

## REPRODUCTIVE TOXICOLOGY

# The male mammary gland: a novel target of endocrine-disrupting chemicals

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## Abstract

In the past several decades, the incidence of two male breast diseases, gynecomastia and male breast cancer, have increased in human populations. Whereas male breast cancer remains a rare disease, gynecomastia, a condition that arises due to abnormal development and growth of the male breast epithelium, is fairly common. In this review, we present the male mouse mammary gland as a potential model to understand human male breast diseases. Even though the male mouse typically lacks nipples, the male retains a small mammary rudiment with epithelium that is highly sensitive to estrogenic chemicals during the perinatal and peripubertal periods. In just the last few years, our understanding of the biology of the male mouse mammary gland has expanded. Researchers have characterized the complexity and size of the male mammary epithelium across the life course. Studies have documented that the male mouse mammary gland has left-right asymmetric morphologies, as well as asymmetries in the responsiveness of the left and right glands to estrogens. Recent studies have also revealed that the effect of xenoestrogens on the male mammary gland can differ based on the timing of evaluation (prior to puberty, in puberty, and in adulthood) and the administered dose. Based on the available evidence, we argue that there is a strong case that estrogenic chemicals promote the growth of the male mouse epithelium, consistent with human gynecomastia. We also argue that these outcomes should be characterized as adverse effects and should be considered in regulatory decision-making.

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## Introduction

Endocrine-disrupting chemicals (EDCs) are compounds that influence the action of hormones in the body, often by mimicking endogenous hormones, blocking the binding of endogenous hormones to their receptors, or altering the synthesis or metabolism of these hormones (Zoeller *et al.* 2012). These chemicals are found in many consumer products including food and food packaging; personal care products including cosmetics, lotions, and soaps; products marketed for the care of infants including teething rings, diapers, and baby bottles; children's toys, sports equipment and medical equipment; fabrics, upholstery, and electronics; pesticides used to control insect populations, weeds, and other unwanted pests; and many others. Human exposure to these chemicals is well documented, and many EDCs are also measured in environmental media such as air, water, dust, and soil.

Although recent work has identified many diverse mechanisms of action, to date, most of the EDCs that have been studied are estrogen receptor (ER) agonists

and androgen receptor (AR) antagonists (Gore *et al.* 2015). Many have more than one mechanism of action (e.g. both ER agonist and AR antagonist behavior). Furthermore, EDCs often produce effects that diverge to differing degrees from the effects of endogenous hormones, creating other challenges to understand the links between EDC exposures and diseases.

In spite of these challenges, a strong case has been made that EDCs affect the health of individuals and populations, including wildlife, domestic animals, laboratory animals, and humans (Gore *et al.* 2015). In 2017, a panel of the US National Academy of Sciences evaluated the evidence linking exposures to phthalates, chemicals used to enhance the flexibility of plastics and also found in many personal care products, to disrupted development of the male reproductive tract (National Academies of Sciences and Medicine 2017). After examining nineteen animal studies and five epidemiology studies, the committee concluded that phthalates alter anogenital distance and fetal testosterone concentrations, consistent with anti-androgenic

properties, which have been well-documented. Analyses of other EDCs have similarly concluded that bisphenol A (BPA), a chemical found in consumer plastics and epoxy resins, alters development of the male and female reproductive tract, consistent with its ER agonist properties (Vom Saal & Vandenberg 2020); that the herbicide atrazine alters hormone synthesis, ultimately disrupting gonadal development and other hormone-sensitive outcomes in species across vertebrate animal classes (Hayes *et al.* 2011); and that chemicals such as the anti-fouling agent tributyltin bind to peroxisome proliferator-activated receptor  $\gamma$ , promoting adipose differentiation and obesity (Heindel *et al.* 2017).

Scientists associated with the Endocrine Society have pointed out that while the number of EDCs identified continues to rise and EDC exposures continue to be documented, trends for numerous hormone-mediated diseases are similarly concerning (Diamanti-Kandarakis *et al.* 2009). In a 2012 report from the World Health Organization and United Nations Environment Programme, which evaluated the State of the Science for EDCs, it was noted that breast cancer incidence has been increasing globally for the past 40–50 years and that exposures to specific classes of chemicals (e.g. polychlorinated dioxins, some solvents, and polychlorinated biphenyls) are associated with increased breast cancer risk. The report also highlighted that there is a trend toward earlier pubertal onset and breast development in young girls, both of which are risk factors for female breast cancer; again, exposures to certain EDCs (e.g. chlorinated pesticides, polybrominated biphenyls) can disrupt pubertal timing. Although evidence for associations between EDCs and breast cancer remains limited, those studies that have been conducted suggest that the mammary gland is likely a sensitive target for EDCs (Rodgers *et al.* 2018, Terry *et al.* 2019).

The mammary gland is a fascinating organ, influenced by several hormones at different stages of development. Although the effects of EDCs on the mammary gland have been the subject of several recent reviews, very little attention has been given to the sexually dimorphic development of the gland. Because the tissue is functional in females, and disease is much more common in females, the male mammary gland has largely been ignored in studies of EDCs. In this review, we examine the male mammary gland and its sensitivity to ER agonists (and other environmental agents). We argue that the male mouse mammary gland is an excellent sentinel to evaluate environmental EDC exposures, and ultimately could shed light on diseases that affect the health of men.

## Introduction to the mouse mammary gland

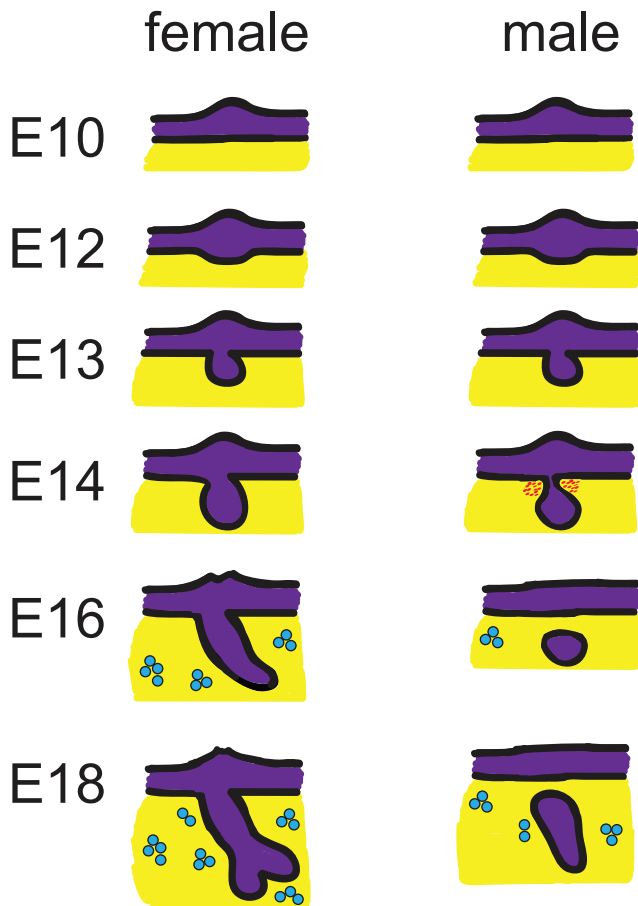
The mouse has five pairs of mammary glands located just under the skin on the ventral side of the animal, positioned from the region close to the neck (with three pectoral/thoracic pairs) down to the region next

to the genitals (two additional pairs of inguinal glands). Development of the gland includes several distinct stages, each controlled by different tissue-based signaling molecules and hormones (Hennighausen & Robinson 1998). Although there are important differences between the development of the mouse and human mammary glands, the mouse remains one of the best surrogate models because of the conserved role of hormones in the development, function and diseases of these tissues (Hovey *et al.* 1999, Hennighausen & Robinson 2001).

In the mouse, mammary epithelium derived from the ectodermal layer and mammary mesenchyme derived from embryonic mesoderm are first detected at embryonic day (E)10, when the epithelium is localized in raised points on the ventral surface of the embryo (Hennighausen & Robinson 1998) (Fig. 1). Over the next 2 days, these epithelial placodes increase to form upraised buds. Then, between E13 and E14, the placodes begin to invaginate into the underlying mesenchyme. Between E16 and E18, the epithelial anlage forms a sprout, and proliferation drives the epithelium into the mammary mesenchyme and precursor fat pad. The epithelium also branches, usually two to three times, and the solid epithelial cord begins to hollow to create a lumen (Hogg *et al.* 1983).

Between birth and the pubertal period, the female mouse mammary epithelium grows within the mammary fat pad at approximately the same rate as the other structures of the body (Hennighausen & Robinson 2001). When ovarian function initiates at approximately 3–4 weeks of age, the mammary epithelium grows at a faster rate, branching and extending the epithelium into the mammary fat pad using terminal end buds (TEBs), for example, large, proliferative structures at the tips of the ducts that penetrate the stroma (Humphreys *et al.* 1996). The presence of TEBs is a hallmark of puberty, and they are typically observed in females from approximately 3 weeks of age until 6–7 weeks of age, when the TEBs reach the edges of the fat pad and regress.

The adult female gland remains relatively unchanged, with modest increases in epithelial branches that develop with each estrus cycle; a small number of alveolar buds, differentiated secretory epithelial structures, will form in the virgin female as she ages (Richert *et al.* 2000). During pregnancy, these alveolar buds will rapidly increase in number, forming lobuloalveolar structures, until the fat pad becomes filled with these structures. At the end of pregnancy, estradiol levels spike and progesterone levels drop, initiating parturition. In the absence of progesterone, estrogen stimulates the secretion of prolactin from the pituitary, inducing lactogenesis (Kuhn 1969). As the pups grow and begin to eat solid food, they consume less milk, eventually leading to natural weaning. With the loss of suckling, mammary involution progresses in two distinct phases: first, massive numbers of secretory cells undergo apoptosis and second, the gland undergoes extensive and irreversible remodeling



**Figure 1** Schematic illustration of sexually dimorphic mammary gland development in the mouse. This diagram demonstrates several features of the male and female mouse mammary gland from embryonic day (E) 10 through E18. Purple structures represent the mammary epithelial anlage, yellow represents the embryonic mammary mesenchyme. Red dots, at E14 in the male, represent androgen receptors bound by testosterone, which is produced by the fetal testis. Light blue circles represent the presumptive mammary fat pad, which begins to develop at E16. The mammary epithelium undergoes similar development in males and females from E10, when the mammary epithelium first appears as upraised nodes on the epidermis, through E13, where the mammary epithelium has invaginated into the underlying mesenchymal tissue. The female epithelium sprouts at E16, and branches at E18. The male epithelium detaches from the overlying epidermis just after E14, preventing the development of the nipple. However, the underlying mammary epithelium, embedded in the mammary mesenchyme, does grow.

and reorganization to return to a non-pregnant appearance (Sutherland *et al.* 2007).

### Estrogens, androgens and the mouse mammary gland

The role of estrogens in the development of the mammary gland was perhaps made most clear after mice with gene knockouts of *Esr1* and *Esr2* became available for study. Development of the mammary gland appears to progress fairly normally in *Esr1* knockout (KO) females

up until puberty. However, at and after puberty, the *Esr1*KO mammary gland continues to have a stunted appearance, similar to the prepubertal mammary gland, indicating that estrogen action is essential for TEB development and ductal elongation (Bocchinfuso & Korach 1997). In contrast, the gross morphology of mammary glands from *Esr2*KO females appear normal at puberty and in adulthood, although there appear to be differences in the stromal organization and in mammary hyperplasias in later adulthood (Warner *et al.* 2020).

Androgens also play a fundamental role in the development of the mammary gland, and, in fact, are essential for the obvious sexual dimorphism observed in the mouse mammary gland. Mammary gland development follows a similar trajectory for both males and females through E13; at this stage, epithelial placodes are formed in both sexes (Fig. 1). On E14, just as the mammary epithelium has begun to invaginate into the underlying mesenchyme, the male testis produces a surge of testosterone (which is obviously absent in females). This testosterone binds to AR in the mammary mesenchyme, which causes the mesenchymal cells to condense around the epithelial sprout, detaching the epithelium from the overlying epidermis (Kratowchwil & Schwartz 1976). The lack of interaction between the mammary epithelium and the epidermis prevents nipple formation; this is why male mice lack nipples. It was long assumed that the underlying mammary epithelial tissue also degrades; however, recent studies suggest that this is not true, at least for several strains of mice (Matouskova *et al.* 2020, Kolla *et al.* 2019).

### EDCs and the male mouse mammary gland

#### Standard measures of toxicity

Despite being understudied, the male mammary gland has been used in standard toxicology studies (sometimes referred to as guideline studies) to identify and evaluate reproductive or developmental toxicants including some EDCs. Because male mice and rats typically lack nipples, nipple retention is a well-established method to identify EDCs with anti-androgenic properties (Foster & McIntyre 2002). Whereas endogenous androgens induce the detachment of the mammary epithelium from the overlying epidermis, preventing the formation of a nipple, exposures to anti-androgens during this critical window of development (usually gestational days 14–18 in the mouse) cause male nipple retention and affect other androgen-sensitive outcomes (e.g. length of the anogenital distance, which is typically longer in males compared to females, but shortened in males after exposure to anti-androgenic compounds) (Hass *et al.* 2007). For example, phthalates, which are EDCs that have been characterized by their ability to block the synthesis of androgens, induce both nipple retention and shortened anogenital distance in male rodents (Boberg *et al.* 2011, Christiansen *et al.* 2009, 2010).

### New lessons on male mammary gland biology

Almost two decades ago, Skarda *et al.* published a series of studies where the male mouse mammary gland (C3H/Di strain) was evaluated for use as a hormone-sensitive bioassay. In the first study, the typical male mammary epithelium was calculated to comprise ~1–2% of the mammary fat pad (see also Fig. 2). The size of the mammary epithelium was unaffected by administration of progesterone alone, but it was increased by estrogens (to fill ~11% of the mammary fat pad); administration of progesterone in combination with estrogen produced the largest ductal tree (filling ~40% of the fat pad) (Skarda 2001). In his second study, Skarda found a non-monotonic relationship between the effect of 17 $\beta$ -estradiol dose and the size of the mammary epithelium; mice administered 0.01–1  $\mu$ g/kg/day 17 $\beta$ -estradiol had the greatest increase in epithelial size, whereas no significant effect was observed after exposures to lower (0.001  $\mu$ g/kg/day) or higher (10  $\mu$ g/kg/day) doses. Again, when estradiol plus progesterone was co-administered, the greatest effect was seen and the dose–response relationship was still non-monotonic (Skarda 2002). Finally, Skarda evaluated the effect of three anti-androgenic substances on the male mammary gland. Unlike males administered anti-androgens during prenatal development, males administered these chemicals for 15 days starting just prior to puberty had no observable changes to the size of their mammary epithelium (Skarda 2003). These studies provided strong evidence that the peripubertal male mammary gland is affected by estrogens, but not anti-androgens, and even low doses are sufficient to induce growth of the epithelium.

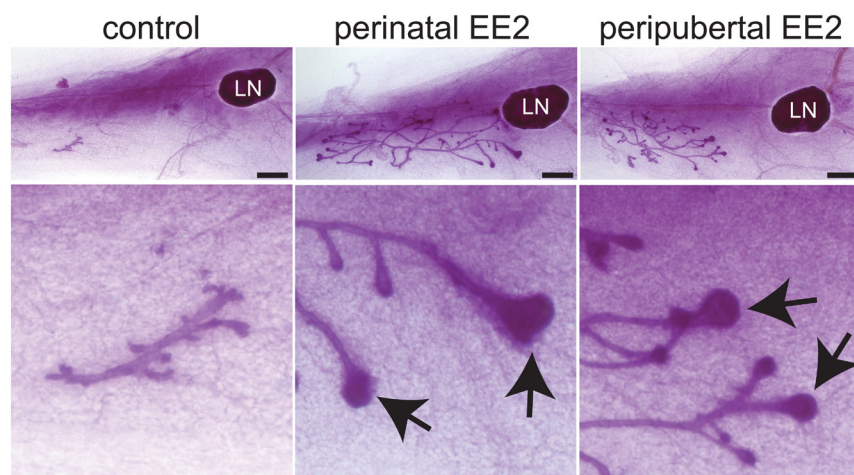
In just the last few years, new studies have revealed more about the basic biology of the male mouse mammary gland (Vandenberg *et al.* 2013, Pokharel *et al.* 2018, Kolla *et al.* 2019). Studies examining the fetal mammary gland have revealed that the male gland, like

the female gland, has a minimal number of branching points prior to birth, and that fat pad differentiation and lumen formation begin at the same time period (e.g. gestational days 16–18). Just after birth, the male mammary epithelium is approximately 0.15 mm<sup>2</sup> in area; just prior to puberty, it has grown to 0.5–1 mm<sup>2</sup>. At puberty, it increases in size to approximately 5 mm<sup>2</sup>, and then in adulthood, its size decreases to 0.5–2 mm<sup>2</sup>, depending on the age of the male.

Another important feature of the male mammary gland that was only recently described is the asymmetric size of the mammary epithelium observed in the left and right mammary glands. This is an example of a ‘cryptic asymmetry’ for example, one that appears to be unrelated to organ function or only present under certain conditions. In both the CD-1 and Balb:C mouse strains, the right mammary gland is consistently larger and more complex than the left (Pokharel *et al.* 2018). This asymmetry can be attributed in part to increased proliferation in the right gland compared to the left. There was no difference in the expression of ESR1 between the left and right mammary epithelia; however, further investigations in the CD-1 mouse revealed that the right gland is much more responsive to 17 $\alpha$ -ethinyl estradiol, with mammary epithelia that nearly double in size after exposures (1  $\mu$ g/kg/day) during the perinatal or peripubertal period (Fig. 2). Studies that fail to consider the natural asymmetry, as well as the asymmetric responsiveness of the left and right mammary glands to estrogens, could potentially prevent the detection of an effect on this sensitive organ.

### Sensitive growth parameters in the male mammary gland

Because the male mammary gland responds to low doses of estrogenic chemicals, it appears to be a good model to detect otherwise unidentified estrogenic pollutants.



**Figure 2** Both perinatal and peripubertal exposures to 17 $\alpha$ -ethinyl estradiol (EE2) promote the growth of the male mammary gland. Whole-mounted mammary glands from male mice. The mammary ductal tree is small but visible in control males. In males born to females that were administered EE2 during pregnancy and lactation (perinatal exposure to the male), the mammary epithelium is larger and more complex. This is also the case for males directly administered EE2 for 10 days, from postnatal days 21 to 30, during the peripubertal period. Scale bar = 1 mm, LN = lymph node. Bottom panels provide higher magnification views of the male mammary epithelium, including structures that resemble terminal end buds (TEBs), highly proliferative structures typically observed in the female mammary gland at puberty (indicated by arrows).

In a recent study, the male mouse mammary gland was used as a sentinel organ, able to distinguish control populations with diverse environmental histories. One population of mice came from a commercial laboratory, where their lifetime environmental chemical exposures were unknown (although standard housing is anticipated to include caging and/or water bottles made with bisphenol A (BPA), an estrogenic EDC). A second population of mice were raised in a controlled animal facility environment with limited exposures to any xenoestrogens (via efforts to screen the cages, bedding and water bottles for estrogenicity). Kolla *et al.* demonstrated that the male mammary glands were morphologically distinct between these two adult CD-1 mouse populations; the males that had been raised in a commercial animal facility had mammary glands that were both larger and more branched than those raised in the controlled facility, consistent with early life exposures to xenoestrogens (Kolla *et al.* 2017).

The male mouse mammary gland has also been used to evaluate the effects of specific estrogenic environmental contaminants. CD-1 mice exposed to low doses of BPA during early development had larger, more elaborated mammary glands in adulthood (Vandenberg *et al.* 2013). Importantly, the effects of BPA on the male mammary gland were non-monotonic, with the greatest effects observed in the mid-dose groups (2.5–25 µg/kg/day), and fewer effects observed at lower (0.25 µg/kg/day) or higher (250 µg/kg/day) doses.

A similar study design was used to evaluate the effects of bisphenol S (BPS), a BPA analogue, on the male mammary gland in CD-1 mice (Kolla *et al.* 2019). Remarkably, exposures to low doses of BPS during perinatal development diminished the size of the left male mammary epithelium prior to puberty, with no visible effect on the size of the right mammary epithelium. Yet, when examined in adulthood, the BPS-exposed mammary glands resembled what had been previously reported in BPA-exposed males, with both left and right mammary epithelial structures that were significantly larger than the epithelia of control males.

A follow-up study evaluated the effect of perinatal BPS exposure on the male mammary gland's response to a second estrogen exposure, administered at puberty. Mice that had been exposed to BPS during the perinatal period had enhanced responses to low doses of 17 $\alpha$ -ethinyl estradiol (Kolla *et al.* 2019). Greater effects were observed in the right mammary gland, consistent with prior reports that the right mammary gland is more sensitive to estrogens than the left. Interestingly, this study reported the presence of TEB-like structures in the male mammary gland. Just as TEBs in the female mammary gland are estrogen-sensitive structures, the number and size of TEBs were increased in males following pubertal estrogen exposures (Fig. 2).

Finally, a study of oxybenzone, a UV filter with complex endocrine-disrupting properties (e.g. ER agonist, ER

antagonist, and AR antagonist effects) was evaluated for its effects on Balb:C male mice (Matouskova *et al.* 2020). Exposures to low and moderate doses of oxybenzone (30 and 212 µg/kg/day), but not high doses of oxybenzone (3000 µg/kg/day), during the perinatal period decreased male anogenital distance, consistent with AR antagonist and/or ER agonist properties. Furthermore, oxybenzone-treated males had significantly smaller mammary epithelial structures prior to puberty, similar to what had previously been observed in males exposed to BPS. In early adulthood, the mammary epithelium in oxybenzone-treated males was similar, or larger than, the epithelia in control males, although these differences were not statistically significant.

Although the number of studies that have been conducted examining the effects of estrogenic EDCs on the male mouse mammary gland remains small, our understanding of the effects of these chemicals on this sensitive tissue continues to grow. We have learned that the male mammary gland is sensitive to ER agonists in both the perinatal and peripubertal periods (Fig. 2); that the left and right glands have different responses and different sensitivities; that the effects of perinatal exposures are dependent on the timing of evaluation, with some xenoestrogens producing opposite effects when the mammary gland is evaluated at or around puberty compared to the effects observed in adulthood; and several studies that suggest non-monotonic responses to estrogens are likely, with the greatest biological responses observed following administration of moderate doses.

### Diseases of the male mammary gland

Even though human males have nipples, other aspects of this organ are clearly sexually dimorphic. The healthy male breast is predominantly comprised of adipose tissue, with a small number of strands of epithelial ducts and connective tissue extending from the nipple (Dershaw 1986). This is distinct from the healthy female breast, where ducts, periductal connective tissue, and glandular tissue comprise the majority of breast structures. Some of the diseases that affect male breast tissue are conditions that affect the female as well, such as dermal lesions (e.g. epidermal inclusion cysts and sebaceous cysts) (Luanow *et al.* 2011). Males can also develop mastitis, an infection of the mammary epithelium that can cause redness, swelling, and pain in the breast tissue. Mastitis can be further complicated by the development of mammary abscesses, where infection occurs in the mammary ductal tissue.

Two breast diseases that might have relevance to studies in the rodent are gynecomastia and male breast cancer. Men are much less likely to be diagnosed with breast cancer than women because the male breast contains a minimal amount of breast epithelial gland tissue, for example, the cells where tumors typically

arise. In women, breast cancer is one of the most common types of cancer and with early detection, it has one of the best survival rates (American Cancer Society 2016). Male breast cancer accounts for less than 1% of all breast cancer cases, and less than 0.2% of all cancer cases in men (Appelbaum *et al.* 1999). Due to the rarity of the disease, men tend to be diagnosed at much later stages thus having worse survival rates.

### Gynecomastia

Gynecomastia is a condition that arises due to abnormal development and growth of the epithelium in the male breast, leading to enlargement of the organ and the occasional secretion of milk (Fentiman 2018). Clinically this disease is characterized by the presence of a mass extending concentrically from the nipple and is usually bilateral but can be unilateral. Pseudogynecomastia is a related condition that presents as increased fat deposition in the breast without glandular proliferation and occurs most frequently in obese men.

Cross-sectional studies suggest that gynecomastia arises in approximately 1% of otherwise healthy boys (aged 2–7 years) (Zhang *et al.* 2019), but its prevalence increases to greater than 30% of men aged 17–58 years, and affects more than 50% of men over the age of 44 years (Nuttall 1979). Gynecomastia is the most common breast disorder in males, and it is often observed during periods when changes to serum hormone concentrations are occurring (Al-Allak *et al.* 2011). At the start of male puberty, estrogen levels rise more quickly than testosterone which leads to an imbalance in the estrogen/androgen ratio, promoting breast tissue growth (Cuhaci *et al.* 2014). As puberty progresses, breast enlargement typically regresses, so most boys do not have persistent gynecomastia. In those who do, the condition usually spontaneously regresses within a few years of onset.

Evidence from clinical medicine indicates that pharmaceuticals that directly impact the ratio of estrogen and testosterone (e.g. anti-androgens used to treat prostate cancer, aromatase inhibitors, gonadotropin-releasing analogues, 5 $\alpha$ -reductase inhibitors, and estrogens) and other drugs that influence the metabolism of endogenous hormones (e.g. opioids, many anti-depressants, alcohol) can induce gynecomastia (Deepinder & Braunstein 2012). Data from a cohort comprised of men working in a factory where oral contraceptives were produced (e.g. men that experienced occupational exposures to 17 $\alpha$ -ethinyl estradiol) similarly suggests an association between exposures to estrogenic chemicals and gynecomastia in men (Harrington *et al.* 1978). Other diseases and clinical conditions that disrupt the balance of estrogens and androgens in men (e.g. liver cirrhosis, starvation, hypogonadism, prolactin-secreting pituitary adenomas, hyperthyroidism, renal failure) can also increase the likelihood that a male will develop gynecomastia (Cuhaci *et al.* 2014).

It has been reported that at least 30% of males will be affected at some point in their lifetime and autopsy data suggest a prevalence rate of 40%, making gynecomastia a very common occurrence. A recent evaluation of Danish health registry data concluded that in the 20 years from 1998 to 2017, there was a five-fold increase in the number of cases of gynecomastia in post-pubertal males (aged 17–20 years) (Koch *et al.* 2020). An 11-fold increase in incidence was observed in older men (aged 61–80 years) over the same period. Because genetic changes are unlikely over such a short period of time, these trends suggest that environmental factors could be contributing to disease risk, yet only a small number of studies have evaluated associations between EDC exposures and gynecomastia.

One case-control study found an association between gynecomastia and plasma concentrations of the phthalate di(2-ethylhexyl) phthalate (DEHP) and its major metabolite mono(2-ethylhexyl) phthalate (MEHP) (Durmaz *et al.* 2010). DEHP concentrations were approximately 50% higher, and MEHP concentrations were 130% higher, in boys with gynecomastia compared to age-matched controls. Although this study provides a compelling case for future investigations, it is limited by the choice of plasma for the evaluation of phthalate exposures, which are typically measured in urine to prevent contamination during sample collection. An older observational study examined an ‘epidemic’ of gynecomastia that was reported in Haitian men (aged 18–53 years) living in US detention centers; the onset of the disease was typically observed within  $130 \pm 45$  days of arrival in the US, implicating a change in environment in onset of the disease (Brody & Loriaux 2003). Further investigation revealed that these men had been exposed to phenothrin, a delousing agent used on bedding and clothing with anti-androgenic properties.

Use of essential oils, specifically lavender oil and tea tree oil, have recently been identified as potential risk factors for gynecomastia. Molecular investigations of both tea tree oil and lavender oil identified individual components that have ER agonist activities (Ramsey *et al.* 2019).  $\alpha$ -terpineol, a compound found in both essential oils, had the highest ER agonist activity in a cell-based assay; 4-terpineol, another compound isolated from both essential oils, is also an ER agonist. Linalool and linalyl acetate, chemicals found only in lavender oil, were also ER agonists. In a 2019 case report, 11 of 19 boys seen with prepubertal gynecomastia reported exposure to personal care and other household products that contained lavender (Ramsey *et al.* 2019). One case, a 7-year-old boy, reported breast enlargement at 4 years of age. The child had been exposed daily to a lavender oil since birth; within 6 months of discontinuation of the oil usage, the gynecomastia had resolved and no further recurrence was reported. An older case study of three young boys with gynecomastia revealed that all three children had been exposed to topical products

containing lavender and tea tree oils (Henley *et al.* 2007). Again, the breast condition was resolved when use of the oils ceased. A 2020 systematic review evaluated the strength of the evidence between lavender and tea tree essential oils and pediatric endocrine disorders, including seven cases of gynecomastia (Hawkins *et al.* 2020). Considering the small number of cases, this systematic review concluded that there was insufficient evidence to evaluate the causal relationship between essential oil use and gynecomastia. Although this conclusion is true when considering the scope of the issue of gynecomastia in the human population, the fact that numerous case studies included an intervention (e.g. ceased use of the essential oil) followed by resolution of the breast condition does provide strong evidence for a relationship between exposures and disease within this small group of patients. The authors of this systematic review emphasize that the lack of evidence for harm should not be interpreted as evidence of safety (Hawkins *et al.* 2020).

Collectively, these studies suggest that it is plausible that EDC exposures could contribute to gynecomastia; increasing rates of the condition suggest that environmental factors are important to disease etiology. Because this disease is not rare, it should be included in future epidemiology studies conducted to evaluate the influence of EDCs on other diseases of the male reproductive tracts, such as prostate and testicular cancer, infertility, and congenital malformations of the reproductive organs (e.g. cryptorchidism, hypospadias) (Sweeney *et al.* 2015).

### **Male breast cancer (MBC)**

Just as national health registry data suggest an increase in gynecomastia rates, several sources show an increase in MBC rates over the last several decades, and epidemiology studies provide some evidence implicating environmental agents in these increasing trends. A recent study utilized data from the Scottish National Health Service that were collected between 1992 to 2017 (Reddington *et al.* 2020). The authors concluded that MBC is typically a disease of older men, with more than 90% of diagnoses occurring after the age of 49 years. The data also revealed that MBC accounted for only 0.36% of all breast cancers diagnosed in the early 1990s, but by 2017 this number had nearly doubled to 0.65%. When the results were evaluated further based on geographic region, it was discovered that more rural areas have higher rates of MBC. The authors hypothesized that this increase in MBC rates in rural areas could be explained by higher exposures to pesticides. Several pesticides have been shown to increase the risk of breast cancer in women, providing biological plausibility to this hypothesis (Macon & Fenton 2013).

To evaluate the relationship between environmental chemical exposure and risk of developing MBC, a

case-control study examined US Marines stationed at Camp Lejeune (North Carolina, USA) from the 1950s through 1985 (Ruckart *et al.* 2015). During this period, it was discovered that the water at Camp Lejeune was contaminated with multiple chemical solvents. In comparing 71 cases with MBC and 373 controls (with cancers in other tissues), the researchers concluded that there was a non-statistically significant increased risk of MBC in men stationed at Camp Lejeune (unadjusted odds ratio=1.45, CI 0.86–2.44; adjusted odds ratio=1.14, CI 0.65–1.97). The risk of MBC was even higher, although still not statistically significant, when taking into account those men with high residential exposures to solvents (e.g. in drinking water).

Another study evaluated whether occupational exposures to gasoline and vehicular combustion products influence the risk of MBC (Hansen 2000). Automotive gasoline is known to contain benzene, butadiene, dibromoethane, dichloroethane, and combusive products that include polycyclic aromatic hydrocarbons, which induce mammary cancers in long-term (female) rodent bioassays (Macon & Fenton 2013). The case-control study examined 230 men whose breast cancer has been diagnosed between 1970 and 1989 and 56 controls per case (12,880 total) (Hansen 2000). After evaluating employment histories, men with occupational exposures to gasoline and combustion products had a significantly increased risk of MBC (odds ratio=2.2, CI 1.4–3.6). Men who were first employed and exposed to gasoline and vehicular combustion products before the age of 40 had a greater risk of MBC (odds ratio=3.7, CI 1.7–7.9).

Although it is not directly related to the question of EDCs, studies evaluating exposure to ionizing radiation suggest that this environmental agent could also contribute to MBC risk. Using data from the Hiroshima and Nagasaki Tumor Registries of atomic bomb survivors, MBC incidence was quantified among 45,880 male participants in the Life Span Study cohort (Ron *et al.* 2005). Nine cases of MBC were diagnosed between 1958–1998 in exposed Life Span Study members and three were diagnosed among nonexposed cohort members. Despite the extremely small sample size, a statistically significant dose-response relationship was observed. A follow-up study found radiation dose-dependent increases in risk of MBC in atomic bomb survivors, and much higher *relative* risk for breast cancer in men compared to women (Little & McElvenny 2017).

## **The male mammary gland is an important target of EDCs**

### **How strong is the case?**

Evidence from rodents is mounting that estrogenic EDCs can alter growth parameters of the male mouse mammary gland, especially when chemicals are

administered during the perinatal period (to the mother) or the peripubertal period (directly to the male). Even the earliest studies evaluating the use of the male mammary gland as a bioassay to detect estrogenicity of chemicals revealed that the male mouse mammary gland is highly sensitive to estrogens; the male mammary gland responded to the same doses of 17 $\beta$ -estradiol that were sufficient to alter the weight of male reproductive organs (i.e. the seminal vesicles) (Skarda 2002). Our own work demonstrates that the growth of the male mammary gland is disrupted by low doses of BPA, BPS, 17 $\alpha$ -ethinyl estradiol, and oxybenzone, all of which have ER agonist activities. Furthermore, our studies reveal that mice exposed to xenoestrogens during the perinatal period have enhanced growth responses in the male mammary gland when a second estrogen exposure is introduced at puberty. Additional studies should evaluate a broader range of EDCs (including chemicals with other mechanisms of action), with the eventual goal of including growth parameters of the male mouse mammary gland in standard hazard assessments.

The evidence linking EDCs to diseases of the male human breast is much weaker. Only a handful of epidemiology studies have even examined the hypothesis that exposures to environmental chemicals (including several with endocrine-disrupting properties) might induce gynecomastia or MBC. National registry data indicate that both diseases of the male breast are increasing in incidence, providing support for a role for environmental agents in disease etiology. At this time, only a small number of case reports and case-control studies of gynecomastia are available; because this condition is relatively common, more epidemiology studies including studies with prospective designs are needed to evaluate the strength of the relationship between EDCs and gynecomastia. On the other hand, MBC remains a rare disease, making it difficult to evaluate in typical cohorts (with a few thousand participants). Yet, understanding the environmental factors that contribute to MBC is important to identify steps that can be taken to mitigate disease risk. Future studies are also needed, likely using animal models, to better identify environmental agents that induce or promote this cancer. Such studies will first require the identification of the most appropriate and biologically relevant animal species and strains to recapitulate the human disease.

### ***Are these diseases of the male mammary gland adverse?***

An important consideration for how data from rodent studies of EDCs might be used for regulatory purposes asks whether the outcome being studied should be considered adverse. The US EPA defines an adverse effect as 'a biochemical change, functional impairment, or pathologic lesion that affects the performance of the whole organism, or reduces an organism's ability to

respond to an additional environmental challenge' (US EPA 2012). Based on this definition, studies showing that early life EDC exposures alter the male mammary gland's response to a secondary hormone challenge would certainly qualify as adverse.

Obviously, cancer is an adverse outcome. But, is the growth of the male mammary epithelium alone sufficient to determine that a chemical induces adverse effects? Although gynecomastia is not a disease with associated mortality, this does not mean that there is no morbidity or other associated costs for individuals that develop this condition. In fact, studies document that the psychological costs associated with gynecomastia can be high (Fentiman 2018). To many men, breast problems are associated with shame, perceived stigma, vulnerability, sadness, anxiety, a sense of unfairness, and loneliness. Prolonged emotional responses to male breast conditions can lead to other physical symptoms such as headaches, chronic fatigue, obesity, and hypertension; the psychological and emotional impact can be even greater in patients who are affected during adolescence and early adulthood (Kipling *et al.* 2014). There are also economic costs associated with the disease, as some men require surgery to correct recalcitrant gynecomastia (Tarallo *et al.* 2019).

Finally, there is evidence from several epidemiology studies that gynecomastia is associated with MBC risk, with one study reporting an odds ratio of 9.78 (CIs 7.52–12.7) for men with a prior history of gynecomastia (Brinton *et al.* 2014). Another found even stronger associations between gynecomastia and MBC in a case-control study focused on alcoholics, with an odds ratio of 21.7 (CIs, 6.67–70.57). These studies suggest that environmental agents that increase risk of gynecomastia (e.g. mammary epithelial growth in the male mouse) could also contribute to MBC risk.

### ***Is EDC-induced growth of the male mammary gland a sensitive parameter to probe other endocrine-mediated effects?***

Finally, it should be considered that growth of the male mammary epithelium in the mouse might be similar to changes in anogenital distance, another outcome that does not cause mortality itself, but is rather associated with diminished health and increased risk of male reproductive diseases (Schwartz *et al.* 2019). More work is needed to demonstrate the relationship between alterations to the male mammary gland and other parameters of health.

## **Conclusions**

We have introduced the male mouse mammary gland as a sensitive organ, with growth parameters that can be disrupted by low doses of estrogenic chemicals administered either during the perinatal or peripubertal



periods. Although nipple retention has long been appreciated as a marker of fetal exposure to anti-androgenic EDCs, here we have described the effects of ER agonists on the size and complexity of the underlying mammary epithelium. These studies build a strong case that the male mammary gland is sensitive to environmental disruptions, and can be used as a fairly simple and straightforward bioassay for evaluating putative EDCs.

In just the last few years, our understanding of the male mouse mammary gland has expanded. We now know that the male gland develops TEB-like structures at puberty, and the number and size of these structures increases in animals exposed to xenoestrogens. We also know that there are natural left-right asymmetries in male mammary gland morphology, as well as asymmetries in the response of the left and right glands to estrogens. Finally, we know that the effect of xenoestrogens on the mammary gland can differ based on the time of evaluation (prior to puberty, in puberty, and in adulthood), and that increasing doses of estrogens can have non-monotonic effects on the growth of the male mammary epithelium.

Human male breast diseases, including both gynecomastia and MBC, are increasing in incidence over the past two to three decades. For this reason, identifying the underlying environmental risk factors for these conditions is an important public health challenge. Although some of the lessons from female breast carcinogens may help us to understand MBC, this rare disease remains poorly studied to date. Development of a biologically relevant animal model is a critical first step to study the effects of EDCs on this rare cancer.

## Declaration of interest

L N V is a member of the US EPA's Science Advisory Board Chemical Assessment Advisory Committee, a scientific advisor (unpaid) to two Horizon 2020 EDC grants and a paid scientific advisor to SUDOC, LLC. Her travel has been sponsored by various government, academic and industry groups to present findings of her research and her EDC-related research has been funded by US government agencies, the University of Massachusetts Amherst, and NGOs including the Cornell Douglas Foundation and the Great Neck Breast Cancer Coalition. G S has no conflicts of interest to disclose.

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## Author contribution statement

G K S and L N V each wrote sections of the first draft of this manuscript. Both authors critically edited the text and reviewed the final version. L N V made the figures.

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