrogenic chemicals may be epigenetically imprinted genes.

Recent studies on uterine leiomyoma supports this. Developmental treatment with DES results in uterine leiomyoma in mice [45], rats [52], and, at least in one study, women [39]. The study in rats used a mutant strain that had a predisposition to uterine fibroids. The authors showed that developmental treatment with DES caused an alteration in gene imprinting as demonstrated by the penetrance of a tumor suppressor

gene in the uterus. Thus, in several model systems gene imprinting by estrogen is associated with later uterine disease.

Finally, the heritability of these changes can be seen by the transmission of disease to the next generation. Mice exposed to DES early in development, when mated to control males, produced offspring that also had increased risk for vaginal adenocarcinomas [53]. Thus, the 'granddaughters' of the treated mice expressed the same rare cancer as the daughters. The implications for transmission of epigenetic traits are obvious, as is the possibility for transgenerational transmission of the DES effect in humans [41]. Transgenerational effects on reproductive capacity may be a common feature of endocrine-disrupting chemicals, since Anway et al. [54] reported very recently that pregnant rats treated with the estrogenic pesticide methoxychlor or the anti-androgenic fungicide vinclozolin have subfertile offspring, and that the male offspring pass the defect through the male germ line for at least four generations. The authors also reported altered patterns of DNA methylation in the male germ line.

We suggest that epigenetic imprinting of key genes during the development of the female reproductive tract plays a critical if not determining role in the function of those tissues in the adult. In order to understand the effect of endocrine-disrupting chemicals on female reproductive health, we will have to start considering what the intrauterine hormone environment was not only for the patient but also for the patient's mother.

CONCLUSION

An abundance of data demonstrates that disruption of reproductive function is occurring in wildlife in response to EDC exposure. Are we, as humans, exempt from such disruption? It would be difficult to imagine that we are, since the cellular and physiological mechanisms by which environmental estrogens exert their effects are similar across vertebrate species. In fact, cases of early thelarche and feminization of males have been reported in response to products that contain compounds with estrogenic activity or in response to phthalate pollutants. It is probable that human exposure to environmental estrogens has greatly increased over the past 40 years with increased agriculture, industry (including plastics manufacturing), popularity of herbal supplements and soy products, and use of pharmacological estrogens. A challenge for future investigators is to determine the extent to which humans are being impacted, as effects may be subtle or may even occur in utero, so that the influence of environmental estrogens is not seen for many years. Such was the case of DES, where some daughters of women who took DES during pregnancy discovered uterine abnormalities and rare cancers as young adults. Evidence of gene imprinting via epigenetic mechanisms suggests that the influence of environmental estrogens may be transgenerational, so that even if a source of exposure is removed, a population may be impacted for decades. The great sensitivity of both breast and uterine tissue to estrogens makes the female reproductive system especially vulnerable to compounds in the environment that mimic estrogen. Thus, as clinicians and researchers, we should consider the possibility that there may often be an environmental basis for disorders of the female reproductive system.

Practice points

- since the cellular biology of estrogens is conserved across vertebrate species, effects of endocrine-disrupting chemicals on wildlife can indicate the impact that endocrine disrupters can have on human reproduction
- industrial contaminants may alter reproductive health, as may plant estrogens (phytoestrogen) and pharmaceutical estrogens
- estrogenic chemicals come in a variety of forms and have effects on every vertebrate species, the effects being similar across species
- estrogenic pharmaceutical agents such as oral contraceptives should be disposed of properly
- in approaching endocrine disrupters in medicine, it is sometimes hard to establish a direct cause and effect, and the effects may be subtle and delayed
- endocrine-disrupting chemicals are much less potent than the ovarian steroid estradiol-17β, ranging from one thirtieth to one ten thousandth as active (keep in mind that the ovarian estrogens estrone and estriol are also only one fortieth as active as estradiol)
- pay attention to women's total soy intake, over-the-counter soy extracts, and derivatives of soy
- be especially cognizant of estrogen action in the breast and uterus
- difficulties of assessing the role of environmental hormones in reproductive health arise because changes may happen early and even in utero as seen in women exposed prenatally to DES
- reproductive health problems such as early onset of puberty, fibroids, endometriosis, and breast disease could be caused by generational exposures

Research agenda

- research in wildlife should be focused towards detecting hot spots for human exposures
- dietary estrogen trials are needed to establish beneficial or harmful effects of these compounds during critical times of development and reproductive life
- research should be directed towards biomarkers of exposure and effect in women to help close the gap of understanding in causality in environmentally associated female reproductive disease

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