Oxybenzone and the Mammary Gland: Impact of an Environmental Pollutant on Health, Disease & Ethical Decision-Making

Klara Matouskova
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OXYBENZONE AND THE MAMMARY GLAND: IMPACT OF AN ENVIRONMENTAL POLLUTANT ON HEALTH, DISEASE & ETHICAL DECISION-MAKING

A Dissertation Presented

by

KLARA MATOUSKOVA

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2022

Environmental Health Sciences
OXYBENZONE AND THE MAMMARY GLAND: IMPACT OF AN ENVIRONMENTAL POLLUTANT ON HEALTH, DISEASE & ETHICAL DECISION-MAKING

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KLARA MATOUSKOVA

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ACKNOWLEDGMENTS

My deepest appreciation to Laura N. Vandenberg, her dedication to science and honesty, her inspiring personality, her enormous support to move my work forward, her nurturing spirit. I have never felt anybody “having my back” so surely.

I’m obliged to my committee members and professors Sallie Schneider, Sasha Suvorov, and Tim Ford.

Many thanks for the intellectual sparks, flames, and fires, and the kindling to feed my own sparks: Tom Zoeller, Dan Goldstein, Richard Jelinek, Theo Colborn, Naomi Oreskes, Erazim Kohak, Jan Sokol, Vaclav Havel.

Sincere thanks to our collaborators and professors: Carol Bigelow, Joe Jerry, Karen Dunphy, Amy Roberts.

To my guardians of the early days in the Vandenberg lab: Durga Kolla, Charlotte LaPlante, Aastha Pokharel, Mary Morcos.

I had great pleasure of collaborating with Shannon Silva, Josh Mogus, Gillian Szabo, Anna Schmidt, Jenny Bugos, Athena Maria, Ruby Bansal, Brenda Medeiros.

And I was lucky to cross many paths with Monika Roy - for her personal vigor and scientific rigor; her levain lives on and starts bread for my family each week. Suzanne Tromara: thank you!
My parents, for their love and support. They had had little clue on my endeavors since I graduated from medical school, but they backed me all the same. My mom did not live long enough to see this day, but my dad stands like a tree, far away still standing. My sister Hana, across the globe, thank you.

My guitar, the faithful companion who awaits silently my fingers to fill the room and my soul with beauty. And Novohradske hory, the shire distant and remote, and its people.

All those whom I met along the way who recognize that our lives are an unearned present and privilege, and live them in truth while ceaselessly examining what the heck that really means.

David, Alex & Adam: I am grateful. Without you I might have done this with more grace. Without you I would not have done it at all.

Bethesda, MD
November 1, 2021

Klara Matouskova
ABSTRACT

OXYBENZONE AND THE MAMMARY GLAND:
IMPACT OF AN ENVIRONMENTAL POLLUTANT ON
HEALTH, DISEASE & ETHICAL DECISION-MAKING

FEBRUARY 2022

KLARA MATOUSKOVA, M.D. CHARLES UNIVERSITY
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The environmental pollutant and common sunscreen compound oxybenzone is a benzophenone type UV light chemical filter used in industrial and consumer goods. This chemical widely contaminates human tissues, non-human species, and environmental matrices. In this dissertation, oxybenzone is investigated for its effects on the mouse mammary gland in the offspring following perinatal exposure; after perinatal and prepubertal exposures as a dual environmental insult during two sensitive times of development; and in adults after exposure during pregnancy & lactation as an environmental factor potentially increasing the tissue susceptibility to mammary tumors. Chapter 1 introduces the mammary gland. Chapter 2 reviews UV screening chemicals with a focus on oxybenzone. Chapter 3 summarizes methods used in this dissertation. Chapters 4 and 5 present two aspects of our studies quantifying the impact of perinatal exposure to oxybenzone on the epithelial and stromal compartments of the mammary gland. Chapter 6 evaluates the effects of exposures during two sensitive periods of development: the perinatal and prepubertal periods. Chapters 7 and 8 present a concise summary of breast cancer,
its trends, its genetic and environmental causes, and findings on tumors acquired in
BALB/c mice transplanted with p53-/- epithelium and exposed to oxybenzone during
pregnancy and lactation. Chapter 9 takes an ethics-based approach to modern-day
pollutants, including endocrine disrupting chemicals, in the process of decision-
making towards sustainable environmental and human health. Finally, chapter 10
summarizes and hints on future directions for oxybenzone research. The results of
this work make a case for oxybenzone’s disruption of the mammary gland’s epithelial
and stromal compartments during several vulnerable periods of development. Our
data also support an argument that animals genetically susceptible to breast cancer
may have heightened sensitivity to environmental chemicals. Lastly, we propose six
principles of environmental health that are imperative in decision-making on
synthetic compounds. The data presented herein contribute to the body of evidence
postulating that oxybenzone is an endocrine disruptor, and that developmental
exposures to everyday synthetic chemicals used for human safety and/or
convenience elicit later-in-life effects on susceptibility to chemical environmental
exposures, on health and disease.
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CHAPTER 1

INTRODUCTION

Human ontogenesis, i.e. the process of individual development by which all of the organs and tissues are formed from conception through adulthood, is a complex series of actions with several discrete programming windows that are ultimately responsible for dictating the individual’s biological destiny. To become a fertile male and to feel like one, three times – early in gestation, shortly after birth and at puberty - testosterone levels must be high and shift the developmental fates of cells, tissues, and organs. The female phenotype, on the other hand, is widely considered the “default” developmental pathway in mammals; and however oversimplified this view, in the reproductive system a person acquires a female-like phenotype in the absence of testosterone signaling, with specific features responsive to estrogens at specific stages of the developmental clock.

Endocrine organs in both males and females (e.g., the ovaries and testes), and sexually dimorphic structures (e.g., the penis and clitoris, and the mammary gland) are indistinguishable between the sexes early in organogenesis. At 6 weeks in utero however, XY human individuals activate the sex-determining region on the Y chromosome (SRY), leading to the production of the testes-determining transcription factor (TDF). TDF is first expressed in the somatic cells that will later develop into Sertoli cells in the testis. These supportive cells prove indispensable for the male reproductive tract as they: 1. Establish the development of the germ cells (that have just moved into the primordial gonads from the mesenchyme of umbilical cord), and 2. produce anti-Müllerian hormone that will cause the paramesonephric ducts - that would otherwise turn into female genital ducts including the uterus - to dissipate. By
gestational week 8 in humans, the Leydig cells have inundated the male embryo-
soon-to-become-a-fetus with the first surge of testosterone. Since female is the
default phenotype in mammals, during embryonic and fetal life, XX individuals do not
require signaling beyond the instructions provided by the genotype to establish the
elementary structure and functional features of the reproductive organs.

The mammary gland, too, represents a sexually dimorphic organ that
undertakes most of its development during the postnatal period. Mammary gland
development in utero begins in both sexes with a mesenchymal-epithelial induction
event: along the mammary ridges [i.e., the two epidermal lines reaching from the
armpits to the inguinal area, dependent on Wnt and FGF signaling (Chu et al., 2004)
(Hens & Wysolmerski, 2005)], the underlying
mesenchyme induces thickenings of the
overlying skin epithelium, creating epithelial
placodes that will eventually form into the
nipples (FIGURE 1.1).

1.1 The male mammary gland:
development & hormonal regulation

In male mice, the first surge of
testosterone occurs around embryonic day
(ED)13. This is the first point where the male
and female mammary gland begins to
develop into dissimilar phenotypes; by ED14
the mammary bud manifests clear dimorphic
structures (Hogg, Harrison, & Tickle, 1983). In the window of ED13 and ED16, the

Figure 1.1: Milk lines. A) Human
mammary ridge/crest forming
seven possible locations of
placodes & mammary glands,
during 5th and 4th week of prenatal
development. B) Mouse mammary
ridge: the five pairs of mammary
glands; embryonic day 11.5. C) Epithelial placode as a thickening
of skin ectoderm.
mesenchyme surrounding the invaginated epithelium becomes responsive to testosterone via the temporary expression of the androgen receptor (Kratochwil & Schwartz, 1976). Thus, the binding of testosterone to the androgen receptors expressed in the mammary mesenchyme activates the fibroblasts to condense around the stemming mammary bud and gradually detach the stem of epithelial cells from its ectodermal placode. This causes both the stem and the placode to regress.

Of note, a series of experiments in genetically androgen-insensitive mice in the 1970s showed that the epithelial regression observed in a typical XY male mouse is achieved due to AR-sensitive mesenchyme cells (Kratochwil, 1971; Kratochwil & Schwartz, 1976). Later, seminal experiments showed that the epithelium induces the receptors in the underlying mesenchyme in a process dependent on parathyroid hormone-related protein (Dunbar & Wysolmerski, 1999; Durnberger & Kratochwil, 1980). Activated parathormone-related protein receptor (PTHrP) in the mesenchyme then activates outgrowth of the epithelium (Hens et Wysolmerski, 2005).

The second testosterone peak, sometimes referred to as minipuberty, takes place in human males between two and four months after birth. Thereafter, concentrations of the hormone decrease to a minimal level until the real puberty around the age of 12 for boys and weeks 3.5-5 in mice (Brix et al., 2019; Falconer, 1984; T. Ma et al., 2021). Even after this second testosterone surge, a male mammary gland below an absentee – in mice, or present – in humans, nipple form rudimentary mammary ducts surrounded by stromal connective tissue, all residing in the mammary fat pad (FIGURE 1.2A). Of course, these epithelial structures are much smaller and less elaborated compared to females, and the absence of a nipple in mice makes them non-functional.
The perinatal male mammary gland does not grow beyond two or three primary and a few secondary branches; in CD1 mice, the area comprised of mammary ducts reaches 0.19 mm$^2$ at ED18 and 0.5 mm$^2$ at PND5 (Vandenberg, Schaeberle, Rubin, Sonnenschein, & Soto, 2013). Around puberty, the size of the mammary epithelium increases about ten times, but in CD-1 males it regresses to about a half of its pubertal area at 7 to 9 months of age (Pokharel, Kolla, Matouskova, & Vandenberg, 2018). BALB/c males will increase the ductal area from about 0.2-0.25 mm$^2$ at PND21 (the right mammary gland being consistently larger) to 0.35 mm$^2$ at puberty, and 0.5 mm$^2$ at 9-11 weeks postnatally (K. Matouskova, Jerry, & Vandenberg, 2019). In terms of complexity, prepubertal male CD1 mammary glands display about ten branches, and BALB/c males typically have about seven.

As discussed more in Section 1.2, mice lacking one or more hormone receptor have been instrumental in determining the role of endocrine signaling in the development, growth, and function of the mammary gland and other organs. For example, in males lacking estrogen receptor α (the ERα KO mice), reproductive fitness is poor due to suppressed mating behavior and reduced sperm count, and later in adulthood the weight of the testis decreases (Couse & Korach, 1999). However, the consequences of the absence of ER on the male mammary gland have not been examined. On the other hand, exposure to ER agonists such as bisphenol A and S, and ethinyl estradiol (S. Kolla, M. Morcos, B. Martin, & L. N. Vandenberg, 2018; Vandenberg et al., 2013), along with oxybenzone (K. Matouskova et al., 2019), and agricultural chemicals (Altamirano et al., 2015) alter the structure and other endocrine-sensitive parameters of the male mammary gland. While rare compared to other diseases in male populations such as prostate and testicular cancers, both gynecomastia and breast cancer affect male populations.
with increasing prevalence (Koch, Brauner, Busch, Hickey, & Juul, 2020). An aggravating widespread pollution with endocrine disrupting chemicals and sensitivity of the mammary gland to those compounds makes research into the male mammary gland and regulatory consideration for the organ a pressing matter (Szabo & Vandenberg, 2021).

1.2 The female mammary gland: development & regulatory signaling

In contrast to the male, the female mouse mammary gland sustains profound re-organizational changes during several periods through the life course. The earliest hint of the mammary gland amounts to no more than a thickening of the epidermis: the placode, which arises at ED11-E12. At ED12.5, a more pronounced structure, the mammary bud, is formed by the mammary epithelium arising from basal keratinocytes which overexpress PTHrP, and the underlying epithelium-induced mesenchyme (Foley et al., 2001). At ED13.5, the surface epidermis shows a slight invagination of the mammary epithelial anlage into the underlying ventral mesenchyme, and at ED15.5, the developing mammary gland is marked by a distinct invagination on the surface and a cleft on the bottom of the mammary epithelial sprout indicative of branching (Hogg et al., 1983; Robinson, 2007). By ED18, the surface epidermis is fully modified into the skin of the nipple and by ED19, 10-15 branches of the ductal tree now advance through a fat pad.

The epithelial cells of the placode and the early mammary bud arise from migrating keratinocytes and indeed, the average mitotic index each day before ED14 is only 0.218% suggesting that proliferation of these cells is rare; yet from ED16 through the end of gestation it increases to over 1% (Balinsky, 1950). Morphogenetic processes of epithelial proliferation and differentiation, tissue elongation and
branching generate a ductal tree of the size of 0.18-0.2 mm² at ED18 with 2-4 branch points, and a considerably larger gland (2.5-4 mm² and 15-20 secondary branches present at birth (Hens & Wysolmerski, 2005). After birth, a typical female mouse mammary gland grows isometrically to the size of the body until just before puberty. Because the female phenotype is considered the default in mammals, and early developmental acquisition of female characteristics are not dependent on the production of ovarian hormones (e.g., estrogens or progesterone) the pre-pubertal mammary gland will have a normal appearance even in females where the fundamental hormonal influence, the ER, is knocked out (Bocchinfuso & Korach, 1997). Embryonic mammary glands devoid of receptors for growth hormone or prolactin – such as IGF-1R and EGFR or PrlR – do not have a distinct phenotype either, a circumstance that further stresses the hormone-independence of embryonic development of the organ (Hovey, Trott, & Vonderhaar, 2002).

Female puberty marks the beginning of extensive changes in the mammary gland, and it is characterized by formation of terminal end buds (TEBs). Microscopically, TEBs consist of a mass of loosely connected, E-cadherin expressing body cells and an exterior line of highly regenerative, P-cadherin-positive cap cells covered with a permeated basal lamina (Paine & Lewis, 2017). Contrary to their name, terminal end buds open the tips of the ducts to an active growth; behind the TEBs, the mammary epithelial network elongates and branches into a complex, fractal geometry (FIGURE 1.2A).

Tissue changes at puberty arise from gonadotropin releasing hormone (GnRH) released by the hypothalamus, further inducing the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the hypophysis. FSH stimulates granulosa cells of the primary (and advanced/older) ovarian follicle to convert
androgens (from the cells of *theca externa et interna*) to estrogens; LH supports the theca cell and triggers ovulation – i.e., releasing the egg and forming the progesterone-producing structure, the *corpus luteum*. In the mammary gland, estrogens bind their respective receptors (see further discussion below) and elicit ductal growth. During puberty, ERα expressed in the mammary epithelium is the primary and essential target of estrogens, whereas the stromal ERα (Mallepell, Krust, Chambon, & Brisken, 2006a) acts in a paracrine manner impacting the neighboring cells (J. Russo, Ao, Grill, & Russo, 1999). Progesterone signaling at puberty supports side-branching of the mammary epithelium by working synergically with IGF-1 whose transcription is activated via growth hormone; IGF-1 activates IGF-1R in epithelial cells (W. Ruan & Kleinberg, 1999).

By the end of puberty (approximately 6 weeks in mice), the TEBs have regressed, and the mammary ductal epithelium spans nearly the entire fat pad. Estrous cycles (in mice) or menstrual cycles (in women) bring about only minor adjustments in the epithelial network: with each cycle there is modest epithelial cell proliferation and occasional tertiary branching, as well as incomplete differentiation of alveolar buds. Each cycle is followed by macrophage influx to remove the cyclically regressed alveolar cells (Richert, Schwertfeger, Ryder, & Anderson, 2000).

Over adulthood and especially during pregnancy and lactation, progesterone along with prolactin plays a lead role in outfitting the ducts with alveolar buds (in mice) or tubulo-alveolar units (in women) namely via paracrine signaling (Slepicka, Somasundara, & Dos Santos, 2021). When oxytocin launches the processes of parturition though uterine contractions, the same pituitary hormone also induces contractibility in mammary myoepithelial cells. Interestingly, dams with mutated
oxytocin were able to deliver normally but could not commence nursing (Young et al., 1996).

Figure 1.2: Mammary Gland Development. A) epithelial ductal mammary tree during lifetime in the female and male. Embryonic stage reproduced from (Hens & Wysołomierski, 2005). B) Pregnancy – Lactation – Involution cycle in the mammary epithelium
At the end of the lactational period, upon weaning, the mammary gland enters two phases of involution: 1) A reversible, 1 to 2-day phase in mice, that reduces milk production. In this phase, epithelial cells in alveolar structures begin to undergo apoptosis coinciding with infiltration of lymphocytes and macrophages. 2) An irreversible phase that protects the ducts from milk accumulation with massive elimination of epithelial cells and remodeling of the mammary gland that includes repopulation with adipocytes (O’Brien, Martinson, Durand-Rougely, & Schedin, 2012). Signaling pathways during the irreversible stage of involution resemble those guiding wound healing processes, namely signals inducing the activation of matrix metalloproteases tasked with degradation of extracellular components and cyclooxygenases activating immune responses in the remodeling period (Slepicka et al., 2021). Interestingly, many of the pathways and genes expressed during involution also contribute to breast cancer development and metastatic processes (Jena, Jaswal, Kumar, & Mohanty, 2019; O’Brien et al., 2012; Wallace, Tarullo, Crump, & Lyons, 2019).

1.3 Hormonal Signaling in the Mammary Gland and beyond

The hypothalamic–pituitary–gonadal axis reaches maturity at puberty and around PND31, the height of puberty in mice, an allometric growth of the mammary epithelial network begins simultaneous with increasing circulations of gonadotropins. Yet proliferation of epithelial cells in the mammary gland requires multiple hormones, growth factors, and their receptors.

Estrogens are ancient hormones that bind evolutionarily highly conserved estrogen receptors; estrogens are required for ductal outgrowth (Korach et al., 1996; Thornton, Need, & Crews, 2003). FSH released from the hypophysis activates
production of estrogens in the ovarian granulosa cells of forming follicles. Other sites of estrogen synthesis include post-ovulation corpora luthea and adipose tissue where estrogens can be synthesized using aromatase, namely in postmenopausal women (Simpson, Ackerman, Smith, & Mendelson, 1981). In estrogen-responsive organs, two classes of nuclear estrogen receptor (ER) are now recognized: ERα and ERβ, and remarkably, the two were discovered more than thirty years apart (Jensen, 1962; Kuiper, Enmark, Pelto-Huikko, Nilsson, & Gustafsson, 1996) although their roles are quite contrasting – and possibly also complementary. During active development of the mammary ductal tree, expression of ERα is high, consistent with the temporal epithelial cell proliferation. While ER β low; on the other hand, the reverse is observed in periods of organ “maintenance” – ERα expression wanes while ERβ increases (Dall et al., 2018). ER β is expressed in adult tissues including the ovaries, vagina, and uterine endometrium, testes and prostate (Taylor & Al-Azzawi, 2000). In the mammary gland ER β has been detected in animals older than 6 weeks in rats and FVB mice (Dall et al., 2018; Saji et al., 2000), and in both active and resting human breast (Taylor & Al-Azzawi, 2000). Despite challenges in producing the ERβ KO mouse model (Warner et al., 2020) and most studies relying on cell cultures, the evidence up to date is leaning towards ER β downstream pathways inhibiting cell proliferation and promoting cell differentiation (or apoptosis) in breast cancer tissues (Leygue & Murphy, 2013), and even in genetically disadvantaged p53 null cells (Thomas, Strom, Lindberg, & Gustafsson, 2011). ERβ KO mice develop a normal mammary gland and can lactate normally (Brown, Manzolillo, Zhang, Wang, & Lamartiniere, 1998). The presence of ERβ might be one of the answers to the long-term protective effect of parity as the ratio of ERα and ERβ cells shifts toward the latter in older parous dams (Dall et al., 2018).
**ERα** is encoded in the mouse by the gene *Esr1* and is well-appreciated for its indispensable, estrogen-driven mediation of proliferation during both normal and pathological conditions in the mammary gland (Couse & Korach, 1999; Hall & Korach, 2002). In their seminal paper, Mallepell and colleagues investigated ductal morphogenesis in ERα⁻/⁻ mice, specifically the role of epithelial vs stroma ERα. They concluded that mammary epithelium is “the prime compartment of ERα signaling both before and during pregnancy” while stromal ERα plays a supportive role (Mallepell et al., 2006a; Mueller, Clark, Myers, & Korach, 2002).

![Figure 1.3: Endocrine Components of Reproductive Organs & Tissues](image)

Classic nuclear ERs regulate expression of target genes via binding the estrogen response element (ERE) on the DNA. Yet, two other ER pathways with different mechanisms of action have been discovered. G protein-coupled estrogen receptors 1 (GPER1), which are structurally unrelated to the nuclear receptors, are
located on the cell membrane. GPER1 is responsible for rapid non-genomic effects of estrogen via a host of second messengers and kinases regulating gene expression through phosphorylated transcription factors (Madak-Erdogan et al., 2008). Signaling via GPER1 is responsible for phenotypic effects including sexually dimorphic differences in a variety of organs including those of the cardiovascular system and gastro-intestinal tract (Doolan & Harvey, 2003; Puglisi et al., 2019). In addition, ER may bind DNA indirectly through various co-factors involving genomic but “non-classical” signaling (Safe & Kim, 2008). Collectively, estrogen actions are no longer seen as simplified measures, equal to uterotrophic ERα-induced responses; rather, estrogens elicit variable effects via actions through more than one estrogen receptor. This is not only true for endogenous estrogens like estradiol: recent research points to xenoestrogens acting via non-genomic receptors often with remarkable intensity. For example, bisphenol A is considered a weak ER agonist judging by its action on nuclear ERs, yet nanomolar concentrations of BPA are capable of increasing calcium oscillation in the pancreas to levels comparable to estradiol (Hall & Korach, 2002; Nadal et al., 2000).

Downstream of estrogen signaling in the mammary gland, amphiregulin binds epidermal growth factor receptor (EGFR) and in a paracrine manner triggers several other local growth factors (Ciarloni, Mallepell, & Brisken, 2007). In both health and disease, amphiregulin is a key transcriptional target of ERα (McBryan, Howlin, Kenny, Shioda, & Martin, 2007) and in initiated ERα-sensitive breast tumors, the absence of amphiregulin significantly extends time-to-tumor in Tp53-/- mice (Meier, Girtman, Lofgren, & Kenny, 2020).

In the mammary gland, estrogens act in concert with **progesterone**, another potent gonadotropin that often stays in the shadow of estrogens in the clinical
setting. Yet it is the synchronous action of the two hormones that supports good health of the female reproductive system, as well as the cardiovascular system, brain, and bones throughout the reproductive years (Prior, 2020). Two main progesterone receptor (PR) isoforms were identified: PR isoform A and isoform B, both encoded on a single gene in both humans and rodents (Kariagina, Aupperlee, & Haslam, 2008). A third isoform has also been recognized, PRC, yet not extensively studied – research from different cell-culture and tissue models provide hints of contradictory effects it may have both inhibiting activation effects on PRA and PRB (Condon, Hardy, Kovaric, & Mendelson, 2006; Wei et al., 1996). Research on the two main isoforms also suggests different distribution and regulation in different organs and tissue cultures (Vienonen, Syvala, Miettinen, Tuohimaa, & Ylikomi, 2002). For example, in MCF-7 cells, 17β estradiol preferentially upregulates PRA while in ZR751 breast cancer cells PRB is preferred.

In the mammary gland, PR is present in both mammary compartments; in the stroma where progesterone signaling promotes ductal outgrowth and in the epithelium in which PRs are required for formation of alveolar buds during pregnancy (Humphreys, Lydon, O’Malley, & Rosen, 1997). Although progesterone plays a mere supporting role during puberty, it gets promoted to a co-lead over adulthood (with a role in the regulation of the estrous cycle) and during pregnancy. Its absence at puberty delays mammary ductal outgrowth, and both side branching and formation of alveolar buds are absent in PR-/- female mice (Brisken & Ataca, 2015). Ovariectomized adult mice administered progesterone alone increased proliferation in luminal and myoepithelial cells although not as significantly as estradiol-treated alone, while in a combined treatment, progesterone enhanced the mitogenic effect of estrogens (Aupperlee & Haslam, 2007). A combined estrogen+progesterone
treatment enhanced also side branching via PRA in adult females and alveologenesis via PRB over pregnancy.

Downstream PR signaling induces a release of Elf5, the “master regulator of alveologenesis” and signature transcription factor in luminal cells (Oakes et al., 2008), as well as RANKL and Wnt4. All three are common targets between progesterone and prolactin (see below). Overlapping with estrogen signaling pathways is amphiregulin. This transmembrane glycoprotein has been found to be critical for mammary ductal development via autocrine and paracrine manner in both normal growth at puberty and disorderly outgrowth in breast cancer (Aupperlee, Leipprandt, Bennett, Schwartz, & Haslam, 2013; McBryan, Howlin, Napoletano, & Martin, 2008).

**Prolactin** is another mammary mitogen required for alveologenesis and milk secretion (Das & Vonderhaar, 1997) (Brisken et al., 1999). However, in females in adulthood, prolactin is also indispensable for nearly every aspect of reproduction including mating and maternal behavior, ovulation, fertilization, and uterine receptivity (Kelly et al., 2002). In males, prolactin is partially replaceable in reproduction; only half of the homozygous Prl-/- males were completely or nearly infertile, leaving half with functional reproduction (Ormandy et al., 1997). The expression of PrlR in macrophages and lymphocytes in human peripheral blood samples, as well as secretion of several cytokines that can be blocked by anti-PRL antibodies, suggest it has an immunomodulating role (Chavez-Rueda et al., 2005).

Prolactin is a peptide hormone synthesized and secreted from the anterior hypophysis and its regulation is predominantly inhibitory – dopamine released by the hypothalamus is its key inhibitor. On the other hand, a number of physiological and pathological factors increase circulating prolactin levels including pregnancy and
nursing, exercise and stress, estrogens and licorice, pituitary tumors and severe renal and liver diseases (Javorsky, 2018). Thyroid hormones, too, exert positive control over prolactin; T3 and T4 regulate binding of prolactin in the mammary gland, and hypothyroidism induces repairable stunted growth of the gland (Vonderhaar & Greco, 1979).

Molecular pathways specific for prolactin signaling include phosphorylation of the prolactin receptor that induces the JAK2 – STAT5 signalizing pathway; dimerized and phosphorylated Stat5 translocates to the nucleus and consequently induces expression of milk proteins (Brisken & Ataca, 2015; Hennighausen, Robinson, Wagner, & Liu, 1997).

The anabolic growth hormone (GH) induces expansion of the mammary ductal tree as well. GH expression is high during puberty and its inhibition stunts growth of the mammary gland, particularly side branching and lobuloalveolar development (Sejrsen, Purup, Vestergaard, Weber, & Knight, 1999). Although their receptors are located in different tissues, GH, prolactin and epidermal growth factor, activate the STAT5 pathway in the mammary gland; prolactin activates the pathway in epithelial cells while GH appears to induce growth via its stromal-bound receptors (Gallego et al., 2001). GH action is mediated directly via the GH receptor and indirectly via insulin like growth factor 1 (IGF-1) (Hu, Li, & Zhang, 2021). IGF-1 is most likely responsible for the interplay between GH and estrogens both in normal development (where estrogens enhance the GH-IGF-1 axis) and in breast cancer (where IGF-1 may serve as prognostic or diagnostic tool and/or possible treatment). Based on their effects on MCF7 cells, a breast cancer cell line, antibodies against IGF-1 receptor may reduce tumor cell proliferation (Laban, Bustin, & Jenkins, 2003).
Lastly, apart from endocrine signaling, paracrine and autocrine signaling growth factors and receptors are involved in the regulation of mammary gland morphogenesis. A clear understanding for the role of paracrine signaling came with the discovery of ER- and PR-positive epithelial cells that were distinct in location from proliferating cells in the mammary ducts (Clarke, Howell, Potten, & Anderson, 1997). Soon after this discovery, the Brisken lab uncovered that both PR (Brisken et al., 1998) and ERα (Mallepell et al., 2006a) act in a paracrine manner. Along with amphiregulin, a molecule regulated via ER, EGF and PR (discussed above), the most common mediators of paracrine communications in the mammary gland include Wnt4, and Elf5 – RANKL; these pathways are associated with PR signaling (Beleut et al., 2010; Brisken et al., 2000) and prolactin-regulated IGF-2 signaling during lactation (Brisken et al., 2002).

Paracrine signaling may take place either within a tissue compartment, i.e. among epithelial cells, or between tissue compartments. In the mammary gland, this would be between the epithelial and stromal compartments. From the earliest stage of placode formation, the mammary tissue compartments induce each other via a
host of local molecules. For example “inhibins and activins” of the transforming growth factor β (TGF β) are required for effective differentiation of epithelial cells, particularly the inhibin βB subunit is involved in stromal induction of epithelium (Robinson & Hennighausen, 1997). Bone morphogenetic proteins (BMPs) are also members of the TGFβ family of signaling complexes and in the mammary gland, the PTHrP – which sensitizes BMP4 in the embryonic mesenchyme - stimulates fetal outgrowth of the epithelial ducts (and inhibits hair follicles in the surrounding epidermis) (Hens et al., 2007). In a recent study, BMP1 was demonstrated to facilitate communication between myoepithelial and luminal cells, and regulating timely formation of alveolar buds during late pregnancy (Shao et al., 2021). In early development, PTHrP induces androgen-responsive peri-epithelial mesenchyme in the male embryonic mammary bud (Hens & Wysolmerski, 2005) however it seems dispensable during puberty and even lactation – contrary to previous hypotheses (Boras-Granic, VanHouten, Hiremath, & Wysolmerski, 2011). Also critical is the role of matrix metalloproteinases in remodeling both the normal and diseased mammary gland (Benaud, Dickson, & Thompson, 1998). Finally, Wnt pathways are perhaps the most challenging and the most complex set of signaling molecules that play a role in practically every stage of pre- and postnatal mammary gland development (Q. C. Yu, Verheyen, & Zeng, 2016). Both canonical and non-canonical Wnt pathway are triggered in formation of the milk ridge, mammary bud, and ductal lumen of TEBs (Paine & Lewis, 2017), and in the stromal and luminal cells in adulthood and throughout pregnancy (Q. C. Yu et al., 2016).
1.4 Disruption to the Mammary Gland Signaling

As depicted in the sections above, mammary morphogenesis and functioning involves a spatially- and temporally-sensitive set of events and interactions. Disruption to either endocrine or paracrine signaling have been investigated in all stages of mammary gland development. While prepubertal development of the mammary gland is not dependent on estrogen signaling, it is indeed sensitive to xenoestrogens.

Evidence from both human and animal studies demonstrates that early exposures to pharmaceuticals and environmental estrogens are associated with increased breast cancer risk later in life. Iatrogenic exposures of human embryos and fetuses to diethylstilbestrol (DES), a pharmaceutical estrogen, exacted an enormous physical and emotional toll on millions of women and men worldwide (Reed & Fenton, 2013). DES is a potent ER agonist that activates both nuclear and G protein-coupled ERs, but its estrogenic disruptions go beyond binding ERs. Like bisphenol A (BPA) - a synthetic estrogen related to DES in structure but not in application (e.g., BPA is used predominately in plastics and related materials requiring durability) – DES is considered an endocrine disruptor, meeting nine out of ten key characteristics as suggested in a recent consensus statement (La Merrill et al., 2020). Endocrine disruptors – including DES, BPA, as well as the pesticide DDT and its metabolite DDE, and industrial chemicals such as polychlorinated biphenyls (PCBs) - are defined as chemicals that act as agonists or antagonists of hormone receptors, as well as compounds that alter secretion, synthesis, or transport of hormones, or alter cell behaviors (e.g., proliferation, apoptosis) in hormone sensitive-cells, as well as compounds that induce epigenetic alterations in endocrine organs.
(Calaf, Ponce-Cusi, Aguayo, Munoz, & Bleak, 2020). Alterations in paracrine signaling has also been suggested as a mechanism of endocrine disruption (Ustundag & Emekli-Alturfan, 2020).

Normal development of the breast is key to mammalian reproduction because this organ is responsible for nursing and nourishing of the young. Beyond breastfeeding, women carry this hormone sensitive tissue around for many years before and many years after pregnancy. Breast cancer incidence has been increasing in human populations both before and long after pregnancy (DeSantis et al., 2019; Lima, Kehm, Swett, Gonsalves, & Terry, 2020). Perturbations to the mammary gland induced by environmental chemicals during sensitive periods of breast development – during gestation, perinatal and pubertal development, pregnancy and lactation – have been associated with permanent adverse alterations to the mammary gland (Macon & Fenton, 2013; Schwarzman et al., 2015; Soto, Brisken, Schaeberle, & Sonnenschein, 2013). In this dissertation, I have evaluated the effects of oxybenzone, an environmental benzophenone-type chemical sunscreen with endocrine disrupting properties, on the mammary gland.
CHAPTER 2
UV SCREENING CHEMICALS

Large portions of this chapter are published as:
Elsevier Academic Press.

2.1 Abstract
UV absorbing chemicals are used in numerous consumer products including over-the-counter sunscreens and other personal care products. Furthermore, UV stabilizing chemicals are now widely used in consumer goods including plastics, fabrics, paints, and building materials to protect the products from UV damage. The widespread use of UV absorbing and UV stabilizing chemicals in consumer goods has led to contamination of environmental matrices (including water, sewage, sediment, and dust), wildlife populations, and human bodies. In this chapter, we review the evidence for this widespread contamination, as well as what is known about the effects of some of these chemicals including the UV absorbing chemical oxybenzone (and its metabolites), and benzotriazole derivatives, a class of UV stabilizing chemicals.

KEYWORDS
benzophenone, skin cancer, endocrine disruptor, liver toxicity, ecosystem, carcinogenesis, coral, pollution
2.2 Introduction

Humans have long protected their skin from prolonged sun-exposure as we understand from ancient Egyptian texts instructing the application of rice bran to absorb the sunlight, and the Native American custom of shielding the skin with powder from aspen trunks (Aldahan, Shah, Mlacker, & Nouri, 2015; Rabinowitz & Tripp, 2015). Still, traditional robes worn by members of desert tribes like the Bedouins and Tuareg Berbers are suggestive of the ancient and judicious use of fabrics to protect human hairless skin (Urbach, 2001). Our ancestors apparently recognized that their skin could become painfully burned when exposed to uncontrolled sunlight. Yet, modern medical science first described the phenomena of the skin deteriorating due to continued exposure “to the weather” at the end of 19th century with the term “sailor’s skin” (Unna, 1896). Within a few years of this description, a number of experimental studies described the first health effects caused by ultraviolet (UV) light (Widmark, 1889). Elegant experiments demonstrated that the burns induced by UV rays, were distinct from burns induced by exposure to “heat”, e.g., infrared spectrum irradiation (Hammer, 1892; Hockberger, 2002).

Experimental discoveries from rodent models soon indicated that extended exposure to UV rays produced both benign and malignant skin lesions. In addition, it was shown that applying a carcinogen – in the early days, tar - directly to the skin caused effects beyond those observed in animals exposed solely to UV irradiation (Findlay,

“Under the name “sailor’s skin,” I have described an affection of those parts of the skin exposed to the weather, which I have chiefly observed in those spending their lives at sea. […] To this first stage of erythema, pigmentation and hyperkeratosis, which may last for years, there succeeds a further one, in which large, warty-like growths, hypertrophies of the sebaceous glands, and papillary and ulcerating carcinomata appear.”
Unna, 1896 p.719
In the clinical field, cancerous diseases of the human skin, too, began to be described as UV-induced skin conditions (Paul, 1918).

In modern times, sun protection emerged as a cultural phenomenon; a pale complexion has been long valued as a societal symbol in the phenotypically fair-skin nations in the Euro-Atlantic region (Etcuff, 2000). Historically, members of non-working classes could seek shade, stay indoors, and protect their skin with clothing and accessories when engaged in outdoor activities. This trend was reversed in the 1930s when pop-culture icons like Coco Chanel and skilled marketers like Eugene Schueller, the founder of L’Oréal, promoted a tanned skin as fashionable and a sign of good health (Cole, 2018). In the US, the eventual establishment of the 40-hour workweek and 2-day weekend boosted popularity of beach-time in the summer and skiing in the winter. Vacationing in those destinations built a niche for convenient and esthetically pleasing skin protection against ad hoc excessive sunlight exposure.

The first formulated chemical sunscreens were based on natural extracts such as quinine, aesculin, and salicin; these were first sold in Europe around 1911 and in the US starting in 1928 (Lowe, 2006; Urbach, 2001). Early sunscreen products blocked or absorbed primarily the UVB light spectrum (wavelength of 280-315 nm) that is known to cause skin burning. Although, UVB constitutes only 5% of the sunlight that reaches the Earth’s surface – 90 % of UVB is absorbed by the ozone layer – the UVB-spectrum waves penetrate into the epidermis, reaching both epidermal cells and melanocytes in the basal layer, straining the epidermal tissue with erythema, e.g., reddened patches, comprised of cells containing DNA mutations (Ikehata et al., 2013). UVA on the other hand, penetrates deeper than UVB, reaching beyond epidermal keratinocytes to dermal fibroblasts and Langerhans cells, and further into the dermal matrix of collagen and elastin fibers. Well-established effects
of UVA rays include suntan due to activation of melatonin – once a desired effect reflected in the trend of indoor tanning facilities consisting mainly of high levels of UVA light. But later evidence has built a case for enhanced photoaging and mutagenicity of UVA light (Marrot, Belaidi, & Meunier, 2005). These findings lead to the classification of indoor tanning radiation as carcinogenic to humans (El Ghissassi et al., 2009) – and a sharp decline in indoor tanning worldwide (Rodriguez-Acevedo, Green, Sinclair, van Deventer, & Gordon, 2020).

The era of synthetized and patented sunscreens began in 1943 with para-aminobenzoic acid (PABA), a UVB absorber whose derivatives have been embodied in a wide range of products while its use as a sunscreen is less common at present (Kluczyk et al., 2002). The first over-the-counter sunscreens targeting UVA rays were approved by the U.S. FDA in the 1970s and with effects primarily advertised as anti-photoaging, UVA filters too have made for an attractive consumer product. As of 2020, FDA approves sixteen over-the-counter sun screening compounds used individually or in a mixture of chemical screens (FDA, 2021). Combining chemicals that individually absorb UVA and UVB rays is common in “broad spectrum” sunscreens, while only a few individual chemicals can screen against both UVA and UVB (Moyal & Fourtanier, 2008; Wahie, Lloyd, & Farr, 2007).

Sun screening chemicals in general have been profitable products and industry data suggest that the outlook remains positive from a market profit perspective (Osterwalder, Sohn, & Herzog, 2014). The use of UV absorbing chemicals [TABLE 2.1] has expanded beyond sunscreens to include other personal care products. Now, UV absorbing and UV stabilizing chemicals are being added to other consumer products including food contact materials, toys, building materials, plastic storage containers, paints, and fabrics. The use of these chemicals is
intended to extend the shelf-life of a material, or to protect the products stored within these materials. With the current global problem of plastic pollution, it is also clear that these chemicals are contaminating many environmental matrices as UV protecting chemicals are released from these products. In this chapter, we focus on two examples of prototypical sunscreens: oxybenzone, a compound commonly used in personal care products including dermal sunscreens and in other consumer goods such as fabrics and inks used in food contact materials, and benzotriazole derivatives, a class of chemicals added to plastics, paints, building products, and other consumer goods to protect against product fading and damage from UV rays.

<table>
<thead>
<tr>
<th>Substance</th>
<th>CAS number</th>
<th>Synonyms</th>
<th>Trade Names</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxybenzone</td>
<td>131-57-7</td>
<td>benzophenone 3 2-hydroxy-4-methoxybenzene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octinoxate</td>
<td>5466-77-3</td>
<td>EHMC ethylhexyl methoxycinnamate octyl methoxycinnamate</td>
<td>Eusolex 2292 Uvinul MC80</td>
<td></td>
</tr>
<tr>
<td>Enzacamene</td>
<td>36861-47-9</td>
<td>4-MBC 4-methylbenzylidene camphor</td>
<td>Eusolex 6300 Parsol 5000</td>
<td></td>
</tr>
<tr>
<td>PBSA</td>
<td>27503-81-7</td>
<td>2-phenylbenzimidazole-5-sulfonic acid</td>
<td>Ensulizole Eusolex 232 Neo Heliopan</td>
<td></td>
</tr>
<tr>
<td>Octisalate</td>
<td>118-60-5</td>
<td>EHS Ethylhexylsalicylate Octyl salicylate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3 Oxybenzone

2.3.1 Sources of exposure to oxybenzone and other chemical sunscreens

Oxybenzone was invented in 1962 and intended as a product that protects human skin on the occasions of excessive sunlight exposure. Among the 16 UV sunscreens approved for use on the U.S. market since the FDA’s Monograph on the subject published in 1999 (FDA, 1999), oxybenzone is in demand for assorted sunscreen formulations. Yet, its producers have conceived many more applications and within a short period of several decades oxybenzone has found its way to countless other personal care products, as well as industrial materials and products such as coatings, ink wetting agents, paints, as well as paint removers (T. D. EPA, 2015). The uses of oxybenzone in this wide range of consumer products means that there are two major routes of exposure to humans: dermal and oral. Dermal exposure to those chemical compounds comes naturally as a consequence of applying sunscreens directly on the skin (Han, Lim, & Hong, 2016) but a number of personal care products including hair sprays, shampoos, lotions, lipsticks, and fragrances represent important and continuous sources of exposure. Oral exposure to oxybenzone occurs due to unintentional consumption of oxybenzone from drinking water, and foods and drinks encased in oxybenzone-treated packaging. Perishable drinks such as fermented liquids or beer must be kept in either dark or UV-protective containers and UV absorbing chemicals can leach into the contents (Markarian, 2004). The industrial inks used on cardboard food packaging are often enhanced with oxybenzone to serve as a photo-initiator and wetting agent (T. D. EPA, 2015). Curiously enough, oxybenzone may be also added to candy as a stabilizing and flavoring agent (T. D. EPA, 2015).
All the above exposures also contribute to high volumes of oxybenzone that are released into the environment. On occasion, additional dermal exposures occur from swimming in surface waters polluted with sunscreens. Several analytical studies indicate that both outdoor and indoor swimming pools and spas contain ng-mg/L concentrations of various sunscreens (Ekowati et al., 2016; Poiger, Buser, Balmer, Bergqvist, & Muller, 2004). Of note, interaction of oxybenzone with chlorine used to disinfect water for recreational activities as well as drinking water may result in the formation of haloacetic acids, with enhanced toxicity and increased mutagenicity compared to the parent compound (Liu, Chen, Wei, & Du, 2014; Manasfi et al., 2019; Teo, Coleman, & Khan, 2015; J. Wang, Gong, & Xian, 2020). These results are not specific to oxybenzone; high concentrations of phenylbenzimidazole sulfonic acid (PBSA), a UVB absorber, were documented in outdoor pools and recreational lakes in South Bohemia – with concentrations as high as 13 μg/L. The documented concentrations of PBSA were considerably higher than those of oxybenzone (620 ng/L) (Grabicova et al., 2013). Despite the ability of some plants – like the umbrella plant, *Cyperus alternifolius* – to remediate oxybenzone contamination in water (F. Chen, Schnick, & Schroder, 2018), it pollutes drinking water because common water treatment facilities are not designed to completely remove chemicals from personal care products and other consumer goods (C. P. da Silva, Emidio, & de Marchi, 2015; Stackelberg et al., 2004) (Rodil et al., 2012; Wu et al., 2018). Even if the water treatment processes were fully successful and all oxybenzone was relegated to sewage sludge, this and similar chemicals would likely find their way back to the food chain. As discussed in later sections of this chapter, organisms in the environment as distant as even earthworms experience negative
impacts of UV screening chemicals on their reproduction and with increased mortality (Casquero, Trigo, Guitarte, & Novo, 2020).

Inhalation is a minor yet not insignificant route of human exposure because oxybenzone and other UV absorbing chemicals have been detected in indoor air and dust. Although this may seem surprising, house dust components commonly include environmental chemicals such as UV filters due to the shedding of keratinocytes treated with personal care products, as well as shedding of fibers from UV-proof clothing, carpeting, and furniture (Mitro et al., 2016). A study from the US demonstrated that homes are not the highest source of indoor air exposures to oxybenzone. Rather, concentrations twice as high as home concentrations were reported in public places such as coffeeshops and libraries, and more than three times higher concentrations of oxybenzone (up to 72 ng/m$^3$) were measured in air collected from the interior cabin of automobiles (Wan, Xue, & Kannan, 2015).

**2.3.2 Human exposure levels**

Nearly every person in the general population (98.9%) in the US has detectable urinary concentrations of oxybenzone at any point in time. The mean concentration in ppb (μg/L) may not seem like a meaningful amount, yet there are several reasons for concern. First, the population urinary concentration has not remained constant over the last decade or more. Rather, the last documented mean concentration from a large biomonitoring study of the US population was 36.3 μg/L – a 58.6% increase compared to the 22.9μg/L mean concentration reported only six years prior (Calafat, Wong, Ye, Reidy, & Needham, 2008; Meeker et al., 2013).

Second, exposures during the critical windows of gestation and the perinatal period have been documented. A biomonitoring study of the US public revealed that
all tested pregnant women (and 98% of non-pregnant women) have detectable urinary concentrations of oxybenzone (Woodruff, Zota, & Schwartz, 2011). In a smaller cohort of postpartum women from North Carolina in the US, 56% of the participants had levels higher than 0.51 μg/L of oxybenzone in breast milk (Hines et al., 2015). Further, 80% of breastfeeding participants had detectable levels of the compound in their urine, and the milk:urine ratio was as high as 1:738. Out of eight UV filters analyzed (including oxybenzone) in a Swiss cohort, 85% of milk samples had detectable concentrations of UV filters (Schlumpf et al., 2010). Although some of the quantitative differences in measured concentrations of oxybenzone in human milk are due to differences in the precision and accuracy of the methods used (Molins-Delgado et al., 2018), detection and concentrations of the parental compound prevail over detection of its main metabolites (Rodriguez-Gomez, Zafra-Gomez, Dorival-Garcia, Ballesteros, & Navalon, 2015).

In addition to the excretion of chemicals in human breast milk, plastic containers used to contain this milk also commonly include various UV filters – namely oxybenzone, 4,4-dihydroxybenzophenone and benzotriazole UV320 – that leach into the contents (Molins-Delgado et al., 2018). Along with data documenting oxybenzone in amniotic fluid and cord blood (Krause, Frederiksen, Sundberg, Jorgensen, Jensen, Norgaard, Jorgensen, Ertberg, Juul, et al., 2018), these data suggest that human neonates - who have arguably never used sunscreens - are exposed to an estimated 69.1 μg/ kg body weight of chemical UV filters daily (Molins-Delgado et al., 2018).

As discussed later in this chapter, a number of chemical sunscreens act as endocrine disruptors. Based on the principles of endocrinology that are also relevant to endocrine disrupting chemicals (Vandenberg et al., 2012; R. T. Zoeller et al.,
2012), low doses of chemicals like oxybenzone may induce significant effects during critical windows of development. In men, UV filters have been detected in semen, suggesting the potential for preconception exposures as well (Leon, Chisvert, Tarazona, & Salvador, 2010).

A third reason that even low levels of human exposure may be concerning is that oxybenzone exposures are difficult to avoid. On the one hand, urinary concentrations of oxybenzone correlate to self-reported frequency of using sunscreens - from an average of 9.3 μg/g creatinine in participants “never” using sunscreen to an average of 116.8 μg/g in individuals reporting “always” using sunscreen (Zamoiski, Cahoon, Freedman, & Linet, 2015). Another study documented the change in urinary concentrations between “never” and “always” groups as 509.8 percent (Ferguson, Colacino, Lewis, & Meeker, 2017). Furthermore, oxybenzone is present in human urine samples year-round, even during wintertime when sunscreen use is generally infrequent (Krause et al., 2012) although exposures to UV filters vary also seasonally within-individuals (Kunisue et al., 2012) and also in environmental matrices (C. P. da Silva et al., 2015; Loraine & Pettigrove, 2006a), with higher exposures measured in the summer months. For this reason, a spot measurement may not correctly represent individual exposures over longer periods (Dewalque, Pirard, Vandeapaer, & Charlier, 2015).

2.3.3 Measurements in the environment

Occurrence of sunscreen chemicals in the environment have been highest in recreational waters and untreated wastewater. Approximately 25% of applied sunscreen is washed off the skin (Danovaro et al., 2008), therefore popular beach areas are especially prone to high concentrations of sunscreen chemicals both in
water and sediments. Coastal waters of Greece (Giokas, Sakkas, & Albanis, 2004), the Canary Islands (Rodriguez, Sanz, & Rodriguez, 2015), the Hawaiian archipelago (Mitchelmore et al., 2019), and the Virgin Islands (Bargar, Alvarez, & Garrison, 2015) have measured exponentially increasing concentrations of sunscreen chemicals with decreasing distance from the coastal line, consistent with a human source.

Fresh surface waters including lakes, lagoons, and rivers typically have a higher presence of oxybenzone metabolites (namely benzophenone 4 and benzophenone 1) than oxybenzone itself (Careghini, Mastorgio, Saponaro, & Sezenna, 2015). Rivers and lakes in Switzerland (Balmer, Buser, Muller, & Poiger, 2005; Fent, Zenker, & Rapp, 2010) contain oxybenzone at concentrations ranging between the detection limit of 2 μg/L to 652 μg/L; rivers in England, Spain, and South Korea also have comparable levels (Kasprzyk-Hordern, Dinsdale, & Guwy, 2008; Rodil et al., 2012; Yoon, Ryu, Oh, Choi, & Snyder, 2010). Overall, it has been estimated that freshwaters worldwide contain anywhere up to 125 μg/L of oxybenzone (S. Kim & Choi, 2014).

Wastewater is a substantial source of oxybenzone and its metabolites for pollution of fresh water. Treated wastewater can account for up to 20% of the water in rivers near urban areas (Rodil et al., 2012), and removal of oxybenzone and other similar contaminants via water treatment technology is not absolute. For example, wastewater treatment plant effluents in Shanghai document divergent removal of different benzophenone sunscreens: 100% of benzophenone 2 was removed versus only 24% of benzophenone 4 (and 82% of oxybenzone) (Wu et al., 2018). Furthermore, sewage sludge and environmental releases from urban industrial coatings (Plagellat et al., 2006; Z. Zhang et al., 2011), sediments, and landfill
leachates (Cuderman & Heath, 2007; Giokas et al., 2004) commonly have detectable concentrations of chemical sunscreens including oxybenzone.

Groundwaters replenish slowly from surface water, and because of natural filtration processes their levels of pollution are typically lower compared to surface waters. For this reason, groundwater is often used as drinking water making their pollution with synthetic chemicals especially alarming (Jurado et al., 2014; Stuart, Lapworth, Crane, & Hart, 2012). Drinking water, too, is often contaminated with residues of UV filters: in southern California, octinoxate and benzophenone have been measured at 260 and 280 ng/L, respectively (Loraine & Pettigrove, 2006a). Saõ Paulo residents drink water with octinoxate and oxybenzone at concentrations up to 101 and 115ng/L, respectively (C. P. da Silva et al., 2015). Further, in Spain, oxybenzone was measured in local tap water at similar concentrations (295 ng/ L) along with four other common UV filters (with concentrations ranging from 35 - 260 ng/L) (Diaz-Cruz, Gago-Ferrero, Llorca, & Barcelo, 2012).

2.3.4 ADME

Adsorption

Chemical sunscreens applied to the skin do not stay on the surface of the skin and instead are transferred into the epidermal and dermal layers and ultimately the underlying blood supply. This movement across the skin barrier has been demonstrated in in vitro studies (Treffel & Gabard, 1996), experimental rodent models (Okereke, Abdelrhaman, & Friedman, 1994) and epidemiological investigations (Janjua et al., 2004; R. Jiang, Roberts, Collins, & Benson, 1999). The rate of a chemical sunscreen’s penetration into the stratum corneum depends on the lipophilicity of the vehicle, the study conditions, and the sunscreen formulation – but
has been estimated at 1-2% (Hayden, Roberts, & Benson, 1997) to 4% (C. Fernandez, Nielloud, Fortune, Vian, & Marti-Mestres, 2002); for formulations containing oxybenzone, dermal absorption of 8% has been calculated for products comprised of 2% oxybenzone and absorption of 9.9% has been observed for products containing 6% oxybenzone (the maximum concentration approved by the U.S. FDA) (T. D. EPA, 2015). Interestingly, both hydro- and lipophilic vehicles promote high penetration of oxybenzone or metoxycinnamate (Gupta, Zatz, & Rerek, 1999). Mixtures of these two UV screening agents resulted in higher retention/penetration rates throughout the basal membrane into the layer of dermis, e.g. the connective tissue containing not only collagen and elastic fibres but also blood vessels – arterioles, venules, and capillaries.

Distribution

Because these chemicals are drawn into the blood stream, sunscreen plasma concentrations can be measured effectively and evaluated over time. A safe threshold of exposure set by the US FDA is 0.5 ng/mL in circulation for all over-the-counter sunscreens approved for the US market: the US FDA “expects that a systemic carcinogenicity study would not be needed to support a [determination of generally recognized as safe and effective] for a sunscreen active ingredient if an adequately conducted human pharmacokinetic [maximal usage trial] resulted in a steady state blood level less than 0.5 ng/mL” (FDA, 2019b). However, a recent FDA study examining six sunscreen ingredients revealed that this threshold level was reached and exceeded 2 hours after application of sunscreen products containing oxybenzone, and between 3-21 hours for the remaining five tested sunscreen chemicals. Blood concentrations were maintained above this threshold for 23 hours in all participants following application of sunscreens containing oxybenzone and
homosalate; more than 75% of participants had these high blood concentrations after using products containing avobenzone, octinoxate, octocrylene, and octisalate. The maximum plasma concentrations exceeded the threshold limit by at least 6 times (3.3 ng/mL for avobenzone in a pump spray) and as much as 500 times (258.1 ng/mL for oxybenzone in a lotion). Moreover, three of the tested sunscreens remained above the threshold for seven days following the last application in the majority of the tested individuals (95% for avobenzone, 75% for octisalate, and 90% for octinoxate), for 10 days in 67% of participants treated with products containing octocrylene, and a full three weeks for 55% of participants treated with homosalate and 96% of participants treated with products containing oxybenzone (Matta et al., 2020). Collectively, these results indicate that sunscreens are easily absorbed across the skin barrier, and remain in circulation for days, or weeks, after application.

Metabolism

Transformation of oxybenzone via two main metabolic pathways, demethylation and hydroxylation, produces several metabolites [Figure 1]. By giving up its methyl group and accepting a hydrogen, oxybenzone (benzophenone 3) is transformed into benzophenone 1 (2,4, dihydroxybenzophenone) (Vione, Caringella, De Laurentiis, Pazzi, & Minero, 2013). The main product of hydroxylation is benzophenone 8 (2,2’ dihydroxy-4-methoxybenzophenone); however, hydroxylation on either of the rings also yields 3-hydroxy-benzophenone-3 or 5-hydroxy-benzophenone-3 (Cheng, 2014; Kamikyouden et al., 2013). Activation of the hydroxylation pathway further produces two oxybenzone metabolites that have been documented in breast cancer cells and isolated rat hepatocytes: 2,3,4-trihydroxybenzophenone (2,3,4 THB) and 2,4,5 -trihydroxybenzophenone (2,4,5 THB) (Kerdivel et al., 2013; Nakagawa & Suzuki, 2002).
Figure 2.1: Metabolism of oxybenzone. Oxybenzone is metabolized through the demethylation (dotted lines) and hydroxylation (solid lines) pathways. After initial transformation, metabolites can be further transformed using both pathways.

The above transformations rely on the family of cytochrome P450 (CYP) enzymes, specifically CYP1A and CYP2D6 (Watanabe et al., 2015). These enzymes are localized in the rough endoplasmic reticulum and mitochondria, primarily in adult hepatocytes, but their expression and activity are protean which proves to be important on two grounds. First, the perinatal period is a particularly vulnerable time of exposure to exogenous substances, including chemical sunscreens, due to underdeveloped detoxication mechanisms involved in phase I of xenobiotic metabolism (de Wildt, Kearns, Leeder, & van den Anker, 1999; Sadler et al., 2016). For that reason – as well as the immaturity of the skin barrier until at least 2 years of age – limited use of products with oxybenzone are recommended for infants. Specific sun protection guidelines have been created for neonates and toddlers by
regulatory agencies that oversee the regulation of personal care products (R. Jiang et al., 1999; Paller et al., 2011). Second, due to circulation of the parental compounds including detection in the uterine environment, and primarily maternal metabolism of chemicals in utero, fetal exposure is likely to be qualitatively as well as quantitatively different from maternal exposure.

Oxybenzone readily metabolizes into products with dissimilar activities and abilities to dissolve in polar vs non-polar solvents. For example, benzophenone 1 is more hydrophilic than oxybenzone owing to the loss of a methyl group (S. Kim & Choi, 2014), and can be detected in surface waters more frequently and in higher concentrations than oxybenzone (Jeon, Chung, & Ryu, 2006). On the other hand, neighboring hydroxyl groups or a 5-hydroxyl group on the A-phenyl ring of oxybenzone seems to decrease some biological activities – e.g., the estrogenic behaviors of oxybenzone metabolites (Watanabe et al., 2015).

**Excretion**

In mice, the free and conjugated forms of oxybenzone are excreted comparably by both the urinary and fecal routes (EC, 2006). In rats, however, the kidneys exclusively excrete the compound (Kadry, Okereke, Abdel-Rahman, Friedman, & Davis, 1995).

In humans, sunscreens chemicals and their metabolites are commonly detected in urine, suggesting a role for the kidneys in excretion. In a study designed to evaluate uptake and excretion of oxybenzone after dermal exposure, participants self-applied 40 g of sunscreen containing 4% oxybenzone (approximately 1.6 g of the active compound) (Gustavsson Gonzalez, Farbrot, & Larko, 2002). At the end of the study (48 hours later), the participants had excreted approximately 11 mg of oxybenzone, less than 1% of the oxybenzone that had been applied to the skin. This
does not mean that the remainder of the oxybenzone bioaccumulated, because this study design could not determine how much of the applied oxybenzone crossed the dermal barrier. Importantly, the researchers concluded that there was large variability in the excretion patterns between human volunteers, although urinary concentrations of oxybenzone were typically highest in the first 24 hours after application. In a second study, lotions containing oxybenzone, octyl-methoxycinnamate (OMC), or 3-(4-methylbenzylidene) camphor (4-MBC) were provided to male and female participants; total lotion was 2 mg/cm² and the concentrations of each sunscreen ingredient were 10% (w/w) (Janjua et al., 2004). Urinary concentrations of oxybenzone were high in women 24-96 hours after application (60 ng/ml) and even higher in men (140 ng/ml). Urinary concentrations of OMC and 4-MBC were much lower (5-8 ng/ml for each compound). At this time, much more work needs to be done to evaluate urinary excretion of oxybenzone and its metabolites after single and repeated applications of products to the dermal surface, as well as controlled oral dosing, using human volunteers.

2.3.5 Aquatic toxicity and effects on wildlife

Chemical UV filters added to personal care products and UV protecting coatings inevitably enter the water systems – rivers, lakes, coastal areas – and the aquatic biota as well as the tissues of aquatic organisms (Balmer et al., 2005; Sanchez-Quiles & Tovar-Sanchez, 2015). From the very bottom of the aquatic food chain to large marine animals, virtually all seafaring species are exposed to human-made and human convenience-driven chemical UV filters [Figure 2.2].
Figure 2.2: Oxybenzone in the aquatic environment. Oxybenzone and its metabolites have been studied in aquatic – and related – species. From the lower trophic levels up to the higher species, aquatic organisms carry a double burden: they accumulate the compounds in their tissues and are affected by the exposures.

Corals are a remarkable species whose multiple stages of development depend on synchrony - and that synchrony relies on environmental clues including lunar cycles, sunlight, temperature and wind (Shlesinger & Loya, 2019). Thus, from the carefully timed event of coral spawning, through the drifting of sperm and eggs with the ocean’s currents, to coral planulae searching for suitable substrates to settle on and polyps beginning to develop their exoskeleton, corals are susceptible to environmental insults (Gleason & Hofmann, 2011; He et al., 2019). Oxybenzone induces premature formation of coral skeletons, increases stress in mature corals that are symbiotic with marine zooxanthellae. Oxybenzone causes the coral to expel their symbionts, which may represent a short-term adaptation yet with long-term consequences for the health of the coral and its’ survival (Downs et al., 2016). The
first evidence on the effects of sunscreens on corals and their symbiont was brought by Danovaro et al (2008). In their earlier discovery the team demonstrated high long-term lytic bacterial activity and oscillating viral abundance in marine bacterioplankton immersed in 500 μL/L of sunscreen (Danovaro & Corinaldesi, 2003). A similar mechanism has been shown between coral and their symbiotic zooxanthellae. The light-sensitive coral was bleached due to a loss of photosynthetic pigment and membrane integrity when ambient seawater was treated with sunscreen; the coral released its zooxanthella rapidly following treatment with oxybenzone, enzacamene, octinoxate and octisalate (Danovaro et al., 2008).

Chemical sunscreens are also detected in other lower aqueous species. Green algae and cyanobacteria take up oxybenzone – in a paradoxical pattern of “the lower the concentration the higher the uptake” (Mao, He, Kushmaro, & Gin, 2017). Following exposures, both species suffer restricted growth rates and reduced production of vital chlorophylls and carotenoids. The effects of four common sunscreen chemicals were tested on plants: all four compounds were absorbed through the roots, inhibited photosynthesis, and decreased the plant’s respiration – effects that increase oxidative stress (Zhong et al., 2020).

Fish tissues tend to concentrate lipophilic environmental contaminants. The bioaccumulation factor in fish depends on the lipophilicity of a specific chemical and can lead to internal concentrations that are hundreds to thousands of times higher than the surrounding environment (Brausch & Rand, 2011). As an example, 3-benzylidene camphor concentrations are 313 times higher in fathead minnow tissues compared to the environment (Kunz, Gries, & Fent, 2006). Oxybenzone is a moderately hydrophobic compound with a partitioning coefficient, logP_{ow}, of 3.64, and it accumulates in fish tissues in their natural environment (Gago-Ferrero, Diaz-
Cruz, & Barcelo, 2013) as well as under experimental conditions (Schlumpf et al., 2001). Octocrylene concentrates up to 2.4 μg/g in estuarial fish (Buser, Balmer, Schmid, & Kohler, 2006), while exposures of experimental fish to concentrations of octocrylene that are described as “practically non-toxic” still accumulate in *Danio rerio* at concentrations up to 17 μg/g wet body weight (Bluthgen, Meili, Chew, Odermatt, & Fent, 2014). Further, 4-methylbenzylidene camphor, oxybenzone, and octyl methoxy cinnamate were demonstrated to bioconcentrate in lake fish (with concentrations of 166, 123, and 64 ng/g lipids, respectively) (Balmer et al., 2005; Buser et al., 2006). Mollusks, unlike fish, are sessile organisms filtering bottom water and surface sediments, and so are known for their ability to accumulated contaminants, including UV filters (Sang & Leung, 2016). Although typically tolerant to stresses, clams, too, responded to 1 mg/L of oxybenzone in microplastics with increased oxidative stress and genotoxicity (O'Donovan et al., 2020).

Of note, both chemical UV filters and UV stabilizers with a substantive bioaccumulation potential also indirectly affect organisms in the upper ranks of the food chain (Langford, Reid, Fjeld, Oxnevad, & Thomas, 2015). Molluscivorous and piscivorous birds – e.g., oystercatchers and cormorants - are particularly susceptible to lipophilic pollutants due to their consumption of a diet consisting of contaminated muscles and fish. Moreover, throughout their life course, birds accumulate large concentrations of pollutants and may dispose the chemicals in their eggs. A study sampled several UV filters in Swiss aquatic ecosystems and measured the lipophilic sunscreen, ethyl-hexyl-methoxy cinnamate, in cormorants at concentrations as high as 701 ng/g lipids compared to concentrations of dozens ng/g lipids in small fish and lake muscles (Fent et al., 2010). Consistently measured concentrations of benzophenone sunscreens and even higher concentration of their metabolites in
unhatched eggs of birds in a natural reservation in Spain suggest bioaccumulation of sunscreen in higher species (Molins-Delgado et al., 2017).

Importantly, aquatic species are affected by multiple stressors, and this may exacerbate the toxic effects of sunscreen chemicals on wildlife. For example, increasing temperatures in the oceans combined with UV filters enhance the expression of stress-induced genes and decrease larvae survival of riparian insects (Muniz-Gonzalez & Martinez-Guitarte, 2020). Also, the increasing acidification of the ocean seems to potentiate new targets of environmental pollutants: oxybenzone *per se* does not affect carbon anhydrase in the digestive tract of a mollusk *Amarilladesma mactroides* after exposures to concentrations of 1 μg/L when the pH was maintained at 8.1, but the chemical increased the enzyme’s activity at lower pH; in fact, a decrease of only 0.5 pH units affected the carbon anhydrase with effects on shell formation (Lopes, de Castro, Barbosa, Primel, & Martins, 2020).

In summary, chemical UV filters are continuously supplied to blue water, fresh and sea waters alike. Compounds of ranging hydro- and lipophilic potentials accumulate in sediments and aquatic biota across the food chain. Importantly, these effects are not only seen in fish; effects have been documented on marine birds and species at the top of the food chain. Yet, equally relevant is that riparian life – namely plants and insects – are affected by these chemicals as well [Figure 2].

### 2.3.6 Acute and subchronic toxicity

**Toxicity Testing in Rats**

The National Toxicology Program (NTP) conducted a series of toxicity tests on oxybenzone using F344/N rats (French, 1992). First, a relatively small study (5 males and 5 females) examined the effects of oxybenzone after 2 weeks of
exposure. The animals were provided chow containing 0, 3125, 6250, 12500, 25000 or 50000 parts per million (ppm) oxybenzone (equivalent to intake of approximately 300, 600, 1200, 2400, and 4500 mg/kg/day). In males, all five doses of oxybenzone increased the weight of the liver and increased liver weight was also seen in females exposed to the four highest doses. Increased relative weight of the kidney was also increased in males exposed to the four highest doses. When evaluated histologically, 4 of 5 males fed chow with 50000 ppm oxybenzone had focal dilation of the renal tubules.

A second two-week toxicity study examined the effects of oxybenzone in male and female rats administered oxybenzone dermally, either in acetone or in a lotion. The applied formulations contained 0, 5, 10, 20, 40 or 80 mg/ml; a total of 0.25 ml was applied to the skin of rats per day, with applied doses of 1.25, 2.5, 5, 10 or 20 mg/animal. Females, but not males, administered the three highest doses in acetone had significantly heavier livers at the end of the administration period. In males dermally administered oxybenzone in lotion, increased liver weights were observed across the treatment groups. No histopathological changes were observed in the livers of rats from either sex.

A thirteen-week toxicity study examined the effects of dietary oxybenzone exposure on rats (n=10 per sex), again with diets containing 0, 3125, 6250, 12500, 25000 or 50000 parts per million oxybenzone. During the conduct of the study, both male and female rats developed discolored urine and stains in the perineal fur, consistent with effects on the kidney. Relative kidney weight was significantly increased only in the highest dose groups, but again, increased liver weight was observed at all five doses in both males and females. There were also effects observed on hematologic evaluations; different doses increased platelet counts,
segmented neutrophils, and reticulocytes at different points in the exposure period. In male rats, the highest dose of oxybenzone decreased the weight of the epididymis and decreased sperm concentrations; in female rats, the highest dose increased the length of the estrous cycle.

Finally, a thirteen-week dermal toxicity study evaluated rats exposed to doses of 0, 12.5, 25, 50, 100 or 200 mg/kg with the oxybenzone applied to the skin in acetone. Females from the four highest dose groups had heavier kidney weights at necropsy; effects on male relative kidney weight were also observed in the 25 and 100 mg/kg groups. Yet, there were no effects on the male or female reproductive organs or on most of the hematologic or clinical chemistry parameters.

Toxicity Testing in Mice

The National Toxicology Program (NTP) also conducted a series of toxicity tests on oxybenzone with B6CF1 mice (French, 1992). Again, the first study was relatively small (5 males and 5 females) and examined the effects of oxybenzone after 2 weeks of dietary exposure. The animals were provided chow containing 0, 3125, 6250, 12500, 25000 or 50000 parts per million (ppm) oxybenzone (equivalent to intake of approximately 1000, 2000, 4000, 8000 or 20000 mg/kg/day). Again, an increase in liver weight was observed in the oxybenzone-treated animals, in all five dose groups for females and in the four highest dose groups for males. There were histopathological changes (e.g., cytoplasmic vacuolarization in the hepatocytes) also observed in these liver samples after exposures to 2000 mg/kg/day or higher doses.

A second two-week toxicity study examined the effects of oxybenzone in male and female mice administered oxybenzone dermally, either in acetone or in a lotion. The applied formulations contained 0, 5, 10, 20, 40 or 80 mg/ml; mice were
administered 0.1 ml per day producing applied doses of 0.5, 1, 2, 4 or 8 mg/mouse. Interestingly, body weight was increased in both males (8 mg) and females (1 and 4 mg) dermally administered oxybenzone. Increased liver weights were observed in males from the two highest dose groups, and in females from all oxybenzone groups (significant in the 1, 4 and 8 mg groups). Increased kidney weight was also observed in males from the two highest dose groups.

A thirteen-week toxicity study examined the effects of dietary oxybenzone exposure on mice (n=10 per sex), again with diets containing 0, 3125, 6250, 12500, 25000 or 50000 parts per million oxybenzone. These diets altered body weight, which was decreased by as much as 16% in males administered the highest dose, and as much as 13% in females administered the highest dose. Increased liver weight was again observed in both males and female mice administered the four highest doses, and microscopic changes in liver histology were observed in the two highest dose groups. Altered kidney weights were also recorded for males in the highest dose group (significantly decreased compared to controls) and in females from the four highest doses (significantly increased compared to controls). Histopathologic lesions of the kidney were observed in numerous oxybenzone-treated animals including several homogeneous eosinophilic casts in renal tubules, dilated renal tubules, and inflammatory cell infiltrates in the renal interstitium. There were also effects of oxybenzone on sperm concentration (decreased after exposures to the highest dose) and the percentage of sperm with cellular abnormalities (increased after administration of feed with 3125, 12500 or 50000 ppm oxybenzone, the only three dose groups evaluated). Length of the estrous cycle was also increased in mice exposed to the highest dose.
Finally, a thirteen-week dermal toxicity study evaluated mice dermally administered 0, 22.75, 45.5, 91, 182, or 364 mg/kg in acetone. In this study, no significant changes were observed in the weight of the kidney or liver in either male or female mice; there were also no abnormal histopathological findings of these organs or skin samples collected from the site where the oxybenzone and acetone were applied. Sperm concentration and was also significantly decreased in all three treatment groups evaluated for this outcome (22.75, 91, and 356 mg/kg) but there were no significant effects on estrous cycle length in oxybenzone-treated females.

Figure 2.3: Toxicity of oxybenzone from acute and subchronic toxicity evaluations. A) Effect of oxybenzone administered in feed on relative liver weight in rats. B) Effect of oxybenzone administered in feed on relative liver weight in mouse. C) Effect of oxybenzone on relative kidney weight after 13-weeks of administration in rats. D) Effect of oxybenzone on relative kidney weight after 13-weeks of administration in mouse. E) Effect of 13-weeks of oxybenzone exposure on epididymal sperm count. M = males, F = females. * p<0.05 compared to untreated controls, as determined by the original study authors. All data from (French, 1992).
NTP’s Toxicity Summary of 2- and 13-week oral and dermal exposures

After this series of 2-week and 13-week studies in mice and rats, evaluating the effects of oxybenzone administered either orally or dermally, the NTP panel came to a number of conclusions (French, 1992). First, there were similar effects across the two species, and similar effects observed in rodents that were administered oxybenzone either orally or dermally. The most common changes were increased weight of the liver (FIGURES 2.3A,B) and kidney (FIGURES 2.3C,D), and in the 13-week studies there were consistent effects on epididymal sperm density (decreased, see FIGURE 2.3E) and estrous cycle length (increased), providing a hint of reproductive toxicity or endocrine disruption.

Based on the 2-week and 13-week studies, the NTP concluded that the no-observed adverse effect level (NOAEL) for microscopic lesions was the 6250 ppm dietary administration for both mice and rats. The NTP panel concluded that it was not possible to identify a NOAEL dose for effects on sperm, because all of the doses tested altered sperm counts. The NTP panel also concluded that oxybenzone is a renal toxicant, with more severe toxicity observed after oral exposures compared to dermal exposures. This provides evidence that the dermal exposures produce lower systemic exposure concentrations. Renal effects were also more severe in rats than in mice and histopathological abnormalities included tubule dilatation with regeneration of tubular epithelial cells, interstitial inflammation, and papillary necrosis.

Skin sensitization

Animal testing to evaluate allergic reactions to dermally applied oxybenzone did not revealed any dermal sensitization in guinea pigs. However, human trials have
given a contrasting picture (EC, 2006). The most recent European photopatch testing ranked chemical UV absorbers, including oxybenzone and octocrylene, as the “second most frequent cause of positive photopatch test reaction” (Subiabre-Ferrer et al., 2019). The common chemical structure of benzophenone-based UV filters suggests the potential for cross-sensitivity and individual clinical cases document indiscriminate allergic reactions across ages and body parts for some individuals, and even an anaphylactoid reaction in a 49-old healthy woman following a sunscreen application (Hanson & Warshaw, 2015; Schram, Glesne, & Warshaw, 2007; Tawfik & Atwater, 2019). Similarly, although earlier animal and human testing made no case for phototoxic or photosensitizing reactions to oxybenzone, individual cases illustrate the potential of oxybenzone to cause allergic reactions, especially in combination with radiant energy (Kiec-Swierczynska, Krecisz, & Swierczynska-Machura, 2005; Knobler et al., 1989; J. P. Russo et al., 2018). Collectively, the evidence to date indicates that oxybenzone can cause both contact allergy and photoallergic reactions in humans.

**Reproductive toxicity testing in mice**

To evaluate whether oxybenzone is a reproductive or developmental toxicant, pregnant Harlan Sprague-Dawley rats were administered oxybenzone in their chow from pregnancy day 6 through the entire lactational period (Nakamura et al., 2015). The concentrations in feed were 0, 1000, 3000, 10000, 25000 or 50000 ppm, equivalent to intake of approximately 0, 65, 220, 700, 2500 and 4500 mg/kg/day (but with somewhat different daily doses based on physiological stage, changes in food intake, and changes in body weight throughout pregnancy and lactation).
In the dams, there were no effects observed on reproductive outcomes including number of pups born, birth weight of pups, number of implantation sites, number of resorptions, or sex ratios in the oxybenzone-treated litters compared to the controls. The mothers did display signs of overt toxicity, with a significant decrease in body weight observed in the highest dose group apparent as early as gestational day 10 and maintained throughout the pregnancy period. Increased liver weight and kidney weight were observed in oxybenzone-treated dams at the end of the lactational period. Interestingly, there were non-monotonic dose responses observed for weight of the ovaries and uterus at lactational day 23, with a significant increase in the weight of both organs in the lowest dose group.

In the clinical chemistry analysis, serum was collected from pregnant mice at gestational days 10, 15 and 20, and from lactating mice prior to weaning (lactational day 23). Although effects were observed across many measures of clinical chemistry, the most consistent effects observed across these four time points were for alanine aminotransferase (ALT), and total bile acids (TBA), which were significantly increased in the two highest doses at all four timepoints. Alkaline phosphatase (ALP) was also significantly elevated at all four timepoints in dams from the three highest dose groups.

Both male and female pups were evaluated throughout the postnatal period as well (Nakamura et al., 2015). Pups from litters exposed to the two highest doses were lighter than controls. Clinical chemistry evaluations also suggested changes in both male and female offspring, with decreases in serum ALT and increases in serum cholesterol levels in the two highest dose groups. At postnatal day (PND) 23, relative liver weights were significantly increased in females from the three highest
dose groups; kidney weights were also lighter in both males and females from the highest dose group.

Measures relevant to reproductive development were also disrupted in mice following perinatal exposures to oxybenzone. First, a decline in male anogenital distance (AGD) was observed at PND 23, even after accounting for differences in body size (statistically significant only at the highest dose). Males from the highest dose group also had lighter testes weights, and remarkably, number of spermatocytes was significantly decreased in males from the four highest dose groups. Serum testosterone levels were decreased by approximately 25-40% in all oxybenzone-exposed males (but these differences were only statistically significant in males from litters from the 3000 and 25000 ppm groups). In female offspring, ovarian weights were significantly decreased at PND 23 in the two highest dose groups, and the number of ovarian antral follicles was also decreased in females from these groups. Uterine weight was also significantly lighter in pups from the highest dose group.

A follow-up reproductive toxicity study (Nakamura et al., 2018) examined additional endpoints in males collected from the developmental and reproductive toxicity study described above. After weaning on PND 28, males from litters exposed to two doses (3000 and 30000 ppm) were evaluated on PND 30. There were some signs of toxicity including decreased body weight, decreased liver weight, and decreased kidney weight, all observed in the higher dose group. Reproductive toxicity was observed with decreased weight of the epididymis, decreased weight of the testis, and decreased weight of the prostate. Gene expression profiles revealed effects of both doses on cell cycle and cancer-related gene expression in the prostate.
2.3.7 Endocrine Disruption

In 2020, the European Union’s Scientific Committee on Consumer Safety released a draft report on benzophenones including oxybenzone. One portion of this report was dedicated to the question of whether oxybenzone is an endocrine disrupting chemical as defined by the World Health Organization and United Nations Environment Programme, i.e. “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations” (Damstra, Barlow, Bergman, Kavlock, & van der Kraak, 2002). This definition of an endocrine disruptor presumes that a chemical has an endocrine mode of action, that it induces adverse effects, and that there is a plausible link between the endocrine mode of action and the adverse effects. This definition is widely used by regulatory agencies, but it relies on problematic definitions of what constitutes an adverse effect, and the use of expert judgement to evaluate biological plausibility (Zoeller et al., 2014). Considering this regulatory definition, and the more simplified definition proposed by experts with the Endocrine Society (an endocrine disruptor is “an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action” (R. T. Zoeller et al., 2012), there is evidence that oxybenzone should be characterized as an endocrine disruptor.

As described in more detail below, UV filters have been commonly studied for their estrogenicity, yet they are arguably even more relevant as estrogen receptor (ER) and androgen receptor (AR) antagonists: out of nineteen sunscreens studied with human ERα and AR assays, only five chemicals exhibited full estrogenic activity while thirteen possessed full anti-estrogenic; and nineteen full anti-androgenic activity (Kunz & Fent, 2006b). Also relevant to its classification as an endocrine
disruptor, oxybenzone and its metabolites affect hormone signaling *in vivo* animals, including humans (Ghazipura, McGowan, Arslan, & Hossain, 2017; Maipas & Nicolopoulou-Stamati, 2015) and demonstrate endocrine activity *in vitro*, as well.

**ER agonist activities**

In competitive binding assays, yeast recombinant or two-hybrid assays (Kawamura et al., 2003; Morohoshi et al., 2005; Schreurs, Lanser, Seinen, & van der Burg, 2002), and reporter cell lines (Molina-Molina et al., 2008), oxybenzone has a weak binding affinity to ER, with a higher affinity for ERα than ERβ (R. H. M. M. Schreurs, E. Sonneveld, J. H. J. Jansen, W. Seinen, & B. van der Burg, 2005). Some other studies have failed to report ERβ agonist activity at all (Schlumpf et al., 2004; Suzuki et al., 2005). In the MCF7 human breast cancer cell assay, oxybenzone induces proliferation up to 95% of the total induced by ethinyl estradiol, a positive control for ER agonist activity (Schlumpf et al., 2001). The EC50 for cell proliferation in the MCF7 cell assay is 3.73 μM, similar to other estrogenic endocrine disruptors, but much lower than the EC50 for 17β estradiol (1.22 pM).

Thus, *in vitro*, oxybenzone itself is often characterized as a weak ER agonist, but some of its metabolites show very strong estrogenic activity. For example, all of the benzophenones tested in yeast hERα assays were estrogenic – although full estrogenic activity was only exhibited when the hydroxyl-group was situated in the para- or, ortho- and para-positions, i.e. benzophenone 1, benzophenone 2, and 2,4,4, trihydroxybenzophenone (Kunz & Fent, 2006b; Suzuki et al., 2005; Watanabe et al., 2015). The main oxybenzone metabolites were further found to exhibit stronger ER agonist behaviors than the parent compound – and when combined with the parent compound, an additive estrogenic effect was observed in the yeast hERα
assays (Heneweer, Muusse, van den Berg, & Sanderson, 2005; L. Wang & Kannan, 2013). In other yeast assays, oxybenzone produced both cytotoxicity and ER agonistic effects (Balazs et al., 2016; Kunz, Galicia, & Fent, 2006). Lastly, transcriptional activity of both ERα and ERβ was increased by $10^{-5}$ and $10^{-4}$ M concentrations of oxybenzone (Schreurs et al., 2002).

Importantly, in vivo effects cannot be always assumed from in vitro data (Schreurs et al., 2002). Exposure to 3 mg oxybenzone/kg/day throughout pregnancy and lactation increased expression of Esr1 in the uterus of ovariectomized Balb/C mice, without increasing uterine weight (LaPlante, Bansal, Dunphy, Jerry, & Vandenberg, 2018) and an acute, 4-day, orally administered exposure to 1525 mg/kg/day oxybenzone in prepubertal rats elicited a significant increase in uterine weight (Schlumpf et al., 2001); both doses and effects are consistent with the characterization of oxybenzone as a relatively “weak” ER agonist. In a rat uterotrophic assay, a 3-day, intraperitoneal exposure to 20, 100 or 500mg/kg doses of oxybenzone did not change the weight of the uteri in ovariectomized females (although increased uterine weight was observed in females administered 50 μg/kg of ethinyl estradiol, a high dose of a positive control for estrogenicity) (Suzuki et al., 2005). A significant increase in uterine weight was, however, observed in rats exposed to 500 mg/kg of benzophenone 1 or 300 mg/kg of 2,4,4’ hydroxybenzophenone - both oxybenzone metabolites that are demethylated and hydroxylated with three hydroxyl groups, respectively. Uterotrophic assays have evaluated other oxybenzone metabolites including 2,4,4’ trihydroxybenzophenone and 4 hydroxy-, 2,4 hydroxy- and 2, 2’,4,4’ hydroxybenzophenones administered to immature Crj:CD (SD) rats. Doses ranging from 40 to 200 mg/kg increased the
blotted uterine weight following three days of subcutaneous injections with the tested compounds (Yamasaki et al., 2003).

Other effects consistent with ER agonist behavior have been documented in mice exposed during early development. For example, the mammary gland exhibits stimulated growth parameters, including increased numbers of differentiated epithelial structures, following perinatal exposure to 3 mg oxybenzone/kg weight/day (administered to the mother during pregnancy and lactation) (K. Matouskova et al., 2019). These effects on the mammary gland are somewhat similar to the effects that have been observed in female mice administered other ER agonists such as ethinyl estradiol and bisphenol S (S. Kolla et al., 2018), i.e. an increased number of alveolar ducts and decreased number of epithelial mitotic events while oxybenzone at 3mg/kg/day but not BPS increases ductal area and number of TEBs.

Estrogenic effects have also been observed in fish. Vitellogenin is the yolk protein that serves as a marker of ER agonist disruption in several fish species. Oxybenzone in wastewater and sediment induces vitellogenin in male medaka, California halibut (Sapozhnikova et al., 2005; Schlenk et al., 2005) and juvenile trout (Coronado et al., 2008). These effects may be due to oxybenzone itself, or the actions of more potent estrogenic metabolites, e.g., benzophenone 1 and 2,4,4, trihydroxybenzophenone.

**ER antagonist activity**

Fewer studies have evaluated ER antagonistic activity of oxybenzone. In one study, yeast reporter assays were used to evaluate anti-estrogenic properties of oxybenzone, other benzophenones, and metabolites (Kunz & Fent, 2006b). To identify ER antagonists, the yeasts were treated with 17β-estradiol at concentrations
that induce 65% of the maximal response, and then co-treated with increasing concentrations of the test compounds. Oxybenzone was the most potent ER antagonist evaluated, showing an antiestrogenic potency only “45 times less potent than the known antiestrogen 4-hydroxytamoxifen” and an IC50 of 1.8 x 10^{-5} M. Other UV filters had ER antagonist properties in yeast reporter assays, including 4-MBC, but only at higher concentrations.

**AR agonist and antagonist properties**

AR agonist and antagonist activities of benzophenone-type UV filters have been evaluated both in *in vitro* assays and *in vivo* assays. Using yeast expressing human AR, benzophenone 2 exhibited “submaximal to full androgenic activity [...] and full antiandrogenic activity” (Kunz & Fent, 2006b). Furthermore, in the yeast assay, oxybenzone, benzophenone 1 benzophenone 4, and 4 hydroxy- and 4,4’ dihydroxybenzophenone were pure anti-androgens without any AR agonist activity. The IC50 value for oxybenzone was 3.7 x 10^{-6} M, similar to the positive control for AR antagonist activity, flutamide (IC50 = 4.3 x 10^{-6} M) (Balazs et al., 2016; Kunz & Fent, 2006b).

Evaluations of AR agonist and antagonist behavior in MDA-kb2 cells similarly concluded that neither oxybenzone or benzophenone 4 displayed any AR agonist action but oxybenzone significantly reduced dihydrotestosterone-induced AR activation, consistent with an AR antagonist (R. Ma, Cotton, Lichtensteiger, & Schlumpf, 2003). Furthermore, in a HELN cell luciferase assay, all tested oxybenzone metabolites as well as the parental compound showed anti-androgenic properties (Molina-Molina et al., 2008).
In vivo, the homology between the rat and human AR ligand binding domain has led to the presumption that analogous effects would be observed between the species (Kelce, Gray, & Wilson, 1998). The Hershberger assay is used to test androgenic and anti-androgenic effects of chemicals. In 8-week old Brl Han: Wistar Jcl(Galas) rats administered 4 hydroxybenzophenone and 4,4, dihydroxybenzophenone via gavage for 10 days had only minor effects on prostate and bulbocavernosus levator ani muscle weight (Yamasaki et al., 2003). On the other hand, 300mg/kg/day 2,4,4’ trihydroxybenzophenone significantly suppressed the effects of testosterone on the prostate and seminal vesicles and so demonstrated its anti-androgenic potential (Suzuki et al., 2005).

Further in vivo, AGD is considered an important indicator of endocrine disruption. The distance between the anus and perineum measures typically about twice as long in males than in females. In both animal and human studies (Bornehag et al., 2015), AGD shortens as a result of disruption of testosterone activity during prenatal development (Salazar-Martinez, Romano-Riquer, Yanez-Marquez, Longnecker, & Hernandez-Avila, 2004). Perinatal exposure to 212 μg/kg oxybenzone induced a lower AGD (adjusted for body weight) in both male and female mice offspring (K. Matouskova et al., 2019). While in males those reductions suggest an anti-androgenic effect of oxybenzone (Fisher et al., 2016; Swan et al., 2005; C. J. Wolf, LeBlanc, Ostby, & Gray, 2000), in females a shorter anogenital distance cannot be quite easily explained. As noted in the sections above, shortened AGD was also reported in male mice in a standardized developmental and reproductive toxicology study, but only after much higher doses (Nakamura et al., 2015).
Finally, the effects of oxybenzone on androgen-sensitive gene expression was examined in adult male zebrafish (Bluthgen, Zucchi, & Fent, 2012). Exposures to 84 μg/L – an environmentally relevant concentration – led to downregulated expression of ar and cyp19b in the brain; interestingly, statistically significant negative fold change in the expression of these genes was not observed after exposure to higher or lower concentrations - 2.4 and 312 μg/L. Effects were also observed for gene expression in the testes, consistent with AR antagonism (Bluthgen et al., 2012).

**Summary of endocrine disrupting properties**

When considering the potential for chemical sunscreens to have endocrine disrupting properties, there are two important aspects to consider. First, there can be multiple, and even opposite, endocrine activities of individual compounds. The fact that personal care products, including UV filters, have more than one endocrine mode of action does not appear to be rare. For example, homosalate, oxybenzone, and 4-methylbenzylidene camphor each exhibited both ER agonistic as well as ER antagonistic actions (Mueller et al., 2003; Schreurs et al., 2002), and alkylphenols elicited both ER agonist and AR antagonist activities in vitro (Sohoni & Sumpter, 1998). Although it may appear counter-intuitive, compounds possessing both agonist and antagonist activity towards a single receptor (so called selective ER modulators – SERMs) are relatively common and have been effective pharmaceutical therapies used for endocrine mediated diseases, e.g., osteoporosis and breast cancer (Jensen & Khan, 2004; Lindsay, Gallagher, Kagan, Pickar, & Constantine, 2009).

Second, there are possible synergistic effects when multiple compounds are applied in conjunction. Traditional approaches to evaluate chemical mixtures,
including UV filters, assume the resulting endocrine effect can be calculated as a sum of the individual endocrine effects measured for individual chemicals in the mixture. Contrarily, synergistic effects of endocrine disrupting compounds are no longer considered rare, particularly in real-life mixtures of chemicals (Kunz & Fent, 2006a; Martin et al., 2021). For example, a mixture of eight compounds, individually considered weak ER agonists (including one of oxybenzone’s metabolites, 2,4, dihydroxybenzophenone) significantly exceeded the estimation of estrogenic effects based on effect summation (Silva, Rajapakse, & Kortenkamp, 2002).

Both of the issues described above (the multiple endocrine modes of action and the additive or synergistic effects of chemical mixtures), have implications on human health and wildlife species. These issues also have potential implications for how endocrine disruptors are addressed in risk assessments and policy.

2.3.8 Genotoxicity & Carcinogenesis

A number of approaches have been used to evaluate oxybenzone’s potential for genotoxicity (National Toxicology Program, 2006). The NTP used E. coli mutagenesis assays with multiple bacterial strains and found no evidence for mutagenesis, either with or without liver extract (to evaluate metabolites as well). Others that have evaluated oxybenzone for mutagenicity reported conflicting results based on the bacterial strain used with either no or weak mutagenic properties of oxybenzone observed in a Salmonella assay (Zeiger et al., 1987).

Genotoxicity tests using other approaches have examined the frequency of micronucleated erythrocytes in the blood of mice from subchronic toxicity studies. In males, there was a modest decrease in the number of micronuclei that were observed, but this trend was not statistically significant. In females, there were no
significant differences between oxybenzone and control groups, but there was a statistically significant dose-response trend for increased number of micronuclei (Witt et al., 2000).

The NTP conducted a two year carcinogenesis study evaluating the carcinogenic potential of oxybenzone in male and female Harlan Sprague-Dawley rats exposed *in utero* starting on gestational day 6 through feed administered to the mother (National Toxicology Program, 2006); post-weaning, the male and female offspring continued to be administered oxybenzone in the feed for 105 weeks. The diets contained 0, 1000, 3000 and 10000 ppm oxybenzone. In the males, an increase in testicular interstitial cell hyperplasia, a non-neoplastic effect, was observed at the highest dose. Malignant meningioma (a brain malignancy) was observed in males from the two lowest dose groups, but this effect was considered equivocal because it was not also observed in the highest treatment group. In female rats, an increase in atypical hyperplasia of the uterine endometrium was observed in all three oxybenzone-treated groups, and an increase in hypertrophy in the adrenal cortex was reported for animals administered 1000 and 3000 ppm. More females from the two mid-dose groups had uterine stromal polyps and females from all three oxybenzone groups were more likely to have thyroid C-cell adenomas, but again, these findings were considered “equivocal”.

The NTP conducted a similar study in B6C3F1/N mice, exposed for 2 years (but without the prenatal exposure that was administered to the rats) via diets containing 0, 1000, 3000 or 10000 ppm oxybenzone. Although there were signs of general toxicity (e.g., a loss of 10% or more body weight) in both male and female mice, there were no neoplastic effects observed. Non-neoplastic changes that were observed included a striking increase in the number of animals with pigmented bone
marrow (an effect observed in every single male and female from the highest dose group), an increase in the number of males and females with altered spleen pigmentation, and kidney metaplasias in 10% of females from the highest dose group. The vast majority of males from the highest dose group also had syncytial alterations in liver hepatocytes and cytoplasmic alterations of the renal tubules.

Based on the outcome of the NTP's rat and mouse carcinogenesis studies, the NTP expert panel concluded that there was equivocal evidence of carcinogenic activity in male and female rats. There was no evidence of carcinogenic activity observed in mice.

2.3.9 Epidemiology

To date, a few dozen epidemiology studies have examined oxybenzone (and/or its metabolites) and associations between exposures and human diseases. These studies have varying strengths based on their study designs (cross-sectional versus case-control versus cohort), the size of the populations evaluated, the quality of the methods used to quantify exposures, and other considerations (e.g., confounding, latency between exposure and effect, etc.) Considering these limitations, we summarize the results of epidemiology studies evaluating endpoints affecting reproduction and endocrine functions.

Endometriosis is an intricate disease characterized by the presence of misplaced functional endometrium-like tissue outside the uterus. The diverse symptoms associated with this disease include chronic pelvic pain, infertility and higher risk of gynecological cancer and cardiovascular disease. Endometriosis affects an estimated 10-15% of women of childbearing age (Eisenberg, Weil, Chodick, & Shalev, 2018). The causal factors are yet to be elucidated but the known
sensitivity of the endometrial tissue to estrogens hints at a potential role for endocrine disrupting chemicals as important factors in the onset or promotion of endometriosis. A case-control study evaluating women undergoing abdominal surgery found an increased risk of endometriosis associated with urine oxybenzone and benzophenone 1 (OR = 4.98 and 5.1, \( p = 0.011 \) and \( p = 0.008 \) respectively) (Peinado et al., 2020). Interestingly, the associations were only observed for women in the second tercile of urine concentrations (e.g., the mid-range of exposures), and the risk was increased when measures of oxidative stress were included in the statistical models. In another study examining a large US cohort, the presence of benzophenone 1, and to some extent also oxybenzone in urine samples correlated with risk of endometriosis (Kunisue et al., 2012). This in vivo outcome is supported by the potent ER agonist features of benzophenone 1 observed in in vitro experiments.

Successful conception may not occur if environmental signaling interferes with the events that coordinate male and female fertility and the health of the gametes. Oxybenzone (along with bisphenol A and triclosan, two common endocrine disrupting chemicals) was measured in the large biomonitoring study, the U.S. National Health and Nutrition Examination Survey (NHANES). In a cross-sectional study utilizing NHANES data, the urinary levels of oxybenzone, bisphenol A and triclosan were clustered together and the triad was positively associated with self-reported female infertility (prevalence ratio = 1.13, 95%CI 1.04-1.24, \( p = 0.007 \)) (Arya, Dwivedi, Alvarado, & Kupesic-Plavsic, 2020). On the other hand, in a prospective cohort study, oxybenzone concentrations were measured in urine samples from women undergoing infertility treatment (Minguez-Alarcon et al., 2019). All three fertility endpoints in the study – probability of implantation, clinical
pregnancy, and live birth – were positively associated with the urinary oxybenzone levels, suggesting that oxybenzone promotes fertility in women experiencing infertility. However, these associations were only observed in the cohort of women who also reported the most time spent outdoors doing “moderate/heavy work”. Sun exposure and physical activity outdoors are frequent confounders in studies evaluating multi-causal conditions. As the authors suggest, their results may suggest that the link between successful conception and pregnancy outcomes may rather point to lifestyle and environmental factors than the sunscreen ingredient. Although the link between excessive UV exposure and skin cancer is well established, moderate sunlight exposure in two Swedish cohorts was negatively correlated with all-cause mortality (Lindqvist et al., 2014a; Yang et al., 2011); and so, the above results point beyond the risks and benefits of sunscreen chemicals only.

In other epidemiology studies, birth outcomes, too, were correlated with urine concentrations of oxybenzone. With higher levels of serum oxybenzone in the mothers, there was a decrease in birth size in female newborns; gestational measures of oxybenzone were positively associated with offspring weight and head circumference in male infants (Philippat et al., 2012; Wolff et al., 2008). In both sexes, maternal urinary oxybenzone measures were also associated with shorter gestation (Tang et al., 2013).

Children’s behavior outcomes were evaluated in a prospective, longitudinal cohort study. Researchers reported inverse associations between maternal urinary concentrations (median 0.37 ug/L, min. 0.08 and max 485 ug/L) and scores on pro-social behavior (Guo et al., 2020). No associations were observed between childhood urinary oxybenzone concentrations (median 1.76 ug/L, min. 0.98 and max 783 ug/L) and pro-social behaviors. An earlier study failed to see differences in
prosocial behaviors associated with oxybenzone in a cohort of 5-year-old boys (Philippat et al., 2017), but important confounders, such as breastfeeding, socioeconomics, and postnatal levels of other chemicals that are closely associated with neurobehavioral adverse effects in humans were not accounted for in this study.

Thyroid hormone balance plays an essential role in neurodevelopment as well as metabolic and energy homeostasis throughout the lifetime. Using NHANES data, a cross-sectional evaluation suggested that oxybenzone had significantly inverse associations with the levels of serum thyroid hormones – both free and total T4 and total T3 (S. Kim, Kim, Won, & Choi, 2017). Oxybenzone exposure levels are also modestly associated with increases in the pituitary hormone thyroid stimulating hormone. These results are consistent with results from in vitro studies, which reveal potent inhibiting activity of benzophenone 2 towards thyroid peroxidase; benzophenone 2 also decreased total T4 concentrations in vivo, but oxybenzone failed to show an effect on these outcomes (Schmutzler et al., 2007).

Figure 2.4: Summary of epidemiology studies of oxybenzone. To date, most of the epidemiology studies examining associations between oxybenzone and health effects have focused on outcomes relevant to fertility, birth outcomes, and other endocrine effects.
In other human organs, oxybenzone has been detected in the adipose tissue of female breasts and its concentration positively correlates with the incidence of tumors in the lateral region of the breast (Barr, Alamer, & Darbre, 2018). In human placenta, oxybenzone concentrations were associated with lower placental weight and placenta-to-fetal weight (Philippat et al., 2019). Further, correlations between oxybenzone exposures have been suggested in the incidence of a rare birth defect associated with failure of neural crest cells to migrate to the intestine (Huo et al., 2016).

Although the number of epidemiology studies evaluating oxybenzone remain limited, these studies suggest that exposures – especially during vulnerable windows of development – may contribute to diseases. To date, the current available evidence for effects on human populations is relatively weak, but the possibility that oxybenzone might cause harm to human populations is particularly concerning because of the ubiquitous exposures that have been documented. These widespread exposures will also contribute to challenges in future epidemiology evaluations, similar to those that have been documented for other non-persistent chemicals.

### 2.4 Benzotriazole UV Stabilizers

UV stabilizers are chemicals added to products to prevent degradation of the product [TABLE 2.2]. These are added to plastics, paints, building products, and other consumer goods to protect against damage from harmful UV radiation; they also can prevent product fading, inhibit corrosion, and minimize fog.
One of the most common classes of chemicals used as a UV stabilizer is benzotriazole and its derivatives. These chemicals are currently produced in volumes that exceed 8000 metric tons per year. Compared to oxybenzone, there are fewer studies that have evaluated the health hazards or environmental impact of benzotriazole-based UV stabilizers. However, because of evidence that one benzotriazole derivative, UV-328, is environmentally persistent, the Swiss

Table 2.2: UV stabilizing chemicals commonly used in plastics and other consumer goods

<table>
<thead>
<tr>
<th>Substance</th>
<th>CAS number</th>
<th>Synonyms</th>
<th>Trade Names</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzotriazole</td>
<td>95-14-7</td>
<td>BTA, BtaH</td>
<td>Tinuvin 234 FF</td>
<td></td>
</tr>
<tr>
<td>2-(2H-benzotriazol-2-yl)-4,6-bis(1-methyl-1-phenylethyl)</td>
<td>70321-86-7</td>
<td>UV-320</td>
<td>Tinuvin 320</td>
<td></td>
</tr>
<tr>
<td>2-(2H-benzotriazol-2-yl)-4,6-di-tert-butylphenol</td>
<td>3846-71-7</td>
<td>UV-320</td>
<td>Tinuvin 320</td>
<td></td>
</tr>
<tr>
<td>2-(5-chloro-2H-benzotriazol-2-yl)-6-(1,1-dimethylethyl)-4-methyl</td>
<td>3896-11-5</td>
<td>UV-326</td>
<td>Tinuvin 326</td>
<td></td>
</tr>
<tr>
<td>2-(2'-Hydroxy-3',5'-di-tert-butylphenyl)-5-chlorobenzotriazole</td>
<td>3864-99-1</td>
<td>UV-327</td>
<td>Tinuvin 327</td>
<td></td>
</tr>
<tr>
<td>2-(2H-benzotriazol-2-yl)-4,6-di-tert-pentylphenol</td>
<td>25973-55-1</td>
<td>UV-328</td>
<td>Tinuvin-328</td>
<td></td>
</tr>
</tbody>
</table>
Government has proposed that its use should be limited and eventually eliminated. Switzerland proposed that this UV stabilizer should be added to the Stockholm Convention, a list of the most hazardous chemical substances that governments agree to restrict to protect human health and the environment.

There are a large number of individual UV stabilizers that are benzotriazole derivatives. In this portion of the chapter, we will review these chemicals as a class and will indicate when the results are specific to a single compound.

2.4.1 Uses in Products / Sources of Exposure

UV stabilizers are added to plastic packaging used to contain food, drugs, and cosmetics. They are also added to plastic products such as toys and sports equipment, to fabrics used in clothing, upholstery and other furnishings, and to building materials including paints. A study that examined new products found detectable levels of UV stabilizers in plastics used in food packaging including plastics made from polypropylene, polyethylene terephthalate, and polyethylene (Rani et al., 2017). UV stabilizers were also measured in plastics used in the fisheries industry including fish traps, ropes, and floats.

Plastics made from polyethylene terephthalate with the UV stabilizer UV-234 are commonly used as food containers for products ranging from beer bottles to food jars to condiment containers (Begley, Biles, Cunningham, & Piringer, 2004). A migration study using food simulants found temperature- and time-dependent migration characteristics; after 10 days at 40°C, the amount of UV-234 that migrated to an ethanol-based simulant was 2 μg per 100 cm².
2.4.2 Measurements in the Environment

Plastic pollution is a serious global environmental challenge. Because UV stabilizers are widely used in different plastic products, one way that their environmental impact has been evaluated is to measure their concentrations in plastics collected from the environment [Figure 2.6]. Marine plastic debris collected from the coast of Korea had measurable concentrations of UV stabilizers including UV-320, UV-326, UV-327, and UV-328 (Rani et al., 2017). When the concentrations measured in the debris were compared with new products, the concentrations differed. UV-320 concentrations were lower in marine debris compared to new products, consistent with release to the environment. In contrast, UV-326 concentrations were typically higher in plastics recovered from the marine environment compared to new plastics, consistent with adsorption of this UV stabilizer from sources in the environment. Another study of plastic fragments collected from beaches in Hawaii measured detectable levels of UV-326, UV-327 and UV-328 in both small (4-7 mm) and large (15-80 mm) fragments (Tanaka, Takada, Ikenaka, Nakayama, & Ishizuka, 2020). One or more of these chemicals were detected in 6 of the 9 small fragments, and in 18 of the 23 large fragments, at concentrations as high as 813 μg/g plastic for UV-326 and 763 μg/g plastic for UV-327. UV-328 was only detected in a single plastic fragment at a concentration of 0.2 μg/g plastic. The authors concluded that fragmentation of plastics does not induce “exhaustive leaching” of UV stabilizers from plastics.

In addition to the measurement of plastics in the environment, UV stabilizers that have leached from consumer products have been measured in environmental matrices including wastewater, sludge, and sediment [Figure 6]. In a study from the Spanish Canary Islands, six benzotriazole derivatives were evaluated in
environmental samples (Montesdeoca-Esponda, Torres-Padrón, Sosa-Ferrera, & Santana-Rodríguez, 2021); more than 60% of all sludge samples contained detectable levels of UV-328 and UV-360. Although detection rates were lower for UV-329, the maximum measurable concentrations of this UV stabilizer in environmental matrices were very high (1933 ng/L in wastewater influent, 57 ng/L in wastewater effluent and 859 ng/L in seawater). Another study from the Canary Islands reported high concentrations of UV-328, UV-329 and UV-360 in sewage samples collected between 2016 and 2017 (Montesdeoca-Esponda, Álvarez-Raya, Torres-Padrón, Sosa-Ferrera, & Santana-Rodríguez, 2019). Results reported from other studies of wastewater treatment plants in Canada (Lu, Smyth, Peart, & De Silva, 2017), China (R. Liu et al., 2014; Song, Ruan, Wang, Liu, & Jiang, 2014), Spain and Portugal (Carpinteiro, Ramil, Rodríguez, & Nogueira, 2012), and sewage sludge from China (T. Ruan et al., 2012) indicate that benzotriazole derivatives are routinely detected in both liquid and solid samples, with differences in the detection rates, concentrations, and identities of specific monomers based on location. These findings indicate that there are geographic differences in the use of benzotriazoles, likely due to their use in different products and the handling of products during disposal. In Norway, four benzotriazoles were detected in wastewater treatment plant effluents and landfill leachates in concentrations that suggest the possible bioaccumulation potential of all compounds (Langford et al., 2015).
UV stabilizers have also been documented in seawater including coastal waters from China, Japan, Korea, Hong Kong, India, Indonesia, the Philippines, and the United States between 2003 and 2007 (Nakata et al., 2012). Although there were differences between geographic locations, UV-328 was measured in seawater samples from both Asian nations and the United States. Other evaluations of the both seawater and sediment from other locations, including the North and Baltic Sea, revealed that these waters are routinely contaminated with benzotriazole derivatives, with UV-360 accounting for the majority of the UV stabilizers measured in northern European waterways (Apel, Joerss, & Ebinghaus, 2018). Further, in the Chinese Bohai and Yellow seas, UV-329 was the most predominant benzotriazole compound, especially around the Shandong Peninsula (Apel, Tang, & Ebinghaus, 2018). River inputs to the Bohai and Yellow seas were determined to be the major source of the UV stabilizers measured in seawater. Importantly, a study evaluating the concentrations of benzotriazoles in coastal areas around a production site on the Northeast coast of the United States found that concentrations remained largely unchanged even after production of the

Figure 2.0.5: Benzotriazole derivatives are measured in environmental matrices. UV stabilizing chemicals are released into the environment during production, and also from the breakdown of products. The widespread reach of plastic pollution, and degradation of consumer goods, has led to the detection of benzotriazole derivatives in coastal waters, wastewater, sewage sludge, freshwater, and house dust.
monomers ceased (Cantwell et al., 2015). The authors concluded from this study that these chemicals persist in the environment and resist degradation in environmental matrices.

Benzotriazole-based UV stabilizers are routinely detected in freshwater including rivers in China (Peng et al., 2017; Z. Zhang et al., 2011) and India (Vimalkumar et al., 2018), and in urban creek waters and sediments collected in Canada (Lu, De Silva, et al., 2016). These chemicals were also detected in dust samples collected from homes in either an urban area or homes located in an area near a municipal dump in the Philippines (J. W. Kim et al., 2012); detection frequencies for five benzotriazoles ranged from 82-94% in urban residences and 65-95% in residences near the municipal dumpsite. Concentrations of UV-234, UV-326, and UV-328 were the highest in the dust samples. The presence of these chemicals in house dust suggest that they are shed from products routinely used in the home.

2.4.3 Measurements in Wildlife and Humans

With benzotriazole derivatives measured in water and sediment samples around the world, it is perhaps no surprise that these chemicals have also been widely measured in fish and other aquatic biota. Fish collected from three rivers in India had detectable levels of six UV stabilizers including UV-9, UV-P, UV-326, UV-327, UV-328, and UV-329 (Vimalkumar et al., 2018). There were differences in the concentrations of each monomer between the three rivers, with an influence of the season (dry versus wet) as well. Benzotriazoles were also measured in fish collected in the Philippines (J. W. Kim, Ramaswamy, Chang, Isobe, & Tanabe, 2011), Canada (Lu, Peart, Cook, & De Silva, 2016), the Canary Islands (Montesdeoca-Esponda et al., 2020), and China (Peng, Jin, Wang, Ou, & Tang, 2015).
Numerous studies have evaluated animals across the kingdom including shellfish (clams and oysters), crustaceans, aquatic predators including marine mammals and sharks, and coastal birds. These studies document benzotriazole exposures in animals collected from Japan (Nakata, Murata, & Filatreau, 2009; Nakata, Shinohara, Murata, & Watanabe, 2010), the North American Great Lakes (Lu et al., 2018) and other locations in Canada (Lu, De Silva, Zhou, et al., 2019) including Arctic sites (Lu, De Silva, Provencher, et al., 2019).

Although exposures have been well documented in animals, human exposures are less-well studied. Two studies have evaluated human breast milk samples collected from four Asian countries (Japan, the Philippines, Vietnam, and Korea. Multiple benzotriazole UV stabilizers were measured in milk samples, typically in the range of 1-75 ng/g lipid when examined individually. In Korea, the sum of eight benzotriazole derivatives measured in human milk was $181 \pm 317$ ng/g lipid (S. Lee et al., 2015). In Vietnam, the Philippines, and Japan, the sum of eight UV stabilizers was $300 \pm 240$ ng/g, $100 \pm 130$ ng/g, and $28 \pm 34$ ng/g lipid, respectively (J. W. Kim et al., 2019).

### 2.4.4 ADME

A rodent study of 2-(2'-hydroxy-3',5'-di-tert-butylphenyl)benzotriazole (HDBB), a UV stabilizer and absorber used in plastics, documented several metabolic outcomes in rats administered 0.5, 2.5 or 12.5 mg/kg/day for 28 days (Hirata-Koizumi et al., 2009). Following intragastric administration, HDBB was rapidly absorbed into the bloodstream, with concentrations that peaked 5-8 hours after daily intake. In animals administered the highest dose, maximal plasma concentrations of HDBB were in the range of 3-5 μg/L with no clear sex-specific effects. Furthermore,
the toxicokinetic parameters were similar after the first administered dose and the 28th administered dose, and no HDBB metabolites were ever measured in plasma samples.

In a human study, a single volunteer was administered a single dose of UV-328 (0.3 mg/kg) and blood samples were collected 2, 4, 6, 8, 10, 24, 34 and 48 hours after exposure. The maximum blood concentration was measured at 8 hours (1.31 mg/L) post-exposure, and five metabolites were measured at this time point (Denghel & Göen, 2021). Analyses of urine samples collected from this individual revealed that urinary concentrations of the parent compound had no detectable levels of UV-328 in samples collected at 24 hours post-exposure, but four metabolites were measured at concentrations ranging from 1.4 – 9.2 μg/L (Denghel & Göen, 2020). A more complete human metabolism study is promised for the future.

2.4.5 Toxicity

The hazards of specific benzotriazole UV stabilizers have been evaluated in invertebrates, fish, and rodents in controlled laboratory studies. Although many of the benzotriazoles have not been studied, and there is a need for studies to evaluate environmentally relevant mixtures, the few studies that are available suggest potential adverse effects across the animal kingdom.

In studies evaluating the freshwater crustacean *Daphnia magna*, neither UV-234 or UV-328 altered neonate number, size, or number of molts (Giraudo et al., 2017). There were also no effects of either benzotriazole on oxidative stress gene expression in this species. Yet, both benzotriazoles altered expression of oxidative stress genes in *C. reinhardtii*, a species of freshwater green algae; production of
reactive oxygen species was also increased after exposure to UV-328 in this species.

Other studies have examined the effects of benzotriazole UV stabilizers on measures of toxicity in zebrafish (Liang, Adamovsky, Souders, & Martyniuk, 2019). Effects of UV-234 were observed on embryo hatching time, measures of mitochondrial and non-mitochondrial respiration, expression of genes involved in oxidative stress, and responses in a photokinesis behavioral experiment. Different effects were observed in zebrafish exposed to UV-320, which did not affect hatching time but altered measures of basal and non-mitochondrial respiration, expression of genes involved in oxidative stress, and genes associate with innate immunity.

In rats, administration of HDBB for 28 days induced numerous changes associated with toxicity (Hirata-Koizumi et al., 2007). Doses at or above 2.5 mg/kg/day altered red blood cell counts, hematocrit and hemoglobin levels, and blood glucose levels. HDBB exposures altered other clinical chemistry measures including blood cholesterol and triglyceride levels, and increased liver and kidney weights at necropsy. Histopathological evaluation revealed degeneration of the cardiac myocardium, cell infiltration in the heart, follicular cell hyperplasia in the thyroid, and hypertrophy of liver hepatocytes in exposed males. A follow-up 28-day study examined the effects of HDBB in castrated male and female rats (Hirata-Koizumi, Matsuyama, et al., 2008). Again, exposures to HDBB increased the weight of the liver at necropsy with effects that were more extreme, and observed at lower doses, in males compared to females. However, the sex-specific effects that were observed in liver histopathology were absent in castrated males, suggesting a role for hormones in the etiology of these effects.
A chronic toxicity study evaluated rats exposed to HDBB daily over a period of either 13 or 52 weeks (Hirata-Koizumi, Ogata, et al., 2008). Significant effects of HDBB (usually observed in animals exposed to 0.5-2.5 mg/kg/day) were observed on red blood cell count, hematocrit measures, and platelet counts in males. Effects were also observed on blood biochemistry, with greater effects on males than on females. In males, long-term effects were observed on organ weights (lungs, testes, kidneys, liver, and heart, which were all heavier than controls) and body weights (which were lighter than controls). In females, effects were only observed on liver weight.

Collectively, these results suggest that the benzotriazole UV stabilizer HDBB is toxic, with effects that are more severe in males than in females. This outcome provides an indication that the actions of HCBB may be mediated by hormone receptors, or there could be sex-specific effects on HCBB metabolism and excretion.

2.5 Risk / Benefit Analysis of Chemical Sunscreens

Chemical sunscreens were designed to be an effective tool to improve public health so that “in the foreseeable future most skin cancer can be prevented” (Urbach, Forbes, Davies, & Berger, 1976). That assurance has not been realized. Quite the contrary, skin cancer both originating in keratinocytes (squamous and basal cell carcinomas) and melanocytes (melanomas) are on a gradual but steady rise (NIH, 2020; Rogers, Weinstock, Feldman, & Coldiron, 2015b).

While the use of chemical sunscreens has increased, especially as these chemicals are added to more and more personal care products, evidence continues to mount that chemical sunscreens are a detriment to the health of humans, wildlife, and whole ecosystems. Popular use of oxybenzone has polluted organisms on
virtually all the branches of the phylogenetic tree. This circumstance with oxybenzone creates two reactions: astonishment & hope. Astonishing is the rate in which oxybenzone has spread everywhere on the planet and the volume in use that allows it to keep polluting. Hope is that the damage can be undone. Because oxybenzone can be metabolized, its more judicious use in the future could mean that pollution of environmental matrices can be mitigated.

2.6 Alternatives to Chemical Sunscreens

Inorganic sunscreens based on metals were once frowned upon due to their unattractive formulation. Yet, mineral sunblockers today are largely designed as nanostructures, most commonly of zinc oxide and titanium dioxide. As pollution with chemical sunscreen has become a concern, nano-sunblockers have been re-examined for their possible market potential and hope as safer sun protection. But nanoparticles are not without risks. When applied to the skin, zinc nanoparticles pass through the epidermal and dermal layers and are absorbed systemically (Gulson et al., 2010) and their hydrophobicity and persistence further raise concerns.

Promising work with plant extracts, e.g., green and white tea, as agents to protect against UV-induced stress – suggests some possibility for use as sunscreens, as measured by the presence of Langerhans cells and absence of oxidative damage in skin cells in vivo (Camouse et al., 2009). Consumption of extracts from the fruit *Garcinia indica* (also known as mangosteen) enhances endogenous UV factors (Dike & Deodhar, 2015). More work is certainly needed to identify safer alternatives, and it should not be assumed that “natural” products are necessarily safe, or that high dose supplements of single compounds can replicate the more complex effects of whole foods.
2.7 Conclusions

Short, habitual exposure to the complex spectrum of sunlight has a multitude of therapeutic effects. First, it effectively enables synthesis of vitamin D3 from a cholesterol metabolite in the skin. Following several more conversions, the active version of vitamin D is then responsible not only for absorption of calcium from the diet, calcium and phosphate homeostasis, and bone metabolism, but also for arterial health, and tumor prevention via inhibited inflammation and proliferation. While supplementation with vitamin D has been heavily promoted (Wacker & Holick, 2013), large epidemiological studies have shown no benefit compared to placebo against several types of cancer or cardiovascular disease (JoAnn E Manson et al., 2019), no effect on the quality of cartilage, bone, or pain in the joints of osteoporotic patients (Jin et al., 2016), and no effect on bone and cartilage health in infants (Gallo et al., 2013). Only in elderly populations does vitamin D supplementation reduce the risk of hip and nonvertebrate fractures, but only after administration of doses twice the recommended 400IU per day (Bischoff-Ferrari et al., 2005). Second, sunlight increases nitrite oxide in the skin and therefore naturally lowers blood pressure (D. Liu et al., 2014), it increases levels of serotonin and dopamine (Lambert, Reid, Kaye, Jennings, & Esler, 2002), and exposure to sunlight is associated with increased life expectancy by lowering overall mortality (Lindqvist et al., 2014b). Conversely, long, ad hoc exposures to sunlight are associated with acute damage of the skin, accelerated skin aging, and skin neoplasias.

Considering the many benefits associated with sun exposure, as well as the risks associated with excess exposure to UV light, humans have responded by producing dozens of chemicals that can be used in sunscreen formulations. The
incorporation of these chemicals into numerous personal care products, beyond traditional sunscreens, has increased their release into the environment. Furthermore, UV filters are no longer limited to use on human bodies; they are also now added to numerous consumer products to provide protection against the harmful effects of UV radiation. This expanded use of UV absorbing and UV stabilizing chemicals has led to contamination of environmental matrixes, wildlife, and the bodies of humans.
3.1 Assessing the Mammary Gland: Whole Mounts

My dissertation projects involve evaluations of the mouse mammary gland in both sexes during several life stages. These approaches allowed for the assessment of a set of well-established variables while developing several new ones. First, in many of our studies, we examine the 4th inguinal mammary gland ductal network in a “whole mount” preparation, i.e., the mammary fat pad is dissected in its entirety and spread on a slide glass, processed and stained, and then visualized using a dissection microscope at magnifications from about 10x to 35x.

In life stages prior to adulthood, we typically image the entire mammary gland and measure in a 2-dimensional view the entirety of the epithelial ductal network. The first quantification we perform is to measure the area subtended by ducts, an endpoint known as the ductal area (FIGURE 3.1A). The next measure of growth tells us how far the ductal tree has grown relative to the centrally located lymph node: the ductal extension (FIGURE 3.1B). Growth of the mammary epithelium during pubertal stages is dependent on the most distal structures of the mammary ducts, the terminal end buds (TEBs), which are defined as bulb-like structures at the tips of ducts that measure equal to or greater than 0.03mm$^2$ (FIGURE 3.1C). These are counted and individually measured.

The number of branching points is the fourth endpoint we measure in early life stages in females and throughout the lifetime of male individuals. Branching points occur because the actively proliferating cells of TEBs are guided not just straight ahead but also to fork, creating secondary and tertiary branches from the primary
stem. Further elongation brings about still more secondary and tertiary branching and counting the forking events provides an estimation of the tree complexity (FIGURE 3.1D). A simple counting method, however, is difficult to use to quantify branching points in the more complex epithelial structures of females starting at pubertal stages. Therefore, we have explored the possibility of using the Sholl analysis, a software tool originally developed to measure the complexity of branching in neurons, and adapted for the rodent mammary gland by our collaborators at NIEHS (Stanko, Easterling, & Fenton, 2015; Stanko & Fenton, 2017). Evaluation of the Sholl analysis was the topic of an undergraduate honors thesis by Gillian Szabo, who used it to quantify the effects of several xenoestrogens on both male and female mouse mammary glands. However, her analyses revealed that this complex, time-consuming method was no better at quantifying effects of xenoestrogens on the mammary gland than the more simplified quantification tools described above.

Adult whole mount mammary glands in BALB/c female mice are generally too large and too complex to be effectively assessed using most of the endpoints described above. For two of my projects that assessed adult mammary tissues – collected either from the F1 generation at 9-11 weeks of age or from post-involution pectoral mammary glands of F0 generation female mice up to 52 weeks old – we applied so called “grid analysis”, a method of unbiased stereology used to quantify the volume fraction of specific structures. We have also quantified mammary gland parameters using optical density, which relies on the spectrophotometric tools of the microscope to compare darkly stained structures (e.g., epithelium) between images (FIGURE 3.1E).

Lastly, aged and disrupted mammary glands suffer from various structural anomalies observable in the whole-mounted mammary glands with a dissection microscope. Intraductal epithelial hyperplasia are a common abnormality with or without malignant potential that may occur either due to a genetic precondition or exposure to excessive endogenous or exogenous estrogens (Bocchinfuso, Hively, Couse, Varmus, & Korach, 1999; K. Mandrup, Boberg, Isling, Christiansen, & Hass, 2016) (Vandenberg et al., 2008a). These structures appear as ducts with a beaded appearance (FIGURE 3.1F). We typically report these kinds of hyperplasia as a binary outcome (present/absent) within each whole-mount and also score their severity. Hyperplastic alveolar structures can also be observed in whole-mounted mammary glands; these appear as heavily stained, and sometimes overgrown, lobular units.
3.2 Assessing the Mammary Gland: Epithelium

The mammary gland is an organ comprised of an interacting collection of tissue types, origins, functions, and regulation. The normal mammary gland has a relatively rigorous organization of ductal epithelial cells compartmentalized by the proteoglycans and a matrix of basal lamina from the surrounding stroma.

The mammary epithelium is a bilayer located ventral to the basal lamina. Facing the ducts is a layer of luminal cuboidal cells connected firmly to an underlying sheet of myoepithelial and/or basal cells in various stages of stemness (Cristea & Polyak, 2018; Slepicka et al., 2021). Despite its rigorous structure, the epithelial cellular environment is highly dynamic throughout the female’s lifetime and the cells of both the epithelial layers display various phenotypes. For example, in the adult murine mammary gland, the basal progenitor cells are abundant in nulliparous females and during pregnancy; myoepithelial and protein c receptor basal cells are detected almost exclusively during lactation; and fewer basal cells appear during involution (Bach et al., 2017). The latest methods using large-scale single-cell profiling underscore the complexity of the long- and short-term progenitor cells during both rapid morphogenesis – puberty and pregnancy – and throughout the “maintenance” periods (Fu, Nolan, Lindeman, & Visvader, 2020). Moreover, transient intermediate phenotypes either between the luminal and basal cells during embryonic development or two luminal types in the adult mammary gland were identified (Pal et al., 2017; Trejo, Luna, Dravis, Spike, & Wahl, 2017).

In the mouse, during puberty, the ducts open into TEBs; these are stem-cell enriched structures that drive branching and elongation of the ductal web (Paine & Lewis, 2017). On the end most distant from the nipple, the TEBs are covered with a
line of cap cells that will eventually become myoepithelial cells. The TEB itself is filled with body cells that beget luminal cells. We evaluate TEBs for markers of proliferation, which allows us to quantify the magnitude of ductal growth, and apoptosis, the morphogenetic process responsible for ductal lumen formation. The mammary gland maintains a balance between these two opposing processes and, although it may seem counter-intuitive, estrogens are responsible for both ductal growth and ductal patency; this balance is potentially disrupted due to abnormal estrogen signaling induced by environmental contaminants (Humphreys, 1999; J. Wang, Jenkins, & Lamartiniere, 2014).

Two types of luminal cells have been identified based on their relative expression of hormone receptors – hormone receptor (HR) high or low. HR-high cells, e.g., hormone sensing luminal cells, also belong to two groups: hormone sensing progenitor cells (present in nulliparous tissues and in post-pregnancy involution) or differentiated cells (identified in nulliparous animals, during involution and pregnancy) (Bach et al., 2017). By using single-cell RNA sequencing, it was discovered that the key genes characteristic for low-HR cells progenitor cells are only expressed in nulliparous animals and in post-pregnancy involution while low-HR alveolar – both progenitor and differentiated -are expressed during pregnancy and lactation; and that high-HR luminal progenitors are present in nulliparous and involuting mammary gland. Fifteen distinct epithelial mammary cells speak of a complex cellular environment where cell populations are transient and commonly express an intermediate phenotype (Bach et al., 2017; Fu et al., 2020).
Many endocrine disruptors are known to alter the expression of hormone receptors, either at the level of mRNA (typically measured with qRT-PCR or RNAseq) or the number of hormone receptors (measured with western blots), or the number of cells expressing hormone receptors (measured with immunohistochemical evaluations) both in vivo and in vitro (La Merrill et al., 2020; H. R. Lee et al., 2013). In the mammary gland, exposures to synthetic estrogens increase the risk of cancer in females in at least two generations, and mammary neoplasia in rodents of both sexes (Hilakivi-Clarke, de Assis, & Warri, 2013; Sweeney, Hasan, Soto, & Sonnenschein, 2015). In our lab, we typically evaluate expression of hormone receptors (including ERα and PR; TABLE 3.1.) in epithelial cells using immunohistochemical methods. Briefly, tissues that have been fixed are embedded in paraffin and sectioned to single-cell thickness, revealing the mammary structures. Nuclear receptors are detected by incubating these tissues with polyclonal rabbit antibodies generated against the receptor of interest; the antibodies are diluted in blocking solution in concentrations optimized for the mammary gland (usually 1:1000 for ERα, 1:500 for PR). The bound antibodies, and thus the hormone receptor expressing cells, are then detected using tagged secondary antibodies.
a colorimetric indicator. The immunohistochemistry method is also used to assess the level of proliferation, which is usually reported as a percent of actively proliferating epithelial cells (Ki67 marker, 1:1000).

**Table 3.1: Antibodies**

<table>
<thead>
<tr>
<th>Protein/Peptide Target</th>
<th>Name of the Antibody</th>
<th>Manufacturer Catalog #</th>
<th>Species/Raised in Polyclonal or Monoclonal</th>
<th>Dilution Used</th>
<th>RRID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>Ki67</td>
<td>Thermo Fisher Scientific RM-9106-S1</td>
<td>Rabbit; polyclonal</td>
<td>1:1000</td>
<td>AB_149792</td>
</tr>
<tr>
<td>Estrogen receptor α</td>
<td>Anti-ERα (C1355)</td>
<td>EMD Millipore 06-935</td>
<td>Rabbit; polyclonal</td>
<td>1:1000</td>
<td>AB_31035</td>
</tr>
<tr>
<td>Progesterone receptor</td>
<td>Anti-PR</td>
<td>Abcam, Cambridge, MA ab131486</td>
<td>Rabbit; polyclonal</td>
<td>1:500</td>
<td>AB_11156044</td>
</tr>
<tr>
<td>F4/80</td>
<td>F4/80 (D259R)</td>
<td>Cell Signaling Technology 70076</td>
<td>Rabbit, monoclonal</td>
<td>1: 500</td>
<td>AB_2799771</td>
</tr>
<tr>
<td>Secondary</td>
<td>Biotinylated anti-rabbit IgG</td>
<td>Abcam, Cambridge, MA ab64256</td>
<td>Goat; polyclonal</td>
<td>Ready to use (5 mg/mL)</td>
<td>AB_2661852</td>
</tr>
</tbody>
</table>

### 3.3 Assessing the Mammary Gland: Stroma

In the mouse mammary gland, stroma refers to any structure located outside the ductal basal lamina. Directly adjacent to the lamina sits the *periductal stroma*, consisting of collagen fibers and proteoglycans, fibronectin and matrix proteinases. All of these components are produced by stromal fibroblasts (Unsworth, Anderson, & Britt, 2014).

The first measure we typically take to evaluate the mammary stroma is to quantify the width of the periductal stroma. Collagen fibers stain bright blue with Masson’s trichrome stain while cytoplasm and muscle fibers are stained a contrasting crimson red. Using the length tool in the ZEN Zeiss software, we take eight measures (FIGURE 3.3A) from six ducts that are cut in a cross-sectional plane per individual. These data are used to produce a single value representative of the average width of the mammary periductal stroma for each individual.
Moving outward from the densely packed periductal stroma stretches the mammary fat pad. In the mouse mammary gland, adipocytes represent the most abundant cells of mesenchymal origin. There are two types of adipose tissue localized to the mammary gland, white and brown adipose cells, and both are crucial for ductal morphogenesis and function. The white adipose cells serve the canonical role of storing energy in the mammary gland while the brown adipocytes are quick to burn it using their unique expression of mitochondrial uncoupling protein 1 (UPC1). Despite what was once a textbook dogma (e.g., it was long believed that the adult mammary gland was comprised only of white adipose tissue), brown fat persists into adulthood with the potential of being mis-regulated due to environmental pollutants (Di Gregorio et al., 2018; Gouon-Evans & Pollard, 2002). Based on their actions, a new group of endocrine disruptors has been identified: metabolism disrupting chemicals or, specifically to the metabolism of the adipose tissues, obesogenic chemicals (Egusquiza & Blumberg, 2020; Heindel & Blumberg, 2019).

We have begun to evaluate animals exposed to estrogenic chemicals for disruption to the mammary adipose tissue. Individual adipocytes of both white and brown fat are assessed for their size/volume and measures of organization (e.g., number of neighboring cells) using formalin-fixed paraffin embedded tissues stained with hematoxylin & eosin (FIGURE 3.3B). Moreover, expression of genes associated with adipose tissue are also examined (see Chapter 5).

The third most abundant cell in the mouse mammary mesenchyme are macrophages (MΦ). As scavengers, macrophages are essential cells of the immune system that reside in the mammary gland throughout the entire life course (Stewart, Hughes, Hume, & Davis, 2019). In fact, the origins of mammary gland macrophages in adulthood can be tracked all the way to the embryonic sac and fetal liver
(Jappinen et al., 2019), and their interaction with the epithelium is proving to be important both in development and involution of the mammary gland (Dawson et al., 2020; Gyorki, Asselin-Labat, van Rooijen, Lindeman, & Visvader, 2009). Two types of MΦ can be distinguished based on their location in the mammary gland: ductal macrophages that form a continuous network between luminal and basal layers (Dawson et al., 2020); and stromal macrophages in close interaction with fibroblasts, endothelial cells, and namely adipocytes (Schwertfeger, Rosen, & Cohen, 2006). In the while fat, the classically activated M1 macrophages seem to promote

**Figure 3.3: Evaluation of the Stromal Structures in the Mammary Gland.**

A) Measuring the width of periductal stroma (trichrome stain). B) Adipose tissues (brown patches encircled); TEB in the bottom left (H&E stain). C) Periductal macrophages – arrows (F4/80 antibody). D) Mast cells in the periductal stroma – arrows (toluidine blue stain)
inflammation via secreting pro-inflammatory cytokines (Chawla, Nguyen, & Goh, 2011) while M2, the alternatively polarized macrophages recognized by the surface receptor CD206, promote transformation of the white to beige type of adipocytes that has been hypothesized as responding to environmental clues including the thyroid hormones (Bertani et al., 2017; Francis et al., 2021; Nguyen et al., 2011). The chemotactic and phagocytic roles are the primary task of macrophages, enabling remodeling of the gland during post-lactation involution (O’Brien et al., 2012). However, macrophages are not only involved in the destruction of mammary structures, but are also involved in normal pubertal mammary development, stromal homeostasis, and in the process of alveologenesis during the estrous cycle and in late pregnancy (Chua, Hodson, Moldenhauer, Robertson, & Ingman, 2010; Dawson et al., 2020; Pollard & Hennighausen, 1994; Y. Wang et al., 2020). Not surprisingly, breast tumors are not devoid of macrophages and their dual and contested role is discussed further in Chapter 8.

In our lab, we visualize macrophages using the F4/80 antibody and evaluate their presence in the periductal stroma (FIGURE 3.3C). Evaluation of F4/80-stained macrophages in induced mouse mammary tumors requires more complex methods of quantification due to the abundance of macrophages in the tissue (FIGURE 8.5); this became one of the topics of an undergraduate honors thesis by Shannon Silva. Using a software named TissueQuant in FIJI, an open-source image processing package by ImageJ, Shannon optimized a protocol that enabled us to isolate DAB-stained pixels (corresponding to the F4/80 signal) and quantify differences in macrophage presence between chemical-exposed animals and controls.

Mast cells are phylogenetically old and phenotypically diverse cells that mature and differentiate in peripheral tissues under paracrine control and regulation.
In the mammary gland, both stromal fibroblasts and epithelial cells promote tissue-specific functional heterogeneity of mast cells. However, mast cells also act in response to *ad hoc* needs of the tissue, producing a context-specific mixture of more than 30 different cytokines (Crivellato, Beltrami, Mallardi, & Ribatti, 2004).

Granules in the cytoplasm of mast cells contain mostly heparin and histamine associated with the primary role of the cells in innate immune responses to agents causing allergic reaction. But the enzymes and cytokines produced by mast cells are regarded as necessary for maintaining homeostasis and tissue repair in various organs (Abe, Kurosawa, Ishikawa, & Miyachi, 2000; Noli & Miolo, 2001). In the mammary gland, other enzymes such as tryptase, chymase, and carboxypeptidase have been identified in mast cells granules and associated with normal development (Galli & Tsai, 2008). During postnatal development, mast cells are well represented in the periductal stroma and near blood vessels, and during puberty they are located directly adjacent to the TEB residual basal lamina and cap cells, and are essential for TEB growth and ductal elongation (Brisken & Ataca, 2015). Mice lacking mast cells grow fewer TEBs, and have fewer proliferating cells within TEBs while their periductal collagen remains intact (Lilla & Werb, 2010). Proliferation and repair are not the only morphogenetic processes mast cells are involved in as they also regulate apoptosis and mammary gland remodeling post-lactation via binding to kallikrein in the plasma (Lilla, Joshi, Craik, & Werb, 2009).

Among other enzymes released from mast cells, tryptase and chymase have been demonstrated to take part in angiogenesis, a multistep process that involves migration of new endothelial cells, proliferation of muscle cells and fibroblasts and hollowing of the new vessels via well-coordinated apoptosis (Blair et al., 1997) (Saarinen, Kalkkinen, Welgus, & Kovanen, 1994). Mast cell pro-angiogenic factors
include angiopoetine-1, IL-18, TNFα, and VEGF that may, indeed, contribute to tumor promotion (Maltby, Khazaie, & McNagny, 2009) (Dyduch, Kaczmarczyk, & Okon, 2012). In the case of breast cancer, mast cells are capable of re-directing the migration and differentiation of T lymphocytes by secreting histamine, proteases, TNF α and other chemokines (Galli, Grimbaldeston, & Tsai, 2008). Lastly, the population of mast cells and serum histamine oscillate based on the stage of the estrous cycle and positively correlate to 17β-estradiol blood concentrations in ovariectomized Wistar rats (Jing, Wang, & Chen, 2012). Collectively, their complex role in proliferation, immunity, and angiogenesis allows mast cells to contribute to breast cancer initiation and progression (E. Z. da Silva, Jamur, & Oliver, 2014).

For their part in normal mammary tissue and neoplasia, we assess the abundance of mast cells in the developing mammary gland, mammary tumors, and in the surrounding stroma (FIGURE 8.5). Due to the content of their intracellular granules, mastocytes stain metachromatically, i.e. toluidine blue stains the cells bright purple with granules clearly recognizable (Ribatti, 2018).

3.4 qRT-PCR

In addition to microscopic evaluation of receptors, cells, and tissue compartments, we evaluate expression of the genes of interest from mammary tissue samples that are snap frozen and stored at -80°C. First, we extract RNA using Trizol reagent and a BeadBug microtube homogenizer, examine the RNA purity as the A260/A280 ratio, and quantify the concentration of RNA in the sample using Nanodrop UV spectrophotometry. 1 mg of RNA from each sample is transcribed to cDNA using reverse transcription. Lastly, 2 µL of cDNA is combined with forward and reverse primers for each target gene (TABLE 3.2) and SYBR green master mix to
amplify and detect the gene of interest (thermal prolife cycles are set as follows: 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 15 seconds at 72°C). A melting-curve analysis is conducted to identify nonspecific products. Finally, quantification of the target gene is evaluated relative to reference genes determined using the ΔΔCt method. Every sample is run in triplicate for each target gene. We use two reference genes for the mammary gland analyses: β actin and β2 microglobulin (TABLE 3.2).

**Table 3.2: Primer sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
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<tr>
<td>β Actin (BA)</td>
<td>CACACCCGCCACCAGTTTCGC (89-108)</td>
<td>TTGCACATGGCCGGAGCGT (162-143)</td>
</tr>
<tr>
<td>β2 Microglobulin (B2M)</td>
<td>CCGGCCTGTATGCTATCCAG</td>
<td>TGTTCGGTTCCATTCTCC</td>
</tr>
<tr>
<td>Progesterone Receptor</td>
<td>AAA GGATCCCGAGGTTTCTC</td>
<td>GTTCCATCTTCCAGCGGATA</td>
</tr>
<tr>
<td>Estrogen Receptor α</td>
<td>TGCAATGACTATGCTCTGG (782-801)</td>
<td>CTCCGGTTCTTGTAATG (921-902)</td>
</tr>
<tr>
<td>PPARδ</td>
<td>GATGGAAGACCACCTGGCATTT</td>
<td>GGAGCAGAAATGTACAGGAGAA</td>
</tr>
<tr>
<td>Fatty Acid Synthase (Fas)</td>
<td>TAAATGGCTCCACCAATCC</td>
<td>CCATGGCTCCAGGAAACAG</td>
</tr>
<tr>
<td>α1 Collage I (Col1a1)</td>
<td>GGAGAGAGCATGACGATGG</td>
<td>AAGTCCCGGTGACTGAGT</td>
</tr>
<tr>
<td>Fibronectin (F1_7)</td>
<td>ATGAAAGCCCTGGATCCC</td>
<td>GAGGCTTCGGCTCCTTGCT</td>
</tr>
<tr>
<td>Adiponectin (Plin2)</td>
<td>GTGAAAGCCCGTCCTCTGC</td>
<td>CCCTGAGGCCATAAGTTTT</td>
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CHAPTER 4

EFFECTS OF PERINATAL OXYBENZONE EXPOSURES ON THE MALE AND FEMALE MOUSE MAMMARY GLAND

This chapter was previously published as:
Matouskova, K., Jerry, D. J., & Vandenberg, L. N. (2020). Exposure to low doses of oxybenzone during perinatal development alters mammary gland morphology in male and female mice. Reproductive Toxicology, 92, 66-77.

4.1 Abstract

Oxybenzone (benzophenone 3) is an ultraviolet radiation filter commonly used in personal care products including sunscreens, textiles and inks, and food and beverage containers, among others. Due to its widespread use, human exposures to oxybenzone are widespread. Oxybenzone is considered an endocrine disrupting chemical due to its antiestrogenic and antiandrogenic properties. We evaluated the effects of oral exposures to oxybenzone on the growth and morphology of the mammary gland, body weight and anogenital distance in BALB/c mice exposed to 30, 212 or 3000 μg/kg/day in utero and during lactation. Developmental exposures to oxybenzone reduced the size and growth of mammary gland in males prior to and during puberty. In exposed females, oxybenzone reduced mammary cell proliferation, decreased the number of cells expressing estrogen receptor α, and altered mammary gland morphology in adulthood. These results suggest that even low doses of oxybenzone can disrupt hormone sensitive organs during critical windows of development.

Keywords: 2-hydroxy-4-methoxybenzophenone, endocrine disruptor; sunscreen; xenoestrogen; anogenital distance
4.2 Introduction

Oxybenzone is a synthetic compound used to protect people and consumer products from solar irradiation. It is added to many personal care products and found at high concentrations in many commercially available sunscreens. Although it was once thought that oxybenzone applied in a sunscreen remains on the skin surface, both animal (R. Jiang et al., 1999) and human studies (Janjua et al., 2004) demonstrate that it penetrates the skin (C. Fernandez et al., 2002; R. Jiang et al., 1999), enters the blood stream (Hayden et al., 1997), and is excreted via urine and feces (Kadry et al., 1995).

In addition to dermal exposure, the growing use and production of oxybenzone has increased its prevalence in consumer goods, resulting in exposures via the oral route as well. Oxybenzone-enhanced glass and plastics protect food, beverages, and other consumer goods from degradation caused by sunlight ("Packaging gets active: Additives lead the way," 2004). Moreover, many textiles include oxybenzone for color stability, and industrial inks contain oxybenzone as a wetting agent and photo-initiator (Danish Environmental Protection Agency, 2015). Finally, oxybenzone is added to perfumes to enhance the fragrance of musk, and to candies and other foods as a flavoring agent. As a result of high production of oxybenzone and only partial ability to remove it from drinking water (Rodil et al., 2012), it has also been detected in surface water (Fent et al., 2010), wastewater and drinking water (C. P. da Silva et al., 2015; Loraine & Pettigrove, 2006b).

Oxybenzone has recently received significant attention as an environmental pollutant suspected to contribute to coral bleaching across the worlds’ oceans (Downs et al., 2016).
Greater than 98% of the U.S. general population has detectable urinary concentrations of oxybenzone, and exposures appear to be increasing; in samples collected between 2003-4, the mean concentration was 22.9 μg/L (Calafat et al., 2008) while six years later the mean concentration was 36.3 ng/L (Meeker et al., 2013). The urinary concentration of individuals who report “always” using sunscreen is more than two times higher than in those who report only “sometimes” using sunscreens and both are strikingly higher from those who reported “never” using sunscreens (Ferguson et al., 2017). Besides urine, oxybenzone has been detected in blood (T. Zhang et al., 2013), semen (Leon et al., 2010), and in amniotic fluid and cord blood, suggesting that the compound can cross the placenta (Krause, Frederiksen, Sundberg, Jorgensen, Jensen, Norgaard, Jorgensen, Ertberg, Juul, et al., 2018). Neonates are further exposed via human milk (Molins-Delgado et al., 2018; Rodriguez-Gomez et al., 2015); concentrations as high as 121 ng/g of lipid were measured in three different cohorts in Switzerland (Schlumpf et al., 2010).

Oxybenzone affects hormone signaling in animals, including humans (Ghazipura et al., 2017; Maipas & Nicolopoulou-Stamati, 2015). Several studies have demonstrated that oxybenzone acts as an estrogen receptor (ER) agonist in yeast assays (Balazs et al., 2016; Kunz & Fent, 2006a), human cell cultures (Schlumpf et al., 2001; Suzuki et al., 2005) and fish (Bluthgen et al., 2012). There is also strong evidence that oxybenzone acts as an ER antagonist in yeast reporter assays (Kunz & Fent, 2006a) and fish (Balazs et al., 2016; Bluthgen et al., 2012). Although the concept of a single compound having both agonist and antagonist activities for a single receptor seems contradictory, this is a common characteristic of other selective ER modulators (often called SERMs) like tamoxifen (Jensen & Khan, 2004). Further, a number of studies point to oxybenzone’s potent anti-androgenic
effects in *in vitro* reporter cell lines (R. Ma et al., 2003), in yeast reporter assays (Balazs et al., 2016; Kunz & Fent, 2006b), and in fish (Bluthgen et al., 2012). Some of these actions may be due to the endocrine disrupting properties of oxybenzone’s metabolites as well (Watanabe et al., 2015).

Estrogens, progesterone and testosterone play a pivotal role in the development of hormone-sensitive organs including the mammary gland. Although it is generally held that the mammary gland in both males and females develops independent of hormones prior to puberty, and only the female hormones produced during the estrous cycle promote epithelial growth (Brisken & O’Malley, 2010), the mammary mesenchyme expresses both ER and androgen receptor (AR) during prenatal development. Further, exposures to ER and AR agonists and antagonists during this critical window of development can affect the growth of the mammary gland, its function and risk of disease (Fenton, 2006). Importantly, the mammary gland is a sexually dimorphic structure, therefore male and female glands not only develop differently, they are affected by endocrine disruptors differently. In both male and female mouse embryos, the mammary band of ectodermal cells begins to form around embryonic day 10.5 and is observed on both the left and right sides, stretching from the upper to lower limb (Stephens, 1982). In female mice, the ectoderm invaginates into the underlying mesenchyme, forming an epithelial cord that branches into the developing fat pad and forms a nipple (five pairs in mice) attached to the skin. Yet, in male mice, the production of testosterone from the fetal testis causes an AR-dependent condensation of the mesenchyme around the stalk of the epithelial cord, ultimately detaching it from the overlying epidermis; the slightly branched epithelial cord does not open to the surface and nipples do not form (Topper & Freeman, 1980). Numerous studies have shown that anti-androgenic
chemical exposures during this critical window of mammary gland development lead to nipple retention in males (Christiansen et al., 2014; C. Wolf, Jr. et al., 1999), and other studies have shown that perinatal exposures to estrogenic chemicals can increase the growth of the male epithelium even when nipples are not present (Kolla, Pokharel, & Vandenberg, 2017; Pokharel et al., 2018; Skarda, 2002; Vandenberg et al., 2013). Other effects of antiandrogenic and estrogenic compounds on the male reproductive system have been reviewed by Hejmej et al. (Hejmej, Kotula-Balak, & Bilińska, 2011).

In this study, we investigated the endocrine-disrupting potential of oxybenzone on male and female BALB/c mice exposed during gestation and the perinatal period. We evaluated offspring at three different life stages: prior to puberty, at puberty and in adulthood. Our results suggest that developmental oxybenzone exposures alter growth parameters in the mouse mammary gland, findings consistent with what would be expected if oxybenzone was a hormonally active endocrine disruptor.

4.3 Methods

Animals

6-8-week-old BALB/c female mice (UMass colony, D.J.J. laboratory, Amherst, MA) were randomly assigned to one of four treatment groups using SPSS statistical software Version 24 (IBM Inc, Armonk, NY) to distribute them evenly according to their body weight. Females were mated and housed in polysulfone cages with bedding and food (LabDiet Chow 5058; LabDiet, St. Louis, MO) and tap water (in polysulfone bottles with rubber stoppers and metal tops) provided ad libitum. Animals were maintained at the University of Massachusetts, Amherst Morrill Animal
Facility, in temperature- and light-controlled conditions (12-hour cycles of light and dark). After weaning, offspring were housed under the same conditions as their mothers, with 4-5 same-sex (and same-treatment) animals per cage. All experimental procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

**Chemical administration**

From pregnancy day zero (when a sperm plug was observed) until the day before weaning (on lactational day 21), females were weighed and orally dosed daily by pipet with either tocopherol-stripped corn oil or oxybenzone dissolved in tocopherol-stripped corn oil. As a result, the offspring evaluated in this study were exposed *in utero* and during the first 21 days of postnatal life via the mother. Three doses of oxybenzone were used: 30 μg/kg/day, 212 μg/kg/day, and 3000 μg/kg/day. All three doses chosen for our study are below the doses used in traditional toxicological studies (μg vs mg) (Tyl, 2009), and the two lowest doses are likely relevant to human intake based on back-calculations of urinary concentrations in pregnant women (Woodruff et al., 2011). Dams were allowed to deliver naturally and litters were not culled. Sample sizes for the treatment groups were: controls, 11 litters; 30 μg/kg oxybenzone, 10 litters; 212 μg/kg oxybenzone, 11 litters; 3000 μg/kg oxybenzone, 9 litters. Only one female and one male from each litter was selected for each endpoint to avoid litter bias, but sample sizes for each endpoint vary based on litter size. Characteristics of litters are reported in (LaPlante et al., 2018) and the litter sizes (mean ± standard deviation) by treatment group were controls: 4.4 ± 1.6; 30 μg/kg/day: 5.0 ± 1.6; 212 μg/kg/day: 3.6 ± 2.9; 3000 μg/kg/day: 3.9 ± 2.6.
Pups were weaned on postnatal day (PND) 21 and co-housed with same-sex animals of the same treatment group for the remainder of the experiment. Animals were ear-tagged upon weaning for identification.

_Euthanasia and tissue collections_

At weaning, we determined the sex of each offspring and assigned them to one of the three age groups: one male and one female from each litter was euthanized prior to the onset of puberty (at PND21), in puberty (PND32-35), or in adulthood (postnatal week 9-11). Results from the dams (the F0 generation) are described in LaPlante et al., 2018 (LaPlante et al., 2018).

On the day of sacrifice, animals were euthanized with CO₂ inhalation followed by cervical dislocation to ensure death. Timing of euthanasia was not limited to specific stages of the estrous cycle. The weight of each animal was recorded and anogenital distance was measured with calipers. Anogenital index (AGI) was calculated as the anogenital distance divided by body weight to account for differences in body size. From every male animal we collected both the left and right fourth inguinal mammary glands, spread both on a glass slide (Thermo Fisher Scientific, Waltham, MA), and fixed them overnight in 10% neutral-buffered formalin (Thermo Fisher Scientific). From every female animal we dissected the right fourth inguinal mammary gland, spread it on a glass slide (Thermo Fisher Scientific, Waltham, MA) and fixed it overnight in 10% neutral buffered formalin. The left mammary gland was fixed in 10% neutral buffered formalin overnight before being washed, dehydrated, and embedded in paraffin for histology and immunohistochemistry.
Whole mounted mammary gland processing and morphometric evaluation

Whole mounted mammary glands were processed and stained according to standard protocols (Markey, Luque, Munoz De Toro, Sonnenschein, & Soto, 2001; Stanko & Fenton, 2017). Briefly, glands were moved through a graded series of ethanols (70%, 95% and 100%, for one hour each) followed by two washes of toluene (one hour the first wash, at least two hours in the second wash) to remove fat from the tissue, rehydrated through a series of ethanols (100%, 95%, 70%) and stained overnight with carmine. Carmine stains all nuclei but allows the ducts to be distinguished because of the close proximity of the epithelial cells to each other compared to the size of the adipose cells in the stroma. Finally, the samples were dehydrated through a series of alcohols (70%, 95% and 100%, one hour each) and xylene for two hours, and sealed in individual k-pax heat-sealed pouches (Fisher Scientific) containing 5ml methyl-salicylate (Acros Organics, Morris Plains, NJ).

The whole mounted mammary glands were imaged with a Zeiss AxioImager dissection microscope (Carl Zeiss Microscopy, Jena, Germany) and a Zeiss high-resolution color camera. All measurements were made with ZEN software (Carl Zeiss Microscopy, Jena, Germany).

In the PND21 and pubertal female mammary glands, and in the PND21, pubertal and adult male mammary glands, measurements included the mammary gland ductal area (e.g., the area subtended by ducts), ductal extension (e.g., the furthest growth of ducts relative to the center of the lymph node, a centralized landmark), number and area of terminal end buds (TEBs, defined as bulb-shaped structures at the end of ducts measuring at least 0.03 mm²). Details about these measurements are provided in (Vandenberg et al., 2006) for females and (Vandenberg et al., 2013) for males. For ductal extension, negative values are
assigned to ductal trees that have not yet reached the midpoint of the lymph node, and positive values are given to ductal trees that have grown past the midpoint of the lymph node; in groups where all animals had negative ductal extensions, the absolute values (positive) are graphed for ease of interpretation. In males at all ages, the number of branching points in the entire ductal tree were counted as well.

**Whole mounted mammary gland evaluation in adult female mice**

The adult female mammary gland is large and complex, so different measurements were made using methods described previously (Vandenberg et al., 2008b). In brief, we imaged the area just anterior to the central lymph node at 30x magnification. Using ZEN software, we placed a 15 by 12 grid on the picture and characterized the ductal structures at each of the 180 crosshairs (e.g. ducts, terminal ends, and alveolar buds). The volume fraction of each structure was calculated by dividing the number of crosshairs hitting each structure by the total number of crosshairs hitting mammary tissue using standard methods from unbiased stereology (Howard & Reed, 2004; Vandenberg et al., 2008b).

**Tissue embedding, sectioning and histological staining**

Fixed mammary glands were washed in 1x phosphate buffered saline, dehydrated through a series of alcohol and embedded with paraffin (Leica Biosystems, Richmond, IL) under vacuum. Paraffin blocks were cut into five micrometer sections on a rotary microtome (Fisher Scientific) and mounted on positively charged slides (Fisher Scientific) for histological evaluation and immunohistochemistry.
For histological evaluations, slides were deparaffinized with xylene and a series of alcohols, stained with Harris' hematoxylin and eosin (Fisher Scientific), dehydrated, and mounted with permanent mounting media (Fisher Scientific). Digital images were collected using a Zeiss Axio Observer.Z1 inverted microscope, a 10X objective, and a high-resolution color camera (Carl Zeiss Microscopy).

**Immunohistochemistry**

Expression of three markers was evaluated using standard methods for immunohistochemistry and commercial antibodies including rabbit anti-ERα (EMD Millipore, Cat# 06-935, Temecula, CA; diluted 1:1000); rabbit anti-Ki67 (Fisher Scientific, Cat# RM-9106-S1; diluted 1:1000), a marker of proliferation; and rabbit anti-progesterone receptor (PR; Abcam, Cat# ab131486, Cambridge, MA; diluted 1:500). Briefly, sections were deparaffinized, hydrated through a series of alcohols, microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval, and treated with hydrogen peroxide to quench endogenous peroxidases. Non-specific binding was blocked with 1% milk protein in 5% normal goat serum (Cell Signaling Technology, Danvers, MA).

Sections were incubated with primary antibodies (diluted in blocking solution) at 4°C for 14-16 h. They were then washed and incubated with secondary antibody (goat anti-rabbit, Abcam, Cat# ab64256) followed by streptavidin peroxidase complex (Abcam, Cat# ab64269). Diaminobenzidine chromogen (Abcam, Cat# ab64238) was used to visualize reactions. Sections were counterstained with hematoxylin (Fisher Scientific). Each immunohistochemical run included a negative control in which the primary antibody was replaced with 5% normal goat serum.

Images were collected of each sample for ERα, Ki67, and PR at 40X
magnification with a Zeiss Axio Observer.Z1 inverted microscope. The fraction of
cells expressing ERα or PR was evaluated by counting at least 500 epithelial cells in
2-4 separate fields. Fraction of cells expressing Ki67 was evaluated by counting at
least 1000 epithelial cells in 3-5 separate fields. Different numbers of fields were
observed to ensure that sufficient cells were available for evaluation; the epithelial
structures in PND21 females are limited, so typically all fields with epithelium were
evaluated whereas in pubertal and adult females, fields were selected at random. All
epithelial cells were evaluated in each field that was imaged. Expression of ERα,
Ki67 and PR were expressed as a percent ratio of the total number of epithelial cells
evaluated.

Statistical analysis

SPSS Version 24 (IBM Inc, Armonk, NY) was used to analyze all data. First,
data were tested for linearity by regression analysis with treatment dose (0, 30, 212
or 3000 µg/kg/day) as the independent variable and the various outcomes
(mammary gland morphometric measurements, expression of markers, etc.) as the
dependent variable. A Pearson correlation is reported in the text when statistical
significance (p<0.05) or a possible statistical trend (p<0.1) was observed.

Next, normally distributed data were evaluated with one-way ANOVA (with
oxybenzone treatment as the independent variable) followed by Fisher’s LSD post-
hoc tests. For body weight measurements, number of pups in the litter was treated
as a covariate as weight has been shown to be affected by litter size (Suvorov &
Vandenberg, 2016). Mammary gland parameters were not corrected for litter size. A
p-value <0.05 was considered statistically significant.
All data are graphed as mean ± standard error. All samples were collected, and all analyses were conducted by experimenters blind to treatment group.

4.4 Results

*Oxybenzone alters anogenital distance in males at PND21 and puberty but does not affect male body weight*

At all three ages, pups were weighed at the time of necropsy. In males, no effect of oxybenzone was observed on body weight at any age (FIGURE 4.1A), even when litter size was treated as a covariate to control for differences in growth parameters (Suvorov & Vandenberg, 2016).

Anogenital distance (AGD), the length between the anus and the genitals, is a sexually dimorphic measure that is known to be affected by prenatal exposures to hormonally active agents. Typically, AGD is longer in males than females due to production of androgens by the fetal testis. We adjusted AGD for the weight of the animal (to calculate anogenital index, AGI) because body size and weight can influence AGD (Swan, 2006). In males, AGI was reduced after exposures to 30μg and 212μg doses (FIGURE 4.1C, ANOVA, p<0.05, Fisher’s posthoc p<0.05) at PND21 and in puberty. In adult males, no differences in AGI were observed.

*Oxybenzone alters body weight in females at puberty and in adulthood and decreases female AGI at puberty*

In females, developmental exposure to oxybenzone did not affect body weight at PND21 (FIGURE 4.1B). However, at puberty, when litter size was treated as a covariate, females exposed to 3000 µg/kg/day oxybenzone were heavier compared to controls (ANCOVA, p<0.05, Fisher’s posthoc p<0.05). Furthermore,
females exposed to 212 µg/kg/day oxybenzone were heavier in adulthood compared to controls (ANCOVA, p<0.05, Fisher’s posthoc p<0.05).

In females, AGI was unaffected at PND21, but decreased by exposure to 212 µg/kg/day when measured at puberty (FIGURE 4.1D, ANOVA, p<0.05, Fisher’s posthoc p<0.05). No effects of developmental oxybenzone exposure were observed on female AGI in adulthood.

![Bar charts showing body weight and anogenital index](image)

**Figure 4.1 Oxybenzone alters body weight and anogenital index in a sex- and dose-specific manner.** A) Perinatal exposure to oxybenzone did not affect male body weight at any age. B) Measurements of anogenital index (AGI; anogenital distance divided by body weight) in males at all three ages. C) Heavier body weights were measured in females exposed to 3000 µg/kg/day oxybenzone in puberty, and in females exposed to 212 µg/kg/day oxybenzone in adulthood, compared to controls. D) Measurements of AGI in pubertal females reveal lower AGI in females exposed to 212 µg/kg/day oxybenzone. In all panels, different letters indicate significant differences, Fisher’s LSD posthoc after significant 1-way ANOVA.
Exposure to low doses of oxybenzone alters male mammary gland morphology

Like other strains of mice, even in the absence of nipples, male BALB/c mice have a small mammary rudiment (FIGURE 4.2A). Our prior work demonstrated that males of this strain also have left-right asymmetric mammary gland development, with a right mammary ductal tree that is typically larger than the left at specific stages (e.g., in puberty and in adulthood) (Pokharel et al., 2018). For this reason, we evaluated the effects of oxybenzone on both the left and right mammary glands.

At PND21, developmental exposure to oxybenzone significantly reduced the size of the male mammary gland epithelium on both the left and right sides (Figure 4.2B, ANOVA, p<0.05, Fisher’s posthoc p<0.05). At puberty, there were no statistically significant effects of oxybenzone. Finally, we examined mammary gland morphology in males in early adulthood (FIGURE 4.2D). There were no statistically significant differences after oxybenzone exposure.

We also examined total branching points (a combined sum for the left and right glands), a measure of mammary gland complexity in each male. At PND21, mammary glands from all three oxybenzone treatment groups had significantly fewer branching points compared to controls (FIGURE 4.2E, ANOVA, p<0.05, Fisher’s posthoc p<0.05). By puberty and in adulthood, the control glands were less complex in their branching, and there were no longer significant differences based on oxybenzone treatment (FIGURE 4.2E).
Perinatal exposure to oxybenzone induces modest alterations in growth of the female mammary gland prior to and at puberty.

We next evaluated growth and development patterns in whole mounted mammary glands collected from females. At PND 21, females exposed to the highest dose of oxybenzone had modestly larger ductal areas compared to controls (ANOVA, p<0.05, Fisher’s posthoc p<0.05) but no significant effects were observed for the extent of growth into the fat pad (ductal extension) (FIGURES 4.3A,B,C).
TEBs were observed in a few animals, with no significant differences in number or size based on oxybenzone treatment (FIGURE 4.3A and data not shown).

At puberty, the females exposed to 30 and 3000 μg oxybenzone/kg/day had a modest increase in the size of the ductal tree and a significant increase in the extent

Figure 4.3: Perinatal oxybenzone exposure induces modest changes to the prepubertal and pubertal female mammary gland. A) Representative whole mounted mammary glands from females from the four treatment groups at PND21. Dotted boxes outline the ductal tree. Scale bar = 2 mm. B) Quantification of ductal area and C) ductal extension in females at PND21. Although not indicated in the graph, ductal extension for all females at PND21 has a negative value because the ductal tree has not yet reached the lymph node. D) Representative whole mounted mammary glands from females from the four treatment groups at puberty. Arrowheads indicate select TEBs. Scale bar = 2 mm. E) Quantification of ductal area and F) ductal extension in females at puberty. G) Quantification of TEB number and H) total TEB area in females at puberty. In all panels, different letters indicate significant differences, Fisher’s LSD posthoc after significant 1-way ANOVA.
of growth into the mammary fat pad (FIGURES 4.3D,E,F). There was also a significant increase in the number of TEBs in females exposed to 3000 μg oxybenzone/kg/day (FIGURE 4.3G, ANOVA, p<0.05, Fisher’s posthoc p<0.05) as well as a trend toward significance for a linear increase in number of TEBs by oxybenzone dose (Pearson correlation 0.324, p = 0.058). There were no significant differences in total TEB area between oxybenzone treated groups and controls (FIGURE 4.3H) although there was a trend toward significance for a linear increase in TEB area by oxybenzone dose (Pearson correlation 0.304, p = 0.075).

**Developmental exposure to oxybenzone alters mammary morphology in female mice in adulthood.**

To determine if developmental exposures to oxybenzone have lasting effects on the female mammary gland, we next quantified mammary gland morphology in young adults. Analysis of adult mammary glands showed a decreased fraction of the mammary gland comprised of ducts as the dose of oxybenzone increased (FIGURES 4.4A,B). The opposite pattern was observed for the fraction of the gland comprised of alveolar buds, with a significant increase observed in females from the 3000 μg oxybenzone/kg/day group (FIGURE 4.4B, ANOVA, p<0.05, Fisher’s posthoc p<0.05); on average, females from this high dose group have more than twenty times more alveolar buds compared to controls. There was also a trend toward significance for a linear increase in volume fraction of alveolar buds by oxybenzone dose (Pearson correlation -0.306, p = 0.065). There were no significant differences in volume fraction of terminal ends based on treatment (data not shown).
Developmental exposure to oxybenzone alters expression of hormone receptors in the mammary gland

ERα is expressed in the mammary epithelium starting just before birth and allows the epithelial cells to respond to estrogens produced in the ovaries (Bocchinfuso et al., 2000), although the role of ER in the prepubertal mammary gland is not clear; ERα is essential for growth and branching of the epithelial ducts at and after puberty (Mallepell et al., 2006a). Interestingly, we observed a decrease in
the fraction of cells positive for ERα in the mammary gland of females exposed to oxybenzone. The decreased fraction of ERα-positive cells was only significant in adult females after developmental exposures to 30 or 212 μg/kg/day (FIGURES 4.5A,B, ANOVA, p<0.05, Fisher’s posthoc p<0.05).

Several previous studies revealed an antagonistic effect of oxybenzone on progesterone receptor in cell cultures (R. H. Schreurs, E. Sonneveld, J. H. Jansen, W. Seinen, & B. van der Burg, 2005), yet the effects of this compound on progesterone receptor expression have not yet been examined. Progestins are important for growth and epithelial branching of the mammary gland via inducing growth hormone secretion (Lombardi et al., 2014). We found no significant effects of oxybenzone on fraction of cells expressing progesterone receptor in mammary glands at PND21 or in adulthood, but a significant decrease in progesterone receptor-positive epithelial cells at puberty in females exposed to 212 μg oxybenzone/kg/day (FIGURES 4.5C,D, ANOVA, p<0.05, Fisher’s posthoc p<0.05).
Developmental exposures to oxybenzone reduce cell proliferation in the mammary gland epithelium

We next examined the effect of oxybenzone on expression of Ki67, a marker of cell proliferation. In controls, approximately 4% of ductal cells are Ki67-positive at PND21 and at puberty; cells within the highly proliferative TEBs were not included in

Figure 4.5: Oxybenzone alters expression of hormone receptors in an age- and dose-specific manner. A) Photomicrograph illustrating expression of ERα (brown cells, indicated by an arrow). Blue cells (indicated by arrowheads) are negative for ERα, counterstained with hematoxylin. Scale bar = 50 µm. Inset is a representative negative control. B) Quantification of epithelial cells expressing ERα in the mammary gland at PND21, puberty, and in adulthood. C) Photomicrograph illustrating expression of progesterone receptor (brown cells, indicated by an arrow). Blue cells (indicated by arrowheads) are negative for progesterone receptor, counterstained with hematoxylin. Scale bar = 50 µm. Inset is a representative negative control. D) Quantification of epithelial cells expressing progesterone in the mammary gland at PND21, puberty, and in adulthood. In all panels, different letters indicate significant differences, Fisher's LSD posthoc after significant 1-way ANOVA.

Developmental exposures to oxybenzone reduce cell proliferation in the mammary gland epithelium

We next examined the effect of oxybenzone on expression of Ki67, a marker of cell proliferation. In controls, approximately 4% of ductal cells are Ki67-positive at PND21 and at puberty; cells within the highly proliferative TEBs were not included in
these measures. At both ages, developmental exposure to oxybenzone reduced the fraction of proliferating cells, although only the females from the 30 µg/kg/day group were significantly different from controls at PND21 (FIGURES 4.6A,B, ANOVA, p<0.05, Fisher’s posthoc p<0.05). There was, however, a significant linear relationship between oxybenzone dose and fraction of cells positive for Ki67 (Pearson correlation 0.447, p = 0.006). In adulthood, proliferation in the mammary epithelium was not affected by developmental oxybenzone exposure (FIGURE 4.6).

**Figure 4.6:** Quantification of proliferation index in mammary epithelial cells. **A)** Photomicrograph illustrating expression of Ki67 (brown cells, indicated by an arrow). Blue cells (indicated by arrowheads) are negative for Ki67, counterstained with hematoxylin. Scale bar = 50 µm. Inset is a representative negative control. **B)** Quantification of epithelial cells expressing Ki67 in the mammary gland at PND21, puberty, and in adulthood. Different letters indicate significant differences, Fisher’s LSD posthoc after significant 1-way ANOVA.
4.5 Discussion

In this study we have examined the effects of perinatal, low-dose exposure to oxybenzone on the growth and morphology of the mammary gland in male and female BALB/c mice at three life stages. To date, studies of oxybenzone on mammals remain limited, and we believe this is the first study to show that perinatal exposures to this compound disrupt development of hormone-sensitive organs. Several broad conclusions may be drawn from our study. First, based on the growth parameters we measured, oxybenzone appears to affect young males more than females, although the effects observed on the adult female mammary morphology (e.g., increases in alveolar buds) were striking. Second, we observed more effects induced by 30 and 212 μg oxybenzone/kg/day than the higher dose of 3000 μg/kg; these two lower doses are relevant to human exposures based on back-calculations of urinary concentrations in pregnant women (Woodruff et al., 2011). Although it is difficult to draw definitive conclusions about dose response shape with only three treated groups, these results may be consistent with non-monotonic effects of many endocrine disrupting chemicals (Vandenberg et al., 2012). Finally, our results stress an urgent need to focus on the consequences of perinatal exposure throughout the life course, from early to adulthood, and on patterns rather than isolated measurements.

The male mouse mammary gland is distinguishable from the female starting from approximately E14, when androgen production in the fetal testis causes a detachment of the mammary tree from the overlying epidermis. Although the male gland fails to develop a nipple, it remains responsive to estrogenic stimuli (Vandenberg et al., 2013). Here we found that when exposed to oxybenzone, the
male mammary gland was initially smaller than in controls; but by adulthood, significant differences were no longer observed between treatment groups. These findings suggest that oxybenzone can produce different effects on the male gland depending on when the animals are evaluated. At this time, we are uncertain of the significance of a smaller mammary gland like those we observed in oxybenzone-treated males. Unlike the female, where ducts are an essential part of the tissue and are responsible for the delivery of milk from lobuloalveolar units to the nipple during lactation, the mammary ducts are a vestigial structure in males. From the perspective of developmental biology, any change in morphology has the potential to be deleterious, and this change might signal effects on other hormone-sensitive outcomes. Considering the responsiveness of the male gland to environmental insults, and the fact that males can develop breast cancer, the vestigial nature of the male mammary ducts does not mean they should be ignored. However, from the perspective of toxicology, we are unaware of any disease condition associated with the disruptions we observed to the male mammary gland after oxybenzone exposures.

In females, the most remarkable effects of perinatal oxybenzone exposure were the high fraction of alveolar buds in adult glands, countered by the decreased rate of proliferation in mammary epithelium at early ages. Alveolar buds are epithelial pouches that form all over the ductal tree in the mid- and late stages of pregnancy in anticipation of milk production. Their presence during non-pregnant adulthood is atypical and suggests an organizational disruption to the tissue. Similar effects on alveolar bud formation have been reported after perinatal exposure to the estrogenic chemicals bisphenol A (Markey et al., 2001), bisphenol S and 17α-ethinyl estradiol (SriDurgaDevi Kolla, Mary Morcos, Brian Martin, & Laura N. Vandenberg, 2018) in
mice, or exposures to a mixture of estrogenic and anti-androgenic chemicals in rats (K. R. Mandrup et al., 2015). Formation of alveolar buds depends primarily on orchestrated action of prolactin and progesterone (Neville, McFadden, & Forsyth, 2002), suggesting the possibility that these environmental chemicals may be acting at the level of the pituitary; additional work is needed to evaluate this hypothesis. We did not control for stage of the estrous cycle stage in these females, and it may have increased variability in the responses we have observed; future studies should also evaluate whether perinatal exposures to oxybenzone alter estrous cyclicity in adult females.

Oxybenzone is hydrophobic (partitioning coefficient, logP_{ow} = 3.64) and accumulates in adipose tissue. This has been demonstrated in fish in their native environment (Gago-Ferrero et al., 2013) and under laboratory conditions (Schlumpf et al., 2010). In humans, oxybenzone has been detected in the adipose tissue of female breasts and its concentration positively correlates with the incidence of tumor in the lateral region of the breast (Barr et al., 2018); the possibility that oxybenzone could be stored in mouse mammary tissue should be evaluated in future studies. We did not attempt to measure oxybenzone in the blood of these pups because they were never directly exposed and, with the exception of the pre-pubertal animals, they were evaluated long after they would have been exposed via mothers’ milk. Based on toxicokinetic data available, we anticipate that the circulating levels in the pups’ blood would always be below the limits of detection in analytical chemistry assays. In tissues and the environment, oxybenzone undertakes two metabolic pathways: demethylation and hydroxylation. Oxybenzone demethylates into benzophenone-1 (BP1, also called 2,4-dihydroxybenzophenone), which further hydroxylates to 2,3,4- trihydroxybenzophenone (2,3,4 THB) or 2,4,5 -
trihydroxybenzophenone (2,4,5 THB). Oxybenzone is hydroxylated on either of its rings forming several different metabolites, e.g. benzophenone-8 (BP-8, also called 2,2’ dihydroxy-4-4-methoxybenzophenone), 3-hydroxy-benzophenone-3 or 5-hydroxy-benzophenone-3 (Cheng, 2014). These transformations require human cytochrome P450 (CYP) 1A and 2D6 (Watanabe et al., 2015), enzymes that are expressed in the adult liver, small intestine and other tissues. CYP expression and activity may change during the life course, with levels that are often lower during fetal development (de Wildt et al., 1999; Sadler et al., 2016). Therefore, exposure of the fetus to the parent compound is likely, and exposure to the major metabolites is likely to occur due to maternal metabolism. BP1, the major metabolite, is also occasionally used in consumer products (S. Kim & Choi, 2014) and it is more hydrophilic than the parent compound, allowing it to be detected in surface waters (Jeon et al., 2006). BP1 is a potent estrogen agonist in yeast assays (Kunz & Fent, 2006a) and a mixture of oxybenzone and BP1 had an additive estrogenic effect on human MCF-7 cells (Heneweer et al., 2005). Because of its metabolism and the biological activity of its metabolites, administering oxybenzone is essentially a test of a chemical mixture. For this reason, the effects of oxybenzone are likely to be complex and to change as the metabolic potential of the individual changes throughout the life course. This is one challenge of mixture studies, although they are likely to be better representative of real-world exposures (Kassotis, Tillitt, Davis, Hormann, & Nagel, 2014; Sapouckey, Kassotis, Nagel, & Vandenberg, 2018).

At different periods of development, the mammary gland is responsive to numerous hormones including estrogens, progestins, prolactin, androgens, growth hormone, IGF, and others (Brisken & Ataca, 2015). As noted above, we observed changes in the fraction of epithelial cells expressing ERα in oxybenzone-treated
females in adulthood. Other effects were observed on the fraction of epithelial cells expressing PR, but only at puberty. We cannot determine if specific effects we observed on the mammary gland were caused by the estrogenic, anti-estrogenic, or anti-androgenic actions of the parental compound or the estrogenic properties of the oxybenzone metabolites, or even non-hormonal pathways. However, many of the effects we observed in the female mammary gland are consistent with those observed after perinatal exposures to estrogenic compounds. Prior studies of xenoestrogens including ethinyl estradiol, diethylstilbestrol, bisphenol A and bisphenol S have shown similar increases in alveolar bud density in young adulthood after perinatal exposures (SriDurgaDevi Kolla et al., 2018; Macon & Fenton, 2013; Markey et al., 2001; Vandenberg et al., 2008b); bisphenol S exposure also decreased Ki67-expression in prepubertal females (SriDurgaDevi Kolla et al., 2018), like we observed here with oxybenzone. Yet, ethinyl estradiol increased the fraction of epithelial cells expressing ERα in adulthood (SriDurgaDevi Kolla et al., 2018), and BPA increased the expression of PR at puberty (Munoz-de-Toro et al., 2005), both of which contrast with what we have observed here for oxybenzone. Further, the effects we observed in the male mammary gland are inconsistent with the effects we have observed after exposures to several ER agonists including ethinyl estradiol, bisphenol A or bisphenol S (Kolla, McSweeney, Pokharel, & Vandenberg, 2019; Pokharel et al., 2018; Vandenberg et al., 2013); in fact, we are not aware of any studies that report smaller, less elaborated ductal trees in males after exposures to hormonally active agents.

One hint at the endocrine properties of administered oxybenzone might come from our measures of AGI. Anogenital distance measures about twice as long in males than in females and depends on androgen action in utero (Salazar-Martinez et
al., 2004). Perinatal exposure to 212 μg/kg oxybenzone induced a lower AGI in both males and females. In males, such reduction suggests an anti-androgenic effect of oxybenzone (Fisher et al., 2016; Swan et al., 2005; C. J. Wolf et al., 2000), while in females a shorter AGI cannot be easily explained. In females, xenoestrogens such as diethylstilbestrol can lengthen anogenital distance (Honma et al., 2002) as do prenatal exposures to testosterone (Rhees, Kirk, Sephton, & Lephart, 1997) although other studies have shown that the xenoestrogen BPA can shorten female anogenital distance, consistent with what we have seen here for oxybenzone (Barrett et al., 2017). Thus, our results suggest that oxybenzone alters numerous hormone-sensitive outcomes, consistent with the effects expected after exposure to an endocrine disrupting chemical with pleiotropic actions. However, the exact mechanism by which it acts remains unclear, and in fact it likely acts via several hormone pathways. Future studies using pharmacologically active positive controls may assist in determining the mechanism by which oxybenzone acts. However, the number of different groups needed to evaluate as positive controls for oxybenzone is large (e.g., an estrogenic, anti-estrogenic, and anti-androgenic positive control as well as mixtures of these positive controls to evaluate pleiotropic effects). While evaluating this range of positive controls is an important endeavor, it was not feasible in the current study.

Finally, the results of this study shed light on the effects of oxybenzone on the mammary gland at different vulnerable periods of development. Our prior study, examining the effects of oxybenzone on the mothers of the mice used in this study, revealed that oxybenzone altered mammary gland morphology and cell proliferation in dams, even weeks after exposures had ceased (LaPlante et al., 2018). Those results suggested long-term or perhaps even permanent effects of exposures during
pregnancy and lactation. Here, our results similarly suggest that the sons and daughters, exposed during perinatal development, are also permanently affected by oxybenzone exposures, with effects that manifest at different periods of postnatal development. Although the specific outcomes between exposed mothers and offspring differ, these studies provide new evidence that exposures to endocrine disrupting chemicals during pregnancy can affect the mammary gland in at least two generations. Future studies are needed to understand the mechanism by which oxybenzone acts on the mammary gland, especially considering its multiple modes of action and its metabolites. Additionally, further studies should address whether oxybenzone alters development of the mammary stroma, and whether these disruptions might play a role in the health or pathology of the mammary gland.

4.6 Conclusion

In this study, we exposed BALB/c mice orally to oxybenzone in utero and through lactation and examined males and females at three different stages: prior to puberty, in puberty, and in early adulthood. Exposures to low doses of oxybenzone altered AGI in both males and females, decreased the size and complexity of the mammary ductal epithelium in males, and increased the appearance of alveolar buds in adult females. Oxybenzone altered the expression of hormone receptors in the female mammary gland in an age- and dose-specific manner and disrupted proliferation in the mammary epithelium.

Recent studies have raised alarm over the use of oxybenzone in sunscreens because of the ability of this compound to penetrate the dermal layer and enter the bloodstream. Human biomonitoring studies confirm that humans are widely exposed to this chemical, that exposures are enhanced after the use of personal care
products containing UV filters, and that exposures are especially high in pregnant women. The results of this study indicate that even low doses of oxybenzone can disrupt hormone-sensitive outcomes in male and female offspring and suggest that more caution is needed when using these products.

Disclosures

LNV has received funding from the Cornell Douglas foundation and Paul G. Allen Foundation. She has been reimbursed for travel expenses by numerous organizations including SweTox, Israel Environment Fund, the Mexican Endocrine Society, Advancing Green Chemistry, ShiftCon, US EPA, CropLife America, BeautyCounter, and many universities, to speak about endocrine disrupting chemicals. KM and DJJ have no conflicts to disclose.

Acknowledgements

The authors thank collaborators and colleagues in the Vandenberg lab for their helpful feedback on this project. We specifically acknowledge assistance and input from Ruby Bansal and our BCERP collaborators Karen Dunphy, Anna Symington and Sallie Schneider. This work was supported by NIH grant U01ES026140 (to LNV and DJJ). The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
CHAPTER 5
EXPOSURE TO LOW DOSES OF OXYBENZONE DURING PERINATAL DEVELOPMENT ALTER MAMMARY GLAND STROMA IN FEMALE MICE

5.1 Abstract

Mammary stroma is a prominent modulator of epithelial development, and its complex network of interactions is either permissive or restrictive in tumor initiation and progression. Exposures to oxybenzone, a common UV filter and environmental pollutant and endocrine disruptor, during perinatal development induce alterations in mammary epithelium. Our earlier research indicates that oxybenzone alters mammary epithelial structures at puberty (i.e. more TEBs and a longer ductal extension) and in adulthood (e.g., a higher volume fraction of alveolar buds). We had also previously observed changes in the expression of hormone receptors at puberty (e.g., oxybenzone induced a decrease in the number of PR-positive epithelial cells) and in adulthood (e.g., oxybenzone induced a decrease in the number of ER-positive epithelial cells), and increased body weight in adulthood. Here, we investigated stromal changes in BALB/c animals exposed during gestation and perinatal development to 0, 30, or 3000 μg oxybenzone/kg/day. In response to exposure to 30 μg/kg/day, we observed morphological statistically significant changes in adulthood (the periductal stroma was thicker and adipocytes were considerably larger). And observed increased number of mast cells in the mammary stroma at puberty may represent a transient influence of oxybenzone exposure. These results provide additional evidence that even low doses of oxybenzone can disrupt hormone sensitive organs during critical windows of development and that developmental
exposure to low doses of oxybenzone can induce abnormalities that manifest in later life.

5.2 Introduction

The etymology of the word stroma comes from the Greek for a “bed” or “layer”. In the mammary gland, the stroma is classified as a “connective tissue” type but neither the role of a “bed” or “connector” portrays the importance of the stroma as it is understood today. Beginning during prenatal development, the mammary gland is established from an interplay between the dermal epithelium and underlying mesenchyme (stroma) (Sakakura, Nishizuka, & Dawe, 1976). In fact, transplantation of mammary epithelium into salivary stroma (and vice versa) indicates the importance of the stroma for establishing the morphology of the epithelium; when such transplantations occur using fetal tissues, the epithelium takes on the morphological characteristics associated with the stroma where it is transplanted. The same is true in carcinogenesis, where changes in the stroma are no longer considered a side effect of epithelial loss of order. Epithelial cells exposed to a carcinogen develop mammary neoplasia only if the stroma was also primed with carcinogen and conversely, normal stroma can prevent a carcinogen-exposed epithelium from tumorigenesis (Maffini, Soto, Calabro, Ucci, & Sonnenschein, 2004).

During normal development, stromal extracellular matrix both stimulates epithelial side-branching via induction of Wnt pathways and suppresses excessive branching via MMPs and TGFβ (Wiseman & Werb, 2002). Beyond para- and autocrine signaling, the stroma may modulate endocrine responses in estrogen-nonresponsive epithelium (Haslam & Counterman, 1991). Other regular mediators of the hormonal crosstalk among the two tissue compartments in the mammary gland
include PTHrP and IGF-1, which is induced by growth hormone (see Chapter 1). Lastly, adipose tissue, vasculature, and immune cells of the stroma can act as either permissive or restrictive towards spreading of tumor cells, and therefore these stromal cells modulate the metastatic potential of epithelial tumors via a complex network of interactions (Hussain et al., 2020; Khamis, Sahab, & Sang, 2012).

Endocrine disrupting chemicals (EDCs) have been investigated for their impact on the mammary stromal structures and components. A new class of chemicals, the metabolism disrupting chemicals, can affect energy balance and fat deposition. This suggests that many EDCs act directly on adipocyte differentiation, or indirectly on central and peripheral organs with a downstream influence on metabolism, storage of triglycerides, and signaling altering hunger and satiety (Heindel & Blumberg, 2019). In various organs, EDCs induce dysfunction in white adipose and brown adipose tissue (Francis et al., 2021). Many of these chemicals are also deposited in lipids, and their presence in adipose cells may contribute to continued dysfunction in the stromal compartment.

As an example, it has been proposed that the primary target for BPA, a common xenoestrogen, in the mammary gland is the stroma. During prenatal development, estrogen receptors are not expressed in the epithelium, thus, during this period of exposure, BPA is most likely acting via receptors expressed in the fetal mesenchyme (Vandenberg et al., 2007). Furthermore, the transcriptomal profile of BPA, compared to ethinyl estradiol (EE2, an estrogenic positive control), also pointed to its effects predominantly located in the stromal compartment (Wadia et al., 2013).

There is also evidence that many estrogenic EDCs can act via the immune cell populations that reside in the mammary stroma. Many of these immune cells
express estrogen receptors and are therefore sensitive to environmental chemicals acting via these signaling pathways. Specifically in macrophages, EDCs decrease phagocytosis in a THP-1 cell culture possibly via ERK1/2 MAPK pathways independent of ERα (Couleau et al., 2015).

In this study, we examined the effects of perinatal exposure to oxybenzone on the stromal structures and cells in the mouse mammary gland. Our study suggests that there are structural impacts of early exposures to oxybenzone that persist to adulthood while effects on immune cells might be transient.

5.3 Methods

Animals

BALB/c mice [UMass colony, D.J.J. laboratory, Amherst, MA, described and analyzed in (LaPlante et al., 2018)] were housed in polysulfone cages, with at maximum of five mice of the same sex per cage, and provided food (soy-free chow, ProLab IsoDiet, Brentwood, MO) and tap water (in polysulfone bottles with rubber stoppers and metal tops) ad libitum. Animals were maintained at the University of Massachusetts, Amherst Morrill Animal Facility, in temperature- and light-controlled conditions (12-hour cycles of light and dark). All experimental procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

Chemical administration

From the first day of gestation (GD0 when a sperm plug was observed) until the day before weaning (on lactational day 21), the mothers were weighed and orally dosed daily by pipet with either tocopherol stripped corn oil or oxybenzone dissolved
in stripped corn oil. As a result, the offspring evaluated in this study were exposed in utero and during the first 21 days of postnatal life via the mother’s milk. Two doses of oxybenzone were used: 30 μg/kg/day and 3000 μg/kg/day (FIGURE 5.1.). Depending on the region, season, and individual use of oxybenzone-based personal care product, the lowest dose is likely relevant to human exposures based on back-calculations of urinary concentrations NHANES studies (Han et al., 2016; Woodruff et al., 2011).

Dams were allowed to deliver naturally, and litters were not culled. Sample sizes for the treatment groups were: controls, 11 litters; 30μg/kg oxybenzone, 10 litters; 3000 μg/kg oxybenzone, 9 litters. Pups were distributed into separate cages upon weaning and kept until the peak of puberty (PND 32-35) or adulthood (PND 65-75). One female from each litter was selected for each endpoint to avoid litter bias, but sample sizes for each endpoint vary based on litter size. Animals were ear-tagged upon weaning for identification.

Figure 5.1: Study Design.
**Euthanasia and tissue collections**

One female from each litter was euthanized at the peak of puberty (PND32-35), or in adulthood (PND 65-75). Results from the dams (the F0 generation) are described in (LaPlante et al., 2018). Characteristics of the F1 females including morphology of the whole mount mammary gland and epithelial endpoints were described in (K. Matouskova et al., 2019).

On the day of sacrifice, animals were euthanized with CO₂ inhalation followed by cervical dislocation to ensure death. From every animal we dissected the right fourth inguinal mammary gland, spread it on a glass slide (Thermo Fisher Scientific, Waltham, MA) and fixed it overnight in 10% neutral buffered formalin. The left mammary gland was fixed in 10% neutral buffered formalin overnight before being washed, dehydrated, and embedded in paraffin for histology and immunohistochemistry. The 3rd pectoral mammary glands were collected (avoiding the lymph node), flash frozen on dry ice, and stored at -80°C for qRT-PCR analyses.

**Tissue embedding, sectioning and histological staining**

Fixed mammary glands were washed in 1x phosphate buffered saline, dehydrated through a series of alcohols and embedded with paraffin (Leica Biosystems, Richmond, IL) under vacuum. Paraffin blocks were cut into five micrometer sections on a rotary microtome (Fisher Scientific) and mounted on positively charged slides (Fisher Scientific) for histological evaluation and immunohistochemistry.

For histological evaluations, slides were deparaffinized with xylene and a series of alcohols, stained with Harris' hematoxylin and eosin (Fisher Scientific),
dehydrated, and mounted with permanent mounting media (Fisher Scientific). Digital images were collected using a Zeiss Axio Observer.Z1 inverted microscope, a 10X objective, and a high-resolution color camera (Carl Zeiss Microscopy).

For quantification of periductal collagen fibers, sections were stained with Gomori’s Trichrome Stain (Richard-Allen Scientific/Thermo Fisher Scientific, REF 87020). In short, deparaffinized samples were fixed with Bouin’s fluid (REF# 88038), and stained with Weigert’s Iron Hematoxylin (REF#s 88028 and 88029) and Gomori’s Trichrome Stain (REF# 88030). Individual dyes in the kit stain the collagen fibers blue, cell nuclei are stained black, while cytoplasm and muscle fibers appear red.

**Mast cells staining and analysis.**

One section from each animal was stained using toluidine blue stain (1% RICCA, Arlington, TX USA). The cover-slipped slides were analyzed using a Zeiss Axio Observer.Z1 inverted microscope, a 10X objective, and a high-resolution color camera. Each age group required a slightly modified approach as the mammary gland differs at puberty and in adulthood. For adult animals, ten to twelve images of ducts comprised of 20 to 100 epithelial cells were chosen. For each image, we counted the number of ducts and number of mast cells associated with ducts. For each animal, the ratio of total number of mast cells divided by the total number of ducts was calculated.

The epithelial compartment of the mammary gland is less developed in pubertal mice. Therefore, the ducts selected for analysis were practically all the ducts present in the section. Terminal end buds were excluded from analysis due to their specific character. Also, extra care was taken to *not* include blood vessels in
the analysis as they often appear near mammary ducts and their walls contain higher numbers of mast cells.

**Immunohistochemistry**

Expression of two markers, Ki67 and F4/80, was evaluated using standard methods for immunohistochemistry and commercial antibodies including rabbit anti-Ki67 (Fisher Scientific, Cat# RM-9106-S1), a marker of proliferation, and F4/80 (a macrophage marker). Briefly, sections were deparaffinized, hydrated through a series of alcohols, microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval, and treated with hydrogen peroxide to quench endogenous peroxidases. Non-specific binding was blocked with 1% milk protein in 5% normal goat serum (Cell Signaling Technology, Danvers, MA).

Sections were incubated with primary antibodies (typically diluted 1:500 or 1:1000 in blocking solution) at 4°C for 14-16 h. They were then washed and incubated with secondary antibody (goat anti-rabbit, Abcam, Cat# ab64256) followed by streptavidin peroxidase complex (Abcam, Cat# ab64269). Diaminobenzidine chromogen (Abcam, Cat# ab64238) was used to visualize reactions. Sections were counterstained with hematoxylin (Fisher Scientific). Each immunohistochemical run included a negative control in which the primary antibody was replaced with 5% normal goat serum.

**qRT-PCR**

Total RNA was extracted from the mammary glands using Trizol reagent and a BeadBug microtube homogenizer. We then examined the RNA purity as the A260/A280 ratio and quantified the concentration of RNA in the sample using
Nanodrop UV spectrophotometry. 1 mg of RNA from each sample was transcribed to cDNA using reverse transcription. Lastly, 2 µL of cDNA was combined with forward and reverse primers for each target gene (TABLE 3.2.) and SYBR green master mix to amplify and detect the gene of interest (thermal prolife cycles were set as follows: 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 15 seconds at 72°C). A melting-curve analysis was conducted to identify nonspecific products. Finally, expression of each target gene was quantified using the ΔΔCt method, which allows each target to be evaluated relative to reference genes. Every sample was run in triplicate for each target gene. Two reference genes were used for the mammary gland analyses: β actin and β2 microglobulin (TABLE 3.2.). These were averaged for the ΔΔCt quantification.

**Statistical analysis**

GraphPad (Prism version 9.1.1) was used to analyze categorical and continuous variables. Normally distributed continuous data were evaluated with one-way ANOVA followed by Tukey post-hoc tests. Categorical variables were evaluated using contingency tables with Chi-square and Fisher’s exact tests and graphed as percent fraction of each experimental group and endpoint.

For all evaluations, a p-value of less than 0.05 was considered statistically significant. All continuous data are graphed as a mean ± standard error. Categorical data are graphed as percentages of each treatment group assigned to each category. All analyses were conducted by experimenters blinded to treatment group.
5.4 Results

5.4.1 The periductal stroma is wider in animals exposed perinatally to oxybenzone when evaluated in adulthood

We began by measuring the width of the loose stroma that typically surrounds the mammary ducts. Of the two doses of oxybenzone tested, 30 µg/kg/day induced the expansion of the periductal stroma in both age groups, although this was only statistically significant in adult animals (FIGURE 5.2 A and B; ANOVA, p values = 0.32 and 0.035 resp, Tukey posthoc = 0.028).

Figure 5.2: Periductal stroma, collagen and fibronectin. A) The width of periductal stroma at puberty. B) The width of periductal stroma in adulthood. C) Relative mRNA expression of Col1a1 mRNA at puberty. D) Relative mRNA expression of Col1a1 mRNA in adulthood. E) Relative mRNA expression of Fn7_1 mRNA at puberty. F) Relative mRNA expression of Fn7_1 mRNA in adulthood. * indicates significance < 0.05: ANOVA, Tukey posthoc test. All qRT-PCR expression data were normalized using the ΔΔCt method.
The loose stroma has a cellular component, the fibroblasts, and a matrix comprised of collagens, glycoproteins, and proteoglycans. We assessed the expression of the gene for type I collagen – its pro-α1(I) chain (Col1_1), the most abundant collagen type. The fold change was slightly greater for both tested doses of oxybenzone in pubertal animals and adults exposed to 3000µg/kg/day, but neither of the differences were statistically significant (FIGURE 5.2 C and D; ANOVA, p value = 0.835 and 0.879 in pubertal and adults resp.). We also evaluated the expression of fibronectin, the matrix glycoprotein. There was no effect on fibronectin expression in either of the age or treatment groups (FIGURE 5.2 E and F, ANOVA p values = 0.954 and 0.273).

5.4.2 Adipocytes in the mammary stroma

Next, we assessed the size of adipocytes in the mammary stroma. In the white adipose tissue, we observed that adult adipocytes were on average more than two times larger in response to oxybenzone (30µg/kg/day) compared to controls (FIGURE 5.3B, ANOVA, p value = 0.0084, Tukey posthoc = 0.077). Furthermore, between puberty and adulthood, the adipocytes in control mammary glands decreased in size by about 1/3 (from an average of 1677 µm² to 1218 µm²). In contrast, white adipose cells from oxybenzone-exposed animals increased in size in the mammary gland from 1502µm² to 2023 µm² in the 30 µg/kg/day group, and from 1473 µm² to 1736 µm² in the 3000 µg/kg/day group. At puberty, the white adipocytes were not significantly different in size between the treatment groups (FIGURE 5.3A, p value = 0.641, ANOVA).

To determine if oxybenzone treatment altered other aspects of adipocyte biology, we also examined the number of cell neighbors for adipocytes, which is a
measure of the organization and packing of adipocytes in the tissue. There was a modest but statistically significant difference between the two oxybenzone treated groups at puberty, suggesting that the higher dose of oxybenzone might be producing subtle effects on the organization of the cells in the fat pad. Although there were modest shifts in this measure, overwhelmingly the adipocytes are organized in hexagons similar to other cells like hepatocytes (FIGURE 5.3C and D; ANOVA, p values = 0.041 at puberty, Tukey posthoc = 0.0491, and 0.6 in adulthood).

![Graphs](image)

**Figure 5.3: White Adipose Tissue.** A) Size of a white adipocyte at puberty. B) Size of a white adipocyte in adulthood. C) Number of an adipocyte adjacent cells at puberty. D) Number of an adipocyte adjacent cells in adulthood. E) Relative mRNA expression of PPARγ mRNA at puberty F) Relative mRNA expression of PPARγ mRNA in adulthood. G) Relative mRNA expression of Plin2 mRNA at puberty H) Relative mRNA expression of Plin2 mRNA in adulthood I) Relative mRNA expression of Fas mRNA at puberty J) Relative mRNA expression of Fas mRNA in adulthood. * indicates significance < 0.05: ANOVA, Tukey posthoc test. All qRT-PCR expression data were normalized using the ΔΔCt method.

We next selected three genes representing different aspects of adipocyte function and evaluated their expression. The transcription factor and abundant fat protein PPARγ is critical for adipogenesis. Although we saw PPARγ downregulation
following exposure to 30µg/kg/day oxybenzone, this difference was not statistically significant (FIGURE 5.3 E and F). Adiponectin is an adipocyte-released cytokines mediating metabolism; adiponectin stimulates β oxidation of fatty acids while fatty acid synthase (Fas) takes part in the opposite process, i.e., the biosynthesis of fatty acids. Again, we saw a non-significant downregulation of Fas in adult animals from the 30µg/kg/day oxybenzone exposure group (FIGURE 5.3J).

In addition to its white adipose tissue, the pubertal mammary gland bears occasional areas of brown fat. Brown adipocytes are smaller than white fat cells (averaging 900 µm² vs 1677 µm²). In our analyses of the brown fat cells in the pubertal mammary gland, we saw a trend toward smaller brown fat cells in mammary glands collected from the 30µg/kg/day group (FIGURE 5.4A; ANOVA, p value = 0.54). In all three treatment groups, the brown adipocytes maintained their hexagonal arrangement (FIGURE 5.4.B).

Lastly, we investigated 5 non-overlapping regions of the mammary gland and compared the proportions of white vs brown adipocytes. Perinatal oxybenzone exposure does not alter the proportion of the fat pad that is comprised of white adipose cells (FIGURE 5.4.C). In all treatment groups, the pubertal mammary gland in BALB/c female mice is comprised of about 90% of white and 10% of brown adipocytes.

Figure 5.4: Brown adipose tissue at puberty. A) Brown adipocyte: size. B) Number of neighboring cells in the brown adipose tissue. C) Percent of white adipocyte tissue compared to brown fat.
5.4.3 Macrophages and Mast Cells in the Oxybenzone-treated Mammary Gland

Scattered throughout the stromal matrix are immune cells that are responsible for maintaining the health of the tissue. In our study, we first focused on quantifying the number of macrophages. Using slides stained with F4/80 antibody, we quantified the number of macrophages present. Macrophages are ample in the periductal stroma in both ages, averaging about 30 per duct at puberty and 19 per duct in adult animals. The number of macrophages in the periductal stroma was not altered by perinatal oxybenzone exposure at either of the ages we evaluated (FIGURES 5.5A and B; ANOVA, p value 0.672 at puberty, and 0.854 in adulthood).

![Graphs showing number of macrophages and mast cells in periductal stroma at puberty and adulthood.](image)

**Figure 5.5: Macrophages, Mast Cells, Proliferation in TEB. A) Number of macrophages in periductal stroma at puberty. B) Number of macrophages in periductal stroma in adulthood. C) Number of mast cells in periductal stroma at puberty. D) Number of mast cells in periductal stroma in adulthood E) Ratio of Ki67 positive cells to cells total in terminal end buds at puberty. F) Ratio of Ki67 positive cells to cells total in terminal end buds in adulthood. ANOVA, Tukey posthoc test.**
We also evaluated the number of mast cells localized to the periductal stroma. Mast cells were less abundant than macrophages, with an average of 0.51 per mm$^2$ at puberty and 1.12 per mm$^2$ in adult animals. We observed an almost doubling of the number of mast cells in females from the 30µg/kg/day group when the evaluation was conducted at puberty (FIGURE. 5.5.C; ANOVA, p value = 0.033, Tukey posthoc = 0.047); however, this effect appears to be transient because no differences were observed in adulthood (FIGURE 5.5.D; ANOVA, p value = 0.73).

5.4.4 Proliferation in the Terminal End Bud Structures

Earlier studies suggest that mast cells are required for normal proliferation in the epithelium as well as in the TEBs specifically (Lilla and Werb, 2010). After seeing the number of mast cells increase in response to a 30µg/kg/day oxybenzone, we next quantified proliferation specifically in the TEB structures in the pubertal mammary gland. The number of cells in the TEB expressing Ki67, a marker of proliferation, was not affected by perinatal oxybenzone exposure (FIGURE. 5.5.E, ANOVA p value = 0.64). These results are consistent with the lack of an effect of oxybenzone on the size of the average TEB in the pubertal mammary gland (data not shown, ANOVA, p value = 0.97).

5.5 Discussion & Conclusion

In this study, we extended our prior work, where we reported on the epithelial features in the first generation (F1) of BALB/c mice exposed to oxybenzone during gestation and the perinatal period (K. Matouskova et al., 2019). In that study, we had observed effects of oxybenzone on the morphology of the epithelial structures at puberty (e.g., more TEBs and a longer ductal extension) and in adulthood (e.g., a
lower volume fraction of alveolar buds). We had also observed changes in the expression of hormone receptors at puberty (e.g., oxybenzone induced a decrease in the number of PR-positive epithelial cells) and in adulthood (e.g., oxybenzone induced a decrease in the number of ER-positive epithelial cells). The results of the current study establish further connections between these effects in the epithelium and alterations to the stroma, and provide additional areas to probe the mechanisms by which oxybenzone alters mammary gland development. Furthermore, the current study provides additional evidence of later-life manifestations (e.g., delayed effects) of developmental exposures to low doses of oxybenzone.

On the cellular and tissue levels, exposure to 30 µg/kg/day of oxybenzone during gestation and perinatal development induced two important alterations to the organization of the stroma in adulthood: hypertrophy in white adipocytes and expansion of the periductal stroma. Despite the larger adipocytes that were observed, the adult body weight of female offspring was not altered by oxybenzone exposure (Matouskova et al., 2019). These results might suggest that the effects of oxybenzone are specific to mammary adipocytes, rather than adipocytes more generally. There is evidence that other estrogenic chemicals have distinct effects on adipocytes isolated from different parts of the body; for example, the effects of bisphenols on the transcriptome of omental fat cells differs significantly from their effects on the transcriptome of subcutaneous adipose cells (Peshdary et al., 2020). Future studies are needed to determine if the effect of low doses of oxybenzone on fat cell size are specific to subcutaneous (mammary) fat.

Similar effects as seen here (hypertrophy of adipocytes in adulthood) have also been shown to be induced by low doses of ERα agonists including EDCs such as dioxins and PCBs, and also high-fat diet (Davis et al., 2013; Le Magueresse-
Therefore, we also explored several molecular mediators of adipose cell size. PPARγ is described as both a necessary and sufficient factor of adipogenesis in mammals and fish (Wafer, Tandon, & Minchin, 2017) due to its ability to stimulate differentiation of pre-adipocytes to adipocytes. PPARγ is a mediator of adipocyte hypertrophy (Kubota et al., 1999). For this reason, we anticipated that the expression of PPARγ would be altered following perinatal exposures to oxybenzone. However, our data do not demonstrate that PPARγ or adiponectin are mediators of oxybenzone-related adipocyte hypertrophy. Similarly, we observed no significant change in the expression of a gene involved in fatty acids biosynthesis, fatty acid synthase, another theoretical mediator of these effects. It has been suggested that unlike hyperplasia (an increase in the number of adipocytes), hypertrophy is a sign of dysfunctional development of the adipocyte cells. Moreover, expanded adipocytes can lead to cellular hypoxia and chronic sub-inflammation along with an increased influx of immune cells and local increase of reactive oxygen species (Bokobza et al., 2021). Therefore markers of inflammation, and possibly, the mechanisms by which inflammation in the mammary stroma affect the long-term health of the epithelium should be evaluated in future research studies.

The periductal stroma was wider in adult animals exposed to the lower dose of oxybenzone. However, two additional endpoints – gene expression of collagen type 1 and fibronectin - we chose to explore the change in greater detail have not yielded any significant insights. We may conclude that a type of collagen fibers other than collagen 1 mediates the change, or that oxybenzone (at the timepoints or doses we investigated) does not alter expression of fibronectin. Several other glycoproteins in the mammary extracellular matrix such as laminin and tenascin, as well as TGFβ and hepatocyte growth factors mediate growth of the matrix (Schedin, Mitrenga,
McDaniel, & Kaeck, 2004). It has also been revealed that activation of fibroblasts leads to increased extracellular collagen, fibronectin, and laminin deposition and ultimately to alterations in the periductal stroma (Vafaizadeh et al., 2010).

Follow-up experiments should also evaluate matrix metalloprotease (MMPs), a group of proteases stored in the periductal stroma. In the mammary gland, the mRNAs for MMPs (MMP2, MMP3, and MP9, gelatinase A, stromelysis, and gelatinase B) are upregulated during involution suggesting their role in physiological healing-like processes, while MMP2 mRNA detected in virgin animals and throughout pregnancy hints at its role acting in physiological maintenance (Schedin et al., 2004). Degradation of MMPs through MMP inhibitors (TIMP1) leads to restricted ductal outgrowth while loss of TIMP1 and MMP upregulation reduces ECM integrity and less restricted TEBs (Fata, Leco, Moorehead, Martin, & Khokha, 1999). In late puberty, MMP2 null mice show delayed ductal outgrowth but increased lateral branching (Wiseman et al., 2003). Evaluating the effects of oxybenzone exposure on MMP expression may elucidate other potential mechanisms by which oxybenzone interferes with the mammary epithelium.

We observed an increase in the number of mast cells at puberty associated with oxybenzone exposure, but this increase was no longer observed in adulthood, suggesting that this effect may be transient. The pubertal increase in the number of mast cells in the periductal stroma might be a consequence of the increased area of the periductal stroma however, we observed no effect on the population of macrophages in the same tissue compartment. Future studies should expand these evaluations to other immune cell populations. Unpublished work from the Schneider lab suggests that oxybenzone can affect the health of several different kinds of
immune cells, but additional work is still needed to determine if these effects are also transient, or can persist long after exposures end.

The doses used in this study are not high doses but instead were selected to be relevant to human real-life exposures. While we have seen most effects at the lower dose (30 µg/kg of body weight, administered daily to the mothers of the offspring in our experiment throughout pregnancy and lactation), other studies such as those conducted by the National Toxicology Program have administered much higher doses (e.g., concentrations of 1000 – 50000 ppm of oxybenzone in feed) (Mutlu et al., 2017; Nakamura et al., 2015). It is difficult to determine based on the two dose groups that we have evaluated whether the effects we have observed are consistent with non-monotonic dose responses, e.g., effects that are greater at lower doses than at higher doses. However, it has been previously demonstrated for other endocrine disruptors that effects observed after exposures to low doses cannot be extrapolated from high exposures and vice versa. However, the effects we have observed in the lower dose group should not be dismissed because of the absence of effects after exposures 100x higher; endogenous hormones produce profound effects in nano- and picomolar concentrations that are also not observed at higher concentrations (Vandenberg et al., 2012).

The effects of environmental chemicals on the mammary gland are often poorly evaluated, if they are evaluated at all, in traditional toxicology studies used by regulatory agencies (Cardona & Rudel, 2020). Unfortunately, the mammary gland stroma is not evaluated as an adverse effect in toxicological studies and in the absence of those data, chemicals causing subtle but consequential effects on this tissue compartment might be falsely concluded as having no negative effect on
human health. Improving evaluations of mammary gland health in regulatory studies will likely have an important effect on public health protections.

In conclusion, we have demonstrated here that the stroma of the mammary gland is a sensitive target of oxybenzone, a UV filter commonly used as a sunscreen in personal care products, packaging of perishable foods and drinks, and in paints, textiles, and plastics. Our data suggest that perinatal exposure to low doses of oxybenzone induces adipocyte hypertrophy and expands the periductal stroma in adulthood. Perinatal exposures to oxybenzone also affect mast cell populations in the mammary gland at puberty, but not in adulthood, suggesting these effects may be transient. Additional work is needed to fully elucidate the mechanisms responsible for these alterations and to better understand their possible correlations with adverse clinical conditions.

**Acknowledgements**

The authors thank collaborators and colleagues in the Vandenberg lab for their helpful feedback on this project. We specifically acknowledge assistance and input from Ruby Bansal. This work was supported by NIH grant U01ES026140 (to LNV). The content of this chapter is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
CHAPTER 6
THE IMPACT OF EARLY LIFE EXPOSURE TO OXYBENZONE ON RESPONSES TO A 17 ALPHA-ETHINYL ESTRADIOL PERIPUBERTAL CHALLENGE

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6.1 Abstract

Early-life exposures to environmental chemicals can contribute to later-in-life susceptibility to hormonally active chemicals. Oxybenzone (benzophenone 3) is an estrogen receptor (ER) agonist and antagonist, and androgen receptor antagonist that has been shown to interfere with development of the mammary epithelium following perinatal exposure. Here, we evaluate the effects of exposures during two vulnerable periods of development: the perinatal and prepubertal periods. We also evaluated the effect of perinatal exposure to oxybenzone on a prepubertal exposure to 17α-ethinyl estradiol (EE2), a pharmaceutical estrogen. We observed effects of perinatal oxybenzone exposure on female offspring including an increase in anogenital distance and in the number of epithelial cells expressing ERα. Contrary to expectations, the female mammary gland did not respond to prepubertal exposure to EE2 in its growth parameters, and perinatal oxybenzone exposure had no effect on EE2 responsiveness. The male mammary gland decreased in both area and complexity in response to prepubertal EE2 but perinatal oxybenzone exposure prevents this effect. Collectively, these results suggest that perinatal exposure to oxybenzone interferes with tissue-specific endpoints in the female, but this exposure has no influence on responsiveness to prepubertal estrogen exposure. Further, oxybenzone interferes with the actions of EE2 in the male mammary gland. These data contribute to the body of evidence suggesting that in vivo oxybenzone exposure
during sensitive periods alters development of the mammary gland and other hormone-sensitive outcomes.

**Keywords:** 2-hydroxy-4methoxybenzophenone, BP 3, endocrine disruptor; sunscreen; xenoestrogen; anogenital distance, two-hit, mice pups, perinatal, prepubertal

**6.2 Introduction**

The perinatal period, which includes the development that occurs *in utero* and the first weeks (in mice) or years (in humans) of postnatal development, is arguably the most critical period in the life of the individual. During this period, all tissues and organs of the body are established in both structure and function, following the instructions of the individual’s genotype. Yet phenotype results from both the genes and the environment and hormone-sensitive tissues such as the mammary gland are prone to disorganization due to environmental contaminants with endocrine disrupting potential.

Regarding the genotype, the direction of perinatal development of the mammary gland depends on the presence or absence of SRY, a sex-determining gene located on the Y chromosome. In its presence, testes are formed, and the male mouse experiences the first surge of testosterone at about embryonic day (ED)13. In response to this testosterone surge, between ED13 and 16 the mesenchyme surrounding the budding mammary epithelium, which temporarily expresses androgen receptor, condenses around the invaginating mammary epithelium, detaching the epithelial bud from its epidermal placode (Kratochwil & Schwartz, 1976). Following the detachment of the epithelial bud, the male mouse fails to form
nipples and the fetal mammary epithelium does not grow beyond two or three primary ducts and a few secondary branches. In CD1 mice, a relatively uncomplex epithelial network is observed in the male a few days after birth – with an average of 10 branching points and a ductal area up to 0.5mm² at postnatal day (PND)5. At puberty, the ductal area of the male mammary epithelium has increased about ten times in size (to approximately 5 mm²), with the epithelium later regressing to about half of its pubertal area in adulthood (Pokharel, Kolla, Matouskova, & Vandenberg, 2018; Vandenberg, Schaeberle, Rubin, Sonnenschein, & Soto, 2013). Similar patterns have been observed in other mouse strains, even when the relative size of the mammary epithelium differs significantly. For example, BALB/c males have a total ductal area of about 0.2-0.25 mm² (the right mammary gland being consistently larger than the left) at PND21, which increases to approximately 0.35mm² at puberty and in early adulthood (Matouskova, Jerry, & Vandenberg, 2019).

Because of the actions of androgens in the male, the female mouse mammary gland becomes visibly distinct from its male counterpart about halfway through gestation. At ED14, as the epithelial anlage invaginates into the underlying mesenchyme, the absence of testosterone in the female allows the epithelium to remain attached to the overlying epidermis (Hogg, Harrison, & Tickle, 1983). The mammary epithelium sprouts at ED16; at ED18, the epithelium has formed a ductal lumen and a well-differentiated nipple along with 10-15 secondary branches in an early differentiated fat pad (Robinson, 2007). In contrast to the response of the male mouse mammary gland to testosterone, embryonic development of the female mouse mammary gland does not depend on ovarian or pituitary hormones, as evidenced by the absence of a mammary phenotype prior to puberty in mice genetically altered to lack hormone receptors (Hens & Wysolmerski, 2005). Yet
prenatal exposure to a number of well-established xenoestrogens is associated with abnormal growth of the gland, as well as increased later-in-life breast cancer risk (Calaf, Ponce-Cusi, Aguayo, Munoz, & Bleak, 2020), suggesting that while estrogens are not necessary for perinatal development of the mammary gland, they are able to disrupt its development (Soto, Vandenberg, Maffini, & Sonnenschein, 2008). A number of mechanisms have been proposed or demonstrated for these effects including increased numbers of estrogen-responsive cells, increased ductal proliferation, altered stromal-epithelial interactions, activation of oncogenes and/or repression of critical tumor suppressing genes, and alterations in the epigenome, among others (Hilakivi-Clarke, de Assis, & Warri, 2013; Soto, Brisken, Schaeberle, & Sonnenschein, 2013).

Ultimately, the growth of female mammary gland is promoted by ovarian estrogens along with progesterone via signaling through estrogen receptor (ER)α and progesterone receptors, respectively. However, the hypophysis-pituitary axis is dormant until just prior to puberty, and at that time both hormones produce a 4- to 5-fold increase in epithelial proliferation (Haslam, 1989). Just prior to the onset of puberty (at approximately PND24), the female CD-1 mammary epithelium is approximately 30-35 mm² and it grows quickly to reach approximately 120 mm² by mid-puberty (PND32-35) (Kolla, Morcos, Martin, & Vandenberg, 2018). Again, similar features are seen in other mouse strains although there are quantitative differences: the female BALB/c ductal network at PND 21 extends to about 5 mm² and grows to approximately 50 mm² by PND32-35 (K. Matouskova et al., 2019). In this strain, terminal end buds (TEBs), the highly proliferative epithelial structures that advance the growth of the ductal network that represent the hallmark of puberty, are not typically seen at PND21, whereas an average of seven TEBs per ductal tree are
observed in BALB/c females at puberty. Compared to the ~20 TEBs that are typical of the CD-1 mouse at puberty, it is evident that there are strain differences in the quantitative features of the mammary gland.

The mammary gland is highly susceptible to hormone signaling throughout life, but especially vulnerable during perinatal development, puberty, and pregnancy & lactation. Throughout these periods, the organ undergoes re-organization, proliferation, induction, and differentiation while relying on a host of molecular signaling (Slepicka, Somasundara, & Dos Santos, 2021). A number of studies suggest that early life exposures to xenoestrogens can alter the response of the mammary gland to subsequent hormone or carcinogen exposures. For example, male mice exposed to bisphenol S (BPS), an estrogenic chemical, have mammary glands that are hyper-responsive to 17α-ethinyl estradiol (EE2), a pharmaceutical estrogen, when it is provided at puberty (Kolla, McSweeney, Pokharel, & Vandenberg, 2019). Furthermore, several studies have demonstrated that female rodents exposed to bisphenol A (BPA) during perinatal development have mammary glands that are hyper-responsive to 17β-estradiol after ovariectomy (Munoz-de-Toro et al., 2005; Wadia et al., 2007).

In this study, we pursued a study design that allowed us to investigate the effects of a “double” environmental insult on the mouse mammary gland, e.g., the first exposure occurred during perinatal development, and the second occurred during the prepubertal period. Here, mice were exposed during perinatal development to oxybenzone, a chemical that has gained popularity as an effective UVA and UVB filter. Initially used as a sunscreen ingredient and in other personal care products, oxybenzone is now also used to stabilize dyes, inks, flavors and fragrances and it pollutes the tissues of both aquatic and terrestrial species including
humans (Calafat, Wong, Ye, Reidy, & Needham, 2008; Downs et al., 2016; EPA, 2015; Molins-Delgado et al., 2017). Oxybenzone is considered an endocrine disruptor because it possesses both ER agonist and ER and AR antagonist activities (Balazs et al., 2016; Kunz & Fent, 2006; Watanabe et al., 2015). Next, during the prepubertal period, mice were exposed to either oxybenzone or to EE2, a synthetic pharmaceutical estrogen commonly used in contraceptives (Stanczyk, Archer, & Bhavnani, 2013).

This study design allowed us to pursue two distinct aims: In the first (see FIGURE 6.1A, “Effects of Oxybenzone”), the mice were exposed to oxybenzone either 1) during the perinatal period or 2) the five-day period just prior to puberty or 3) over both the perinatal and prepubertal periods. A fourth group was unexposed to oxybenzone at all times. This aim allows us to determine which period of life (perinatal vs prepubertal) is most sensitive to oxybenzone.

In the second approach (see FIGURE 6.1B, “Oxybenzone & EE2”), mice were exposed to either vehicle or oxybenzone during the perinatal period, and then administered EE2 during the 5 days prior to puberty. This study design allowed us to determine whether perinatal exposure to oxybenzone might potentiate the effects of xenoestrogens administered later in life. This second study involved three experimental groups: 1) controls unexposed to either oxybenzone or EE2 or 2) mice exposed to EE2 during the prepubertal period (but unexposed during the perinatal period) or 3) a group of mice exposed to oxybenzone during the perinatal period followed by EE2 exposure during the prepubertal period.
6.3 Methods

Animals

BALB/c mice were kept in polysulfone cages with bedding, fed soy-free chow (ProLab IsoDiet, Brentwood, MO) and tap water (in polysulfone bottles with rubber stoppers and metal tops) provided *ad libitum*. Animals were maintained at the University of Massachusetts Amherst Animal Facility in temperature-, humidity-, and light-controlled conditions. All experimental procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

Chemical administration

Female BALB/c mice were mated and then exposed to either tocopherol-stripped corn oil (negative control) or 3000 μg/kg/day of oxybenzone dissolved in
corn oil from pregnancy day 0 through lactational day 21. Each day, the mothers were weighed and orally dosed daily by pipet. As a result, the offspring evaluated in this study were exposed in utero and during the first 21 days of postnatal life via the mother (e.g., throughout the perinatal period). At weaning, one male and one female pup from each litter was randomly assigned to a prepubertal experimental group to avoid litter bias. The animals were ear tagged for identification and co-housed with the same-sex and same prepubertal treatment peers.

Prepubertal exposure ensued on the five days following weaning – from PND21 to 25. The pups were weighed and orally dosed daily by pipet with stripped corn oil, 2.5 μg/kg/day EE2, or 3 mg/kg/day oxybenzone. Sample sizes were 10-12 animals per treatment group including the control group.

Euthanasia and tissue collections

One day after the last administered prepubertal dose (e.g., on PND26), animals were euthanized with CO₂ inhalation followed by cervical dislocation to ensure death. The weight of each animal was recorded and anogenital distance was measured with calipers. Females were inspected to determine whether there was an opening in their vagina, a sign of the onset of puberty.

From every male animal, we collected both the left and right fourth inguinal mammary glands, spread both on a glass slide (Thermo Fisher Scientific, Waltham, MA), and fixed them overnight in 10% neutral-buffered formalin (Thermo Fisher Scientific). We also dissected and weighed the seminal vesicles. From every female animal, we dissected the right fourth inguinal mammary gland, spread it on a glass slide (Thermo Fisher Scientific, Waltham, MA) and fixed it overnight in 10% neutral buffered formalin. The left mammary gland was fixed in 10% neutral buffered
formalin overnight before being washed, dehydrated, and embedded in paraffin for histology and immunohistochemistry. Finally, from each female, the uterus was carefully dissected and weighed.

**Whole mounted mammary gland processing and morphometric evaluation**

Whole mounted mammary glands were processed through a graded series of alcohols followed by two washes of toluene to remove fat from the tissue. Whole mounted glands were rehydrated through a series of alcohols and stained overnight with carmine. Finally, the samples were dehydrated through a series of alcohols and xylene and sealed in individual k-pax heat-sealed pouches (Fisher Scientific) containing 5ml methyl-salicylate (Acros Organics, Morris Plains, NJ).

The whole mounted mammary glands were imaged with a Zeiss AxioImager dissection microscope (Carl Zeiss Microscopy, Jena, Germany) and a Zeiss high-resolution color camera. All measurements were made with ZEN software (Carl Zeiss Microscopy, Jena, Germany). In females, the mammary gland measurements included the mammary gland ductal area (e.g., the area subtended by ducts), ductal extension (e.g., the furthest growth of ducts relative to the center of the lymph node, a centralized landmark), number and area of TEBs (defined as bulb-shaped structures at the end of ducts measuring at least 0.03 mm²). Images of female mammary glands were captured at 15x magnification. In males, the number of branching points in the entire ductal tree in both the right and left mammary gland were measured along with the ductal area and TEB-like structures (not limited by their true size but based on the ratio of the diameters in their center and the respective duct). Images of male mammary glands were captured at 18x magnification.
Tissue embedding, sectioning and histological staining

Fixed mammary glands were washed in phosphate buffered saline, dehydrated through a series of ethanols and embedded with paraffin (Leica Biosystems, Richmond, IL) under vacuum. Paraffin blocks were cut into five micrometer sections on a rotary microtome (Fisher Scientific) and mounted on positively charged slides (Fisher Scientific) for histological and immunohistochemical analyses.

For histological evaluations, slides were deparaffinized with xylene and a series of alcohols, stained with Harris’ hematoxylin and eosin (Fisher Scientific), dehydrated, and mounted with permanent mounting media (Fisher Scientific). Digital images were collected using a Zeiss Axio Observer.Z1 inverted microscope, a 10X objective, and a high-resolution color camera (Carl Zeiss Microscopy). Periductal collagen fibers were stained with Gomori’s Trichrome Stain (Richard-Allen Scientific/Thermo Fisher Scientific, REF 87020). In short, deparaffinized samples were fixed with Bouin’s fluid (REF# 88038), stained with Weigert’s Iron Hematoxylin (REF#s 88028 and 88029), and Gomori’s Trichrome Stain (REF# 88030). Individual dyes in the kit stain the collagen fibers blue, cell nuclei are stained black, while cytoplasm and muscle fibers appear red.

Immunohistochemistry

Expression of three markers was evaluated using standard methods for immunohistochemistry and commercial antibodies including rabbit anti-ERα (EMD Millipore, Cat# 06-935, Temecula, CA); rabbit anti-Ki67 (Fisher Scientific, Cat# RM-9106-S1), a marker of proliferation; and rabbit anti-progesterone receptor (PR; Abcam, Cat# ab131486, Cambridge, MA). Briefly, sections were deparaffinized, hydrated
through a series of alcohols, microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval, and treated with hydrogen peroxide to quench endogenous peroxidases. Non-specific binding was blocked with 1% milk protein in 5% normal goat serum (Cell Signaling Technology, Danvers, MA).

Sections were incubated with primary antibodies (typically diluted 1:500 or 1:1000 in blocking solution) at 4°C for 14-16 h. They were then washed and incubated with secondary antibody (goat anti-rabbit, Abcam, Cat# ab64256) followed by streptavidin peroxidase complex (Abcam, Cat# ab64269). Diaminobenzidine chromogen (Abcam, Cat# ab64238) was used to visualize reactions. Sections were counterstained with hematoxylin (Fisher Scientific). Each immunohistochemical run included a negative control in which the primary antibody was replaced with 5% normal goat serum.

Images were collected of each mammary sample for ERα, Ki67, and PR at 400X magnification with a Zeiss Axio Observer.Z1 inverted microscope. Expression of ERα and PR were evaluated by counting at least 500 epithelial cells in 2-4 separate fields. Expression of Ki67 was evaluated by counting at least 1000 epithelial cells in 3-5 separate fields. Expression of ERα, Ki67 and PR were expressed as a percent ratio of the total number of epithelial cells evaluated.

Statistical analysis

GraphPad Prism statistical software (version 9.1.1.) was used to perform statistical analyses. One-way ANOVA tests were used to determine the variance of the means among the treatment groups (using perinatal + postnatal treatment groups as the independent variables), and Tukey’s post hoc test was used to evaluate the between-groups significance, correcting for multiple comparisons.
When needed, the Kruskal-Wallis ANOVA test was utilized for non-normally distributed outcomes. Categorical data were analyzed using a Chi-square test for trend. A p-value less than 0.05 was considered significant. Graphs represent means ± standard error unless indicated otherwise.

6.3 Results

EFFECTS OF OXYBENZONE

6.3.1 Oxybenzone does not alter animal weight but modestly changes endocrine sensitive endpoints in both males and females

We started by quantifying the effects of oxybenzone on growth and overt signs of endocrine activity in the male. Neither perinatal nor prepubertal exposures to oxybenzone affected the body weight of the male at PND26 (FIGURE 6.2A). However, there was a trend observed in the oxybenzone exposure groups, which showed an increased anogenital index compared to controls (FIGURE 6.2B, Kruskal Wallis test, p value = 0.0834). The weight of seminal vesicles (corrected for body weight) was not significantly changed by oxybenzone treatment (FIGURE 6.2C, ANOVA, p value = 0.2434).

We next evaluated growth and overt endocrine endpoints in the female at PND26. The body weight of female pups was not altered due to exposure to oxybenzone at either stage of development (FIGURE 6.2D, ANOVA, p value = 0.74). On the other hand, we observed a significant increase in the female anogenital distance in response to perinatal exposure to oxybenzone (FIGURE 6.2E, ANOVA, p value = 0.0517). Uterine weight was not changed following either perinatal or prepubertal exposure to oxybenzone (FIGURE 6.2F, ANOVA, p value = 0.5665).
Finally, females exposed to oxybenzone during prepubertal development were less likely to have the vaginal aperture open at PND26 compared to controls, although this effect was not statistically significant (FIGURE 6.2G, Chi-square test for trend, p value = 0.2793).

Figure 6.2: “Effects of Oxybenzone” Male and Female morphometrics at PND26.  A) Male animal weight.  B) Male anogenital index.  C) Weight of seminal vesicles relative to the body weight (arrows on the inserted icon indicate seminal vesicles in the male reproductive system in the mouse).  D) Female animal weight.  E) Female anogenital index (ANOVA, p value = 0.0517; Tukey posthoc p value = 0.0302);  F) Uterus to body weight ratio (inserted icon of a mouse uterus without ovaries).  G) Vaginal opening.
6.3.2 Female mammary gland growth parameters at pubertal onset are not affected by perinatal or prepubertal exposure to oxybenzone

We next evaluated the effect of oxybenzone on the female mammary gland, measuring several growth parameters at PND26. We observed no significant effects of oxybenzone treatment, regardless of the period of administration, on ductal area (FIGURE 6.3A), ductal extension (FIGURE 6.3B), number of TEBs (FIGURE 6.3C), average TEB size (FIGURE 6.3D), total TEB area (FIGURE 6.3E), or TEB density (FIGURE 6.3F). These results suggest that oxybenzone treatment alone does not affect these aspects of mammary gland morphology at this age.

6.3.3 The male mammary gland exhibits consistent asymmetry, but neither the left nor the right gland are affected by oxybenzone exposure alone

We next turned our attention to the morphology of the male mammary gland. Prior studies of the male mouse mammary gland revealed a distinct left-right asymmetry both after exposure to a synthetic estrogen and in controls (Pokharel et al., 2018). The right mammary gland has been reported to be larger than the left, and more sensitive to ER agonists administered either during the perinatal or peripubertal period. Consistent with those findings, we observed that the right mammary gland was more likely to have epithelial structures present and the epithelial network was larger, more branched and had modestly higher number of TEB-like structures compared to its left counterpart (FIGURES 6.4A, ANOVA test, p value <0.001; 6.4B-D vs 6.4E-G).

For the remainder of our analyses of the male mammary gland, all variables were evaluated separately for the right and left glands. In the right mammary gland, ductal area was decreased with prepubertal and perinatal exposure to oxybenzone although this difference was not statistically significant (FIGURE 6.4.B ANOVA, p value = 0.1910). Other parameters in the right mammary gland followed similar patterns after oxybenzone exposure (e.g., fewer branching points and fewer TEB-like structures, see FIGURES 6.4C,D). These responses are not statistically significant but are consistent with our previous findings (Matouskova et al., 2019).
Figure 6.4: “Effect of Oxybenzone”: Male Whole Mount Mammary Gland analyses at PND26. A) Ductal tree presence in the right vs left male mammary gland (MG). B) Ductal area in the right MG. C) Number of branching points in the right MG. D) Number of pre-terminal end buds in the right MG. E) Ductal area in the left MG. F) Number of branching points in the left MG. G) Number of pre-terminal end buds in the left MG.
As for the left mammary gland, nearly half of the males were lacking an outgrowth on the left side, therefore the statistical power is diminished, making interpretation of the results less reliable than in the right mammary gland. In spite of this limitation, we observed that the effect of oxybenzone on ductal area, branching points, and TEB-like structures follows a similar pattern as we observed for the ductal tree on the right (FIGURES 6.4E,F,G).

**EE2 and OXYBENZONE**

**6.3.4 Five days of exposure to EE2 during prepuberty does not alter male body weight, anogenital distance, or weight of the seminal vesicles in BALB/c mice**

The next step we took was to examine the effect of prepubertal EE2 exposure on growth and endocrine endpoints in the male. We found that this short-term exposure to EE2 does not affect body weight (FIGURE 6.5A) or anogenital distance (FIGURE 6.5B). We were surprised to find no effect of prepubertal EE2 exposure on the relative weight of the seminal vesicles (FIGURE 6.5C).

**6.3.5 Five days of exposure to EE2 during prepuberty does not alter female body weight, anogenital distance, uterine weight, or the timing of vaginal opening in BALB/c mice**

Prior studies have documented numerous effects of prepubertal EE2 exposure on the female mouse, so we next turned our attention to the female pups at PND26. Not surprisingly, there was no effect of prepubertal EE2 on body weight (FIGURE 6.5D) or anogenital index (FIGURE 6.5E); this was expected because this parameter is established during prenatal development. Interestingly, where we saw that perinatal exposure to oxybenzone altered anogenital index (see FIGURE 6.2E),
when these animals were administered a postnatal estrogen challenge, the effect of perinatal exposure to oxybenzone was no longer observed (FIGURES 6.2E. vs 6.5E).

Perhaps most surprising is the absence of an effect of EE2 on uterine weight. The uterotrophic response has been well documented in prepubertal female mice at lower doses of EE2 than what we administered here. We observed that the mean weight of the uterus was higher in females perinatally exposed to oxybenzone and later to EE2 (0.1725g) than in controls (0.1336g), but this difference was not statistically significant (FIGURE 6.5F, ANOVA, p value = 0.38). Vaginal opening also did not significantly differ among the treatment groups (FIGURE 6.5G, Chi square = 0.2116, p-value = 0.6455).

6.3.6  Modest evidence that perinatal exposure to oxybenzone diminishes the effects of prepubertal EE2 treatment on the female mammary gland

Prior studies have demonstrated that the mammary gland responds to estrogentic stimulation at prepubertal stages with quantifiable changes (usually increases) in morphological parameters. Transient prepubertal exposure to EE2 increased growth parameters of the mammary gland as measured in the whole mount, but we were once again surprised to find that these effects were not statistically significant. These included effects on ductal area, (FIGURE 6.6A, ANOVA test, p value = 0.1897), ductal extension (FIGURE 6.6B, ANOVA test, p

![Diagram showing statistical analysis of ductal area and ductal extension.]

**Figure 6.6: “Oxybenzone & EE” Female Mammary Gland Whole Mount Analyses.** A) Ductal area. B) Ductal extension. C) Average number of terminal end buds (TEBs). D) TEB average area. E) TEB area total. F) TEB density.
value = 0.6009), number of TEBs (FIGURE 6.6C, ANOVA test, p value = 0.0938) and average size of the TEBs (FIGURE 6.6D, ANOVA test, p value = 0.6414). There was some indication that perinatal oxybenzone exposure interferes with the modest estrogenic stimulation; ductal area (and number of TEBs) in females exposed perinatally to oxybenzone followed by prepubertal EE2 treatment was smaller than in females stimulated with EE2 alone (FIGURES 6.6A,C).

6.3.7 Effect of perinatal oxybenzone and postnatal EE2 on histological parameters of the female mammary gland

In response to perinatal exposure to oxybenzone, female luminal cells express ERα in proportionally greater numbers than controls (FIGURE 6.7A, ANOVA, p value = 0.0023); this effect was more pronounced in females exposed to oxybenzone during both the perinatal and prepubertal periods (FIGURE 6.7A, ANOVA p value = 0.0267). Interestingly, a short-term prepubertal exposure to EE2 alone does not significantly alter the number of ERα expressing luminal cells whereas a combined exposure (perinatal oxybenzone plus prepubertal EE2) increases the number significantly (FIGURE 6.7B, ANOVA, p value = 0.0017).

We next used Ki67 as a marker to evaluate proliferation in luminal cells. We observed no effect of oxybenzone at either period of development on the number of cells in mitosis (FIGURE 6.7C). There was also no statistically significant effect of perinatal oxybenzone exposure combined with a short term prepubertal exposure to EE2 on measures of mitosis (FIGURE 6.7D).

Finally, we examined the effect of oxybenzone and/or EE2 on the width of the loose connective tissue located around mammary ducts (also known as the periductal stroma). In females, exposure to EE2 during the prepubertal period does
not significantly alter periductal stromal width. This was observed regardless of perinatal exposure to oxybenzone (FIGURE 6.7F, ANOVA test, p value = 0.7169).

Figure 6.7: Immunohistochemistry analyses in the female mammary gland at PND 26. 
6.3.8 The right, but not the left, male mammary gland is responsive to postnatal exposure to EE2, and this effect is abrogated by perinatal exposure to oxybenzone.

Consistent with our earlier findings and studies [FIGURES 6.4A-G and (Pokharel et al., 2018)], the right male mammary gland is present more often and is more sensitive to exposure to oxybenzone compared to the left (FIGURE 6.8A). However, peripubertal exposure to EE2 lessens the naturally occurring right-left asymmetry in the male mammary gland. Contrary to what was observed in controls, the left mammary gland exhibits no difference in ductal tree occurrence once stimulated with EE2.

When exposed to EE2 during peripubertal development, the right male mammary gland is smaller (FIGURE 6.8B, ANOVA, p value 0.0599), with a significant reduction in complexity as measured by the number of branching points (FIGURE 6.8C, ANOVA, p value = 0.033) compared to controls. Yet, males exposed to oxybenzone during perinatal development are insensitive to a peripubertal estrogen challenge, with no effect on number of branching points compared to untreated males (FIGURE 6.8C). The number of TEB-like structures were decreased after EE2 exposures (with or without perinatal oxybenzone exposure), but these effects were not statistically significant (FIGURE 6.8D).

In contrast, we observed no significant effects of prepubertal EE2 treatment on the left mammary gland, regardless of whether the male was exposed to oxybenzone during the perinatal period, although the trends were similar as in the right (FIGURES 6.8E,F,G). The absence of a left mammary ductal tree in a number of the samples likely contributed to the lower statistical power for measures of the left gland.
Figure 6.8: “Oxybenzone & EE2” Male Whole Mount Mammary Gland analyses at PND26. A) Ductal tree presence in the right vs left male mammary gland (MG). B) Ductal area in the right MG. C) Number of branching points in the right MG. D) Number of pre-terminal end buds in the right MG. E) Ductal area in the left MG. F) Number of branching points in the left MG. G) Number of pre-terminal end buds in the left MG.
6.5 Discussion

In this study, we observed that a small number of outcomes were altered in animals exposed to oxybenzone during the perinatal period, consistent with its actions as an endocrine disruptor. For example, anogenital distance was longer in females exposed to oxybenzone during the perinatal period compared to controls. Also, expression of ERα in the female mammary gland was significantly increased in animals exposed to oxybenzone. These results are consistent with several prior studies that investigated the reproductive toxicity and endocrine-disrupting effects of oxybenzone in rodents. An NTP study aimed at evaluating the toxicity of oxybenzone showed that high doses (up to 50000 parts per million in feed) lowered sperm concentration, increased the frequency of sperm abnormalities, and increased the length of the estrous cycle in female mice and rats (French, 1992). Reproductive abnormalities observed in a later toxicological study included decreased anogenital index in the male rat at PND 23, reduced weight of testes and the number of spermatocytes, decreased weight of the ovaries and uterus, and decreased numbers of antral follicles following exposure to 25000 and 50000ppm (in feed) of oxybenzone during development (Nakamura et al., 2015). Recent work also from our laboratory revealed that short-term oxybenzone exposure in adult female mice increased the expression Esr1 at doses that had no effect on uterine weight (LaPlante, Bansal, Dunphy, Jerry, & Vandenberg, 2018). Hormone-sensitive outcomes are not limited to rodents, as studies in zebrafish have also revealed that oxybenzone exposures alter vitellogenin expression in fish (Kariagina et al., 2020). Collectively, the results of the current study, together with the results of these prior studies, suggests that oxybenzone meets at least four of the ten key characteristics
of an endocrine disrupting chemical: it is a hormone receptor agonist, a hormone receptor antagonist, it alters expression of hormone receptors, and it alters the fate of cells in hormone-responsive organs (La Merrill et al., 2020).

The effects of oxybenzone have now been demonstrated in several studies specifically examining the mammary gland. In human breast epithelial cells and in the mammary epithelium of mice treated with oxybenzone, 1 µM and 5µM concentrations or 3000µg/kg/day respectively, increased DNA damage as measured by a double-strand break assay and in the quantification of R-loops, a three-dimensional structure that is associated with DNA damage (Majhi et al., 2020). The damage was ERα-dependent and analogous to adverse effects on DNA caused by endogenous estrogens (e.g., 17β-estradiol). Yet, in a toxicological study that evaluated the carcinogenic potential of oxybenzone in the Sprague-Dawley rat, the incidence of mammary gland fibroadenomas and carcinomas were decreased in females administered food containing 10,000ppm oxybenzone compared to unexposed controls (NTP, 2020). These results suggest that, at this high dose, oxybenzone might prove to be protective against some kinds of mammary tumors. Our own work has shown that developmental exposure to oxybenzone is associated with greater ductal growth and number of TEBs in the female pubertal mammary gland, and an increase in the number of alveolar buds in the female adult mammary gland (K. Matouskova, Vandenberg, L.N., 2022). In contrast, males exposed to oxybenzone during development had smaller and less elaborated mammary epithelial networks compared to controls at PND21.

The study design used in the “EE2 & Oxybenzone” experiment was devised based on our hypothesis that exposure to xenoestrogens during early organ development would increase susceptibility of the mammary tissue to pre-neoplastic
changes later in life. The suggested mechanisms of augmented susceptibility include interference with the number of nuclear and non-classical membrane receptors, enhanced activation of these receptors, as well as non-genomic epigenetic alterations – specifically DNA methylation of estrogen-sensitive genes (Prins, Tang, Belmonte, & Ho, 2008). For example, perinatal exposure to bisphenol A (BPA) enhances responses to estradiol in the mammary gland – exposures to 25 or 250 ng/kg/day BPA potentiated the number and size of TEBs when estradiol was administered at puberty to ovariectomized females (Munoz-de-Toro et al., 2005). In another study, mice were administered bisphenol S (BPS) during early development and then given an additional EE2 challenge at puberty. Whereas control females responded to the EE2 challenge as expected, e.g., with increases in the number of ovarian follicles in all stages, this estrogen-induced stimulation was not observed in females that had been exposed to BPS (Hill, Sapouckey, Suvorov, & Vandenberg, 2017). These studies suggest that early life exposures to xenoestrogens can enhance, or abrogate, the effects of estrogens administered around puberty.

We were surprised to find that early life exposure to oxybenzone enhanced the responses of the mammary gland to a peripubertal estrogen challenge in females, as measured by an increased expression of ERα (FIGURE 6.7B). This was especially surprising because there were no significant effects on the morphology of the mammary gland in the females of any treatment groups (FIGURE 6.6). However, we did note that EE2 exposure alone had no effect on morphology of the mammary gland, or weight of the uterus (FIGURE 6.5F, Figure 6). These results suggest that the female BALB/c mice used in this study may be unusually insensitive to estrogenic stimuli either because of a genetic- or age-specific factor. This
insensitivity has been observed previously for BALB/c mice, in contrast to other strains (Haslam & Counterman, 1991; Kleinstreuer et al., 2016).

EE2 is a well-accepted positive control for estrogenicity, and its inclusion is suggested as a routine practice. The inclusion of such a positive control in studies of potential endocrine disruptors is necessary to distinguish between “true negative” effects of test chemicals and negative effects that are observed because the test system is unresponsive or insensitive to stimulation (vom Saal & Welshons, 2006). Animal models should have demonstrated sensitivity to low doses of positive control chemicals, e.g. EE2 (vom Saal et al., 2010). As our results suggest, such responsiveness cannot be assumed. Lastly, doses of test chemicals should be chosen based on knowledge of the relative receptor binding affinity whenever possible. For example, if the test chemical has a receptor binding affinity that is 100 to 1000 times lower than the positive control, then doses of the test chemical should be administered at least 100 to 1000 times higher than those that elicit a response in the positive control (Timms et al., 2005). To illustrate the point, an extensive, multi-laboratory effort failed to consistently detect effects of BPA on uterine weight at doses below 300 mg/kg/day (Kanno et al., 2003). However, the rat strains that were used are not appropriate for such studies because, based on their relative binding affinities and the absence of uterotrophic responses to 0.1 µg/kg/day EE2, doses in the range of 1000µg/kg/day BPA are predicted to alter uterine weight (Kanno et al., 2001).

Our results provided additional evidence on the left-right asymmetry of the male mammary gland that is consistent with previous findings in the same and other mouse strains. We found that the mammary epithelium was present in significantly greater numbers of male mice on the right side compared to the left fat pad. Our
prior studies revealed that the right mammary gland is also consistently more commonly affected by exposure to endocrine-disrupting chemicals (Pokharel, Kolla, Matouskova, & Vandenberg, 2018). Here, we found that perinatal oxybenzone exposure altered the response of the right male mammary gland to a prepubertal EE2 challenge. In the males, both ductal area and complexity of the right gland were significantly reduced upon prepubertal exposure to EE2, but these effects were no longer observed in the males exposed to oxybenzone (FIGURE 6.8B,C). No effects of either oxybenzone or EE2 were observed in the left male mammary gland. These results suggest that early life oxybenzone exposure stunts the response of the right male mammary gland to estrogens administered later in life.

6.6 Conclusion

The results in this study reveal modest but significant effects of perinatal exposure to oxybenzone, a common active ingredient in sunscreens and personal care products and UV-resisting agent in packaging, textiles, and industrial paints and coatings. Female mice exposed to oxybenzone in utero and during lactation had a longer anogenital distance. Perinatal exposure to oxybenzone also increased the ratio of cells expressing ERα by 25% compared to controls. In males, short-term prepubertal exposure to EE2 reduces both the area and complexity of the right mammary ductal tree. Interestingly, perinatal exposure to oxybenzone reduces the effects of EE2 in the male mammary gland as well. This study contributes to an existing body of evidence suggesting that oxybenzone is an endocrine disruptor, and suggests that disruptions during perinatal development can persist beyond the period of exposure, and that the right mammary gland in the male is a sensitive target of endocrine disruption.
7.1 Breast Cancer Incidence and Trends

Worldwide, breast cancer is the leading cause of female neoplasia in 154 out of 185 countries; of all cancers diagnosed in women, 24 percent occur in the breast (Bray et al., 2018). Furthermore, despite advances in diagnostics and treatment, breast cancer leads female cancer mortality in more than 100 countries around the world.

In the United States, according to the database of Surveillance Epidemiology and End Results (SEER), which includes data from patients with tumors dating back to 1975, breast cancer in women in all age categories has increased over the past four decades – by an average annual percentage change in 55-69 year old women of 0.14% (95% CI = -0.18% to 0.46%) and 0.93% (95% CI= 0.48 % to 1.41%) in women 70-89 years old at the time of diagnosis (Kehm, Yang, Tehranifar, & Terry, 2019). Population-based incidence data from the Connecticut Tumor registry offer data dating back to the 1930s. This registry documents a shocking increase in incidence of breast cancer from 16.5 cases per 100 000 women in 1935 to 38.5 cases per 100 000 women in 2015 (Lima et al., 2020); this is equivalent to an increased relative rate of 2.4. Importantly, the authors accounted for parity trends and concluded that changes in parity explain no more than 4% of the variability in breast cancer incidence over the 80 years that have been studied.

7.1.1 Disparity in Breast cancer Incidence and Mortality

As a disease with multiple causative factors, breast cancer incidence varies across age, race and ethnicity, but also depends on social, economic, and
environmental determinants of health. In the period of 2012-16, incidence of breast cancer in non-Hispanic white and black women was reported as 130.8 and 126.7 cases per 100 000 women respectively (DeSantis et al., 2019). These rates are much higher than that of American Indian/Alaska Native, Asian/Pacific Islander, and Hispanic female populations, which are reported as 94.7, 93.2, and 93.7 cases per 100 000 women, respectively.

Similarly, race, ethnicity, and social, economic and environmental determinants of health also impact breast cancer mortality rates in the US. Mortality ratio rates collected between 2013 and 2017 identified 42 states with breast cancer mortality rate disparities based on ethnicity, and in all 42, non-Hispanic black women are more likely to die from breast cancer than non-Hispanic white women. The District of Columbia (relative risk = 1.83), Louisiana (relative risk = 1.63), and Mississippi (relative risk = 1.59) rank as the top three states with ethnicity-driven disparities in women’s death from breast cancer. Overall, during this same period of time, breast cancer mortality rates were 20.3 deaths per 100 000 women in non-Hispanic white populations and 28.4 cases per 100 000 women in non-Hispanic black populations; this compares to 14.6, 11.5. and 14.0 deaths per 100 000 women in American Indian/Alaska Native, Asian/Pacific Islander and Hispanic female populations respectively (DeSantis et al., 2019).

### 7.2 Protective Effect of Parity

The mammary gland of a parous adult female rodent is distinct from that of a nulliparous animal. Similarly, breast tissues of women who have had a full-term birth deviate from tissues of those who have not. As discussed below, these changes can
include the types of differentiated epithelial cells and structures, the composition and characteristics of the periductal stroma, and the immune cell populations, among others. Thus, on a microscopic and molecular level, both the mammary epithelia and stromal structures bear signs of post-partum involution if full-term pregnancy occurred in the body. These differences suggest that pregnancy is a critical period for long term protection against breast pathology. Yet estrogen levels that increase to nearly a hundred times higher compared to regular ovarian cycles pose a paradox: on the one hand, the high levels of estrogen associated with pregnancy induce protective changes in the breast, but on the other hand, in the treatment of breast cancer, lowering the circulating level of estrogens is typically the first line of defense. This paradox has been investigated at least since Sir Beatson published an observation of a breast cancer in his patient regressing in response to removal of her ovaries (Beatson, 1896).

Early epidemiological data clearly showed that pregnancy, especially at younger ages, lowers the long-term risk of breast cancer (MacMahon et al., 1970). Later still, data began to show that the long-term benefit takes some time to be achieved because pregnancy first conveys a 2 to 15 year long period of increased risk (Albrektsen, Heuch, Hansen, & Kvale, 2005). Using more sophisticated statistical analyses, the latest large collaborative “pooled” evaluation to examine the relationships between age, parity, and breast cancer concluded that parous women do acquire significant protection against the disease but only after about 20 years of slightly higher risk of breast cancer (2.2% in women who had given birth vs 1.9% in those who had not); the period of increased risk is longer, the later in life her pregnancy has occurred (Nichols et al., 2019).
Hypotheses proposed on the underlying cellular and molecular mechanisms of the protection afforded by parity have focused on the two mammary compartments: the epithelium and stroma. In the epithelium, parity induces permanent changes consistent with lower susceptibility to carcinogenesis such as decreased epithelial proliferation, fewer undifferentiated epithelial cells (J. Russo & Russo, 1997), and also fewer luminal progenitor cells (Bach et al., 2017). ERα-positive epithelial cells tend to become less common following postpartum involution, but the number of ERβ-expressing cells increase in older parous animals compared to age-matched nulliparous controls (Dall et al., 2018). Further, parous animals have lower PR expression in the mammary epithelial cells, as well as downregulated expression of genes in the Wnt/β-catenin pathway (Muenst et al., 2017), and higher expression of other genes involved in cell adhesion, DNA repair, and apoptosis compared to age-matched nulliparous controls (Incassati, Chandramouli, Eelkema, & Cowin, 2010; J. Russo, Balogh, & Russo, 2008).

The crucial role of stroma in carcinogenesis has been described in classic experiments in the 1970s and 1980s: normal or carcinogen-treated mesenchyme were transplanted with normal or carcinogen-treated epithelium in various organs, revealing that the treatment of the stroma dictated the disease phenotype in the epithelium (Sakakura et al., 1976). Thus, treatment of the stroma alone with a carcinogen was sufficient to induce epithelial-derived tumors in the cross-transplanted samples.

Considering this essential role of the stroma, further experimentation has worked to identify which features of the stromal compartment are responsible, at least in part, for the protection afforded by parity. The changes, before all else, seem highly conserved amongst rat strains; there is a conserved signature in the stroma,
including alterations in activity of Tgf-β, immune cells, and extracellular matrix (Blakely et al., 2006). Interestingly, a parity-associated gene expression signature revealed conserved effects of parity in the epithelium, including downregulation of amphiregulin and the GH/Igf-1 axis. In human breast tissue, a recent study revealed 286 genes that were differentially expressed – mostly upregulated - in women who experienced full-term pregnancy compared to nulliparous women, although the study did not discern in which effects were attributed to the epithelium or stroma (Santucci-Pereira et al., 2019).

Experimentally, the extracellular matrix from parous rodents – specifically its protein components and enzymes - restricts ductal elongation and bifurcation more than matrix isolated from nulliparous females, and in 3D organoid cultures it suppresses development of alveolar structures (Schedin et al., 2004). Pregnancy-associated changes in the immune cell populations including macrophages, lymphocytes, and mast cells are addressed in Chapter 8, however, the mammary stromal compartment also includes adipose tissue and vascular systems where potential targets of parity-related changes are largely still to be investigated.

Of note, beyond effects that are directly observed in the mammary tissue, parity in the rat animal model is associated with other effects that can indirectly impact mammary gland health. For example, parity permanently decreases the levels of growth hormone (GH) in the pituitary, and GHRH in both the hypothalamus and hypophysis; parity also permanently alters the levels of prolactin in the pituitary which can be induced by lower THR and higher dopamine levels in the hypothalamus (Subramani et al., 2021). Both of these hormones (prolactin and GH) are key regulators of mammary gland morphogenesis (see Chapter 1). The role of prolactin in regulating the morphology and function of the gland is observed for the
most part during late pregnancy and lactation. In contrast, GH, which can bind to prolactin receptors in addition to growth hormone receptors, acts throughout the lifetime and can influence mammary carcinogenesis (X. Chen, Wu, Zheng, Liu, & Wang, 2020; Katz, 2016; Subramani, Nandy, Pedroza, & Lakshmanaswamy, 2017).

7.3 Genes Associated with Breast Cancer

Like the organ itself, breast cancer risk is strongly sexually dimorphic, with much higher risk observed in the female sex. Lifetime risk for women in the U.S. is approximately 1 in 8 (13%) and more than 280,000 women are expected to be newly diagnosed with breast cancer in 2021. The lifetime risk of the disease in men in the U.S. is 1 in 833, corresponding to 2650 male patients that will receive the diagnosis of breast cancer annually (SEER, 2021a).

Age is the next most critical risk factor for breast cancer, and age has been associated with damage to the genome because somatic mutations accumulate over time. This relationship between age and accumulation of mutations does not appear to hold true for stem cells, which means that older age is not the only factor that can influence the frequency of mutations in the cells that may participate in carcinogenesis (Brazhnik et al., 2020). Interestingly, in addition to the number of age-related mutations, the number of mutations calculated in tumors are predictable and can be seen as a clock for tumor development and progression (S. Jones et al., 2008). Thus, genomic instability appears to be a common feature of tumors, and because these mutations accumulate in a stereotypical manner, they can be used to calculate the time since the initiation of the tumor.

Although much work has been done to identify genes known to be tumor suppressors or oncogenes, it is now understood that a single mutation, either
somatic or germline, will not produce a neoplasia (Hanahan & Weinberg, 2011). It is instead estimated that somewhere between two and eight – and in some cases as many as twenty - mutations in respective genes must be altered for a tumor to manifest clinically (Vogelstein et al., 2013). The smorgasbord of available genes is abundant: an extensive collaborative study identified 990 cancer-associated mutations in 51 distinct genes mutated in the twelve most common types of cancer (Pan et al., 2014); over 400 unique mutations in the tumor suppressor gene Tp53 alone are listed in the IARC database (Bouaoun et al., 2016).

In breast cancer, 10% of cases are associated with inherited germline mutations. 6% of those are due to mutations in the alleles of the major breast cancer susceptibility genes, BRCA 1 and BRCA2 (Tung et al., 2016). Mutations in BRCA1 and BRCA2 increase the risk of breast cancer for the carriers from 13% seen in the general population to 55-65%, and 45% respectively (S. Chen & Parmigiani, 2007). After these genes are accounted for, the next most common breast cancer related mutations occur in TP53. The frequency of TP53 germline mutations has been estimated from as high as 1 in 5000 to 1 in 23,000 (Gonzalez, Noltner, et al., 2009; Laloo et al., 2003), and the likelihood of a mutation is increased in individuals with a family history of early onset Li-Fraumeni syndrome related cancers. Additional research quantifying the frequency of somatic TP53 mutations suggests a role for ethnicity in individual risk of breast cancer. High profile genetic profiling of populations in different parts of the world revealed that mutated TP53 accounts for 46% of somatic mutations in triple negative breast tumors isolated in African American patients, 27% of tumors collected from Caucasian patients, 39% of Saudi Arabian patients, and 19% of tumors from Chinese patients (Ademuyiwa, Tao,
Other genes that increase the likelihood of breast cancer to a more modest degree include *PTEN, PIK3C, ATM* or *CHEK2*. Once again, the frequency of mutations in these genes is highly dependent on ethnicity and tracking their origin might be challenging (H. A. Yu et al., 2014). These low/moderate-penetrance genes often have a role as a modifier of cancer manifestation, or a larger number of mutations in those genes may produce a higher lifetime risk of breast cancer compared to a smaller number of mutations in high penetrance genes (Thier et al., 2003; Weber & Nathanson, 2000).

The risk of breast cancer risk has increased over time in both the general population and in women carrying germline mutations. Ashkenazi Jewish females are carriers of several ancient *BRCA1/2* mutant alleles specific to this population and therefore their risks and cancer manifestation can be studied with unique precision. Comparing the risks of breast cancer between women born before and after 1940, i.e. before the boom of the petrochemical industry and after, revealed that non-genetic factors have significantly increased the penetrance of the high-risk, *BRCA1/2*, mutations in Ashkenazi Jewish women (King, Marks, Mandell, & New York Breast Cancer Study, 2003). These results suggest that there is a substantial influence of non-genetic, environmental factors superimposed on the genotype.

In conclusion, awareness of the genetic factors that contribute to increased risks of cancer allow sensitive populations to be identified. *De novo* mutations in *BRCA1/2* are rare but testing for the presence of familial mutations in these genes is common. Furthermore, testing for somatic mutations in *TP53* may be beneficial as *de novo* mutations in these genes can be present in up to 20% early onset cancers.
(Gonzalez, Buzin, et al., 2009). Individuals who are carriers of these mutations might be the first in their family diagnosed with Li-Fraumeni syndrome, an autosomal dominant disease manifesting in early-onset tumors, often even in childhood, as well as breast cancers that develop before the age of 30 (Ruijs et al., 2010).

Because an individual cannot change their genetic risk factors, it has been widely discussed amongst environmental health scientists that more effort is needed to identify modifiable risks for cancers. Yet, there is increasing understanding that genetics and environment should be viewed in concert. Certainly, there is evidence that some genetic backgrounds can increase the sensitivity of individuals to environmental carcinogens and other environmental risks (Colletti et al., 2014; Jerry et al., 2018). Genetic screening is important because it offers the opportunity for earlier interventions. Cancers diagnosed in early stages can usually be treated more effectively, therefore identification of vulnerable individuals using modern methods of genetic testing represents a highly consequential effort toward secondary prevention attributed to advances in medical science and technology.

7.4 Environmental Chemicals Associated with Breast Cancer

The origin of as many as 80% of breast cancers does not rely on genetic mutations. The argument that the culprits of increasing incidence of hormone-sensitive cancers are likely environmental is constructed from three clues: 1/ the incidence of hormone-sensitive cancers has increased over just two or three generations, therefore a profound genetic shift is unlikely; 2/ human exposure to environmental chemicals including many endocrine disruptors has increased enormously over the past three or four generations; and 3/ the available evidence from experimental in vitro and in vivo studies, well as mechanistic research and
epidemiological studies, builds a strong case of environmental causes of endocrine cancers.

As noted above, estrogens pose a paradox pertaining to breast cancer; women are exposed to fluctuating levels of endogenous estrogens during ovarian cycles reaching as high as 700pg/mL mid cycle (compared to serum levels less than 16pg/mL and 10pg/mL prior to puberty and after menopause respectively), and even higher levels are measured during late pregnancy (up to around 7000pg/mL) (Gardner, 2018); however, 87% of women in the general U.S. population remain breast cancer-free throughout their lifetime. In spite of the high estrogen levels associated with parity, pregnancy and lactation are long-term protective factors against the risk of breast cancer (Nichols et al., 2019). On the other hand, women who enter menopause later in life, and those who start menstruating earlier in life, experience increased risk for breast cancer; these results suggest that lifetime exposures to estrogens, including natural ones, are a major risk factor for mammary cancer (Soto, Vandenberg, Maffini, & Sonnenschein, 2008). Furthermore, exposures to various xenoestrogens have recently been associated with higher risks of breast cancer and a wider range of adverse reproductive outcomes (Rodgers, Udesky, Rudel, & Brody, 2018). Evidently, estrogens alone are “necessary but insufficient” players in breast cancers and a number of modifying and contributory factors must also be considered (Cohn, 2011).

The first indication that exogenous estrogens could contribute to breast cancer risk came from the iatrogenic exposures of countless expectant mothers to diethylstilbestrol (DES) between 1940s and 1971 (in the U.S.). The DES cases represent a tragic probe into the effects induced by a potent synthetic nonsteroidal estrogen in human populations. Mothers exposed to one dose or more of DES
during pregnancy experience a long-term increased risk of breast cancer (Titus-Ernstoff et al., 2001); their offspring, both daughters and sons, exposed in utero are more likely to be diagnosed with a range of uterine and vaginal malformations; ovarian, uterine, breast and testicular cancers as well as other benign tumors in these tissues compared to unexposed individuals (Al Jishi & Sergi, 2017). Animal experiments have supported the epidemiological data pertaining to DES (Kawaguchi et al., 2009; Newbold, Padilla-Banks, & Jefferson, 2006). While clinicians have learned that “more estrogens” during the estrogen-rich time of pregnancy contradicts the desired outcome, the effects of environmental estrogens have largely been unexplored in females exposed during adulthood (including during pregnancy) because of assumptions that adults would be less sensitive to hormone disruption.

Common environmental xenoestrogens linked to mammary cancer in rodents or humans include the well-studied plasticizer bisphenol A (BPA) (Keshavarz-Maleki et al., 2021; Vom Saal & Vandenberg, 2021), organochlorine pesticides such as DDT, non-organochlorine pesticides such as atrazine, industrial persistent pollutants such as polychlorinated biphenyls (PCBs), and dominantly air-bound contaminants such as dioxins and polycyclic aromatic hydrocarbons (PAHs) (Gore et al., 2015). The group of environmental chemicals associated with breast cancer is diverse in molecular structure and most lack the typical 4-ring configuration associated with endogenous steroid receptor ligands. Yet all of them interfere with estrogen action either via binding to or by activating estrogen receptors through genomic or non-genomic pathways, or they alter the synthesis of endogenous estrogens; several of these chemicals also induce epigenetic modifications in the cells that produce estrogens or respond to them (La Merrill et al., 2020).
Prolonged exposure to estradiol (E2) initiates tumorigenesis in human breast epithelial cells via structural and genomic changes: the exposed epithelium no longer differentiates or forms duct-like structures. Rather, it aggregates into spherical masses and proliferates rapidly (J. Russo & Russo, 2006). These changes are accompanied by genomic transformations including both gains in 1p and 5q and deletions in 3p, 8p, and 18q. Formation of reactive estrogen metabolites are another path towards cancer initiation. Endogenous estrogens transform into catechol estrogens, and these are oxidized into quinones. Upon reaction with DNA, the quinones form depurinating adducts capable of initiating cancer because DNA with apurinic sites is prone to errors in repairs, and thus likely to mutate and eventually, form cancers (Cavalieri & Rogan, 2016). The formation of DNA adducts has been previously shown as a tumorigenic effect of DES (Saeed, Rogan, & Cavalieri, 2009).

There is additional evidence that estrogens are involved in the initiation of breast tumors. An imbalance in estrogen metabolism, largely due to dysregulation of CYP enzymes, has been demonstrated in tumors of ERKO mice (Devanesan et al., 2001) and breast tissue of carcinoma patients (Rogan et al., 2003). Furthermore, endogenous estrogens and other ER agonists may induce direct DNA damage via interaction with chromatin and the formation of R loops, the three-stranded hybrid DNA-RNA structures formed by cellular RNAs that are normally removed in a timely manner and carefully regulated (Majhi et al., 2020; Niehrs & Luke, 2020; Stork et al., 2016).

A number of indirect mechanisms, sometimes referred to as epigenetic or non-genomic effects, enhance susceptibility to carcinogenesis and enable tumor progression. In a truly epigenetic manner, exposure of epithelial cells to E2 or BPA induced hypermethylation of several genes involved in branching processes and
enhanced their invasion and migration capacity (S. V. Fernandez & Russo, 2010). BPA was also demonstrated to increase the number of proliferating cells and stimulate abnormal cell proliferation, and produce pre-neoplastic lesions in the mammary gland (Bosquiazzo, Varayoud, Munoz-de-Toro, Luque, & Ramos, 2010; Murray, Maffini, Ucci, Sonnenschein, & Soto, 2007). Further, endogenous estrogens and other ER agonists interfere with apoptosis in breast cancer cells (Bailey, Shin, Westerling, Liu, & Brown, 2012; J. Wang et al., 2014). Based on the role of the mammary stromal components discussed above, these components are likely one of the indirect mechanisms that permit cancer progression. Experimental therapies that suppress aromatase in the adipose tissue have been used in obese women based on a theory that estrogens can induce adipokines in the adipose tissue, leading to increased risk of breast cancer (O'Flanagan, Bowers, & Hursting, 2015; Subbaramaiah et al., 2011).

Much work still remains to be done to understand how xeno/estrogens contribute to susceptibility to breast cancer. What is clear from recent work by breast cancer advocates is that the current methods that have been used to evaluate environmental chemicals for their contributions to breast cancer risk are insufficient, and even when there is evidence that xenoestrogens disrupt mammary gland health, these findings are often ignored by regulatory agencies (Cardona & Rudel, 2020).
CHAPTER 8
EXPOSURE TO OXYBENZONE DURING PREGNANCY AND LACTATION
ALTERS TUMOR CHARACTERISTICS IN A TP53-/‐ MURINE MODEL OF BREAST CANCER

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8.1 Abstract

Background: Oxybenzone is a UV filter commonly used in sunscreens, and countless consumer and industrial products. Prior studies indicate that oxybenzone is an estrogen receptor (ER) agonist, and it can induce ER-dependent DNA damage. In vivo studies demonstrate that exposures during sensitive periods of development increase proliferation and expression of ERα. Based on these findings, we hypothesized that oxybenzone would increase the susceptibility of the mammary gland to develop cancer in a genetically susceptible mouse model, either by decreasing tumor latency or increasing tumor severity.

Methods: We transplanted Tp53‐/‐ epithelial cells into cleared mammary fat pads of BALB/c mice, and exposed the females to either 0 (control) or 3000 µg/kg/day oxybenzone throughout pregnancy and lactation. We surveilled these mice until they reached 52 weeks of age to investigate tumor latency. Follow‐up analyses examined tumor characteristics in the Tp53‐/‐ tissues, and mammary gland characteristics in wild type tissues.

Results: Exposure to oxybenzone during pregnancy and lactation modestly decreased the latency of mammary neoplasia in a Tp53‐/‐ model. In the tumors, oxybenzone exposure enhanced tumor vascularization and macrophage infiltration.
Evaluation of the morphology of the wildtype (non-transplanted) glands confirmed that exposures to oxybenzone during pregnancy and lactation produce permanent alterations in the morphology of the mammary gland.

**Conclusions:** Exposures to oxybenzone during pregnancy and lactation may pose an additional challenge to mammary gland health in genetically vulnerable individuals. Mutations in the tumor suppressor gene *Tp53* in the mammary epithelium combined with exposures to oxybenzone, an endocrine disrupting chemical, elicit modest but significant changes in tumorigenesis outcomes that may be consistent with worse clinical prognosis.

**Keywords:** 2-hydroxy-4methoxybenzophenone, endocrine disruptor; sunscreen; xenoestrogen; parity

**Figure 8.1:** Study Design.
8.2 Introduction

Pregnancy and breastfeeding provide a unique set of protections to the mother including a decreased incidence of metabolic syndrome and cardiovascular diseases as well as a much-discussed protection against breast cancer (Gunderson et al., 2010). Initially observed in large human datasets in 1970 and followed up with epidemiological cohort studies, early pregnancy and multiple parity reduce later-in-life risk of breast cancer (MacMahon et al., 1970). Yet even though pregnancy provides significant protection during the post-menopausal period, the risk of breast cancer in parous women peaks six years after giving birth (Albrektsen et al., 2005). According to a recent meta-analysis, childbirth increases the risk for breast cancer for 20 years postpartum, regardless of the length of lactation (Nichols et al., 2019).

Many studies evaluating the molecular mechanisms involved in breast cancer have revealed the role of germline mutations, somatic mutations and/or epigenetic alterations in three central groups of genes: proto-oncogenes [e.g., HER2 (Krishnamurti & Silverman, 2014)], DNA repair genes [e.g., BRCA 1 and BRCA 2 (Winter et al., 2016)], and tumor suppressor genes [e.g., the classic Rb, colorectal cancer associated APC, or the master regulator Tp53 (Sherr, 2004)]. As investigation of these genes continues to progress, understanding of their specializations has revealed that their actions can be pleiotropic, e.g., the products of Tp53 or BRCA can both suppress malignant growth of tumor cells and repair damaged DNA (Boutelle & Attardi, 2021; Matsuda et al., 2020).

It is also becoming more widely appreciated that development of breast cancer is a multiple step process that is more complicated than an event involving a single gene mutation. For example, among BRCA1 and BRCA2 mutation carriers,
46% and 52% of women respectively will develop breast cancer at some point in their lives, indicating that about half of women with these mutations will not develop the disease (Rebbeck et al., 2015). The prevalence of *BRCA1* and *BRCA2* mutations in the U.S. population is estimated as occurring in 1:300 to 1:500 women (Moyer & Force, 2014), leaving approximately 99% of all breast cancer diagnoses unexplained by these specific prototypical inherited mutations (Tung et al., 2016). Twin studies conducted in Scandinavian countries found that 27% of breast cancers can be attributed to inherited causes (Lichtenstein et al., 2000), providing additional evidence that most cases of the disease occur due to accumulated mutations in moderate and low penetrance genes, epigenetic alterations, and other environmental risk factors. Furthermore, women in two different cohorts were investigated for frequency of germline or somatic mutations in *BRCA* and other high penetrance genes; in both cohorts, those mutations were estimated to be responsible for about 10% of breast cancer cases (Cybulski et al., 2019; Tung et al., 2016). The relatively modest role of mutations in prototypical cancer genes contributes to a renewed interest to identify environmental determinants that can either promote breast cancer outright or interact with genetic susceptibility factors to increase cancer risk indirectly.

Commonly recognized environmental factors involved in breast cancer etiology include obesity, lack of physical activity, and use of tobacco or alcohol (Hiatt & Brody, 2018). Other environmental exposures associated with increased risk of breast cancer include nitrogen oxides from air pollution, industrial chemicals such as congeners of polychlorinated biphenyls (PCBs), and legacy pesticides like dichlorodiphenyltrichloroethane (DDT) and its metabolites (Andersen et al., 2017; Cohn et al., 2015; Cohn, Terry, Plumb, & Cirillo, 2012). Further, both endogenous
and exogenous estrogens are associated with an increased risk for breast cancer as seen in women who enter puberty earlier and menopause later (Soto et al., 2008), as well as in women administered some hormone therapies at menopause (J. E. Manson et al., 2013). The tragic story of increased breast cancer risk observed in women exposed to pharmaceutical estrogens such as diethylstilbestrol (DES) during organizational periods of mammary gland development similarly highlights the sensitivity of the breast to exogenous hormonally active agents (Palmer et al., 2006; Titus-Ernstoff et al., 2001).

Studies of women exposed to DES led scientists to hypothesize that other endocrine disrupting chemicals, including chemicals found in everyday consumer products, contribute to the mixture of quotidian estrogenic exposures and thus may increase risk for breast cancer as well (Hiatt & Brody, 2018; Macon & Fenton, 2013; Schwarzman et al., 2015; Soto & Sonnenschein, 2010). PCBs, DDT, and DDT metabolites, all chemicals associated with increased breast cancer risk, are hormonally active agents, and their endocrine disrupting properties are suspected to be responsible for their actions (Gore et al., 2015; Rodgers et al., 2018; Rudel, Fenton, Ackerman, Euling, & Makris, 2011). Importantly, several xenoestrogens have been described as “epigenetic carcinogens” because of their ability to promote uncontrolled growth of breast cells via estrogen receptor (ER) mediated processes and formation of estrogen-DNA adducts (Cavalieri & Rogan, 2016).

Oxybenzone (benzophenone 3) is an ultraviolet filter used commonly in personal care products, fabrics, resins, and plastics. Because of its widespread use, it has been documented to pollute a number of environmental matrices, and it has been repeatedly measured in human biological samples including urine, blood, semen, amniotic fluid, and breast milk (Hines et al., 2015; Krause, Frederiksen,
Oxybenzone is an ER agonist and androgen receptor antagonist, and its metabolites, namely benzophenone 1 and 2,4,4’ trihydroxybenzophenone, are also ER agonists, making its effects on the mammary gland important to evaluate (K. Matouskova, Vandenberg, L.N., 2022). In our previous study, we found that mice exposed to oxybenzone during pregnancy and lactation had significantly higher proliferation rates in their post-involution mammary epithelium, even several weeks after exposures had ceased (LaPlante et al., 2018). We also found that oxybenzone induced ER-dependent DNA damage in the mammary glands of exposed mice, as well as in T47D breast cancer cells (Majhi et al., 2020). Collectively, these results led us to hypothesize that exposures to oxybenzone would alter the risk of mammary cancer in a mouse model with an unfavorable genetic background, e.g., mice with mammary epithelium lacking Tp53.

Here, we transplanted BALB/c mice with Tp53−/− epithelial cells, and then exposed these females to 0 or 3000 µg/kg/ body weight oxybenzone during pregnancy and lactation. The mice were observed for tumor development until the females reached 52 weeks of age. Once tumors emerged, they were investigated for clinically relevant characteristics. We found that oxybenzone modestly shifted the latency to develop tumors and altered some but not all aspects of tumor biology (e.g., necrosis and macrophage infiltration). Oxybenzone also permanently altered the morphology of the BALB/c wildtype (non-transplanted) mammary epithelium.
8.3 Materials and Methods

Animal Model

BALB/c female mice were raised in the UMass colony and housed under standard conditions, e.g., in polysulfone cages with sanitary bedding. Chow (ProLab IsoDiet, Brentwood, MO) and tap water (in polysulfone bottles with rubber stoppers and metal tops) were provided *ad libitum*. Animals were maintained in temperature- and light-controlled conditions (12-hour cycles of light and dark) throughout the study. All experimental procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

At 3 weeks of age, female mice were anesthetized and epithelium from the left and right 4th mammary glands was removed, and the cleared fat pads were injected with mammary epithelial tissue fragments previously collected from animals lacking Tp53 [see FIGURE 8.1] (Yan et al., 2010). The genotype of the transplanted cells was verified with PCR.

At 6 weeks of age, each female was randomly assigned to a treatment group using statistical software to distribute them evenly according to their body weight; 22 females were assigned as controls (CTRL) and 20 female mice formed the oxybenzone-treated group (OXY). The females were then paired with untreated breeder males. The control females were orally administered tocopherol stripped corn oil and the oxybenzone group was exposed to 3000 μg/kg body weight/day (dissolved in tocopherol stripped corn oil) via pipette feeding throughout pregnancy and 21 days of lactation (approximately 42 consecutive days).
Tumor Surveillance

After weaning at 21 days post-partum (at approximately 12 weeks of age), female mice were palpated for mammary tumors and their general condition was examined. This occurred every two weeks for the first 24 weeks of surveillance, and then every week from 24 weeks post-transplant until tumors were detected, or 52 weeks of age. When tumors were detected, they were closely surveilled and surgically removed before they reached a diameter of 1 cm in any dimension. The animals continued in the experiment if tumors were observed unilaterally. Tumor regrowth or identification of bilateral tumors required the females to be euthanized.

Euthanasia and Tissue Collection

Animals were euthanized with CO₂ inhalation followed by cervical dislocation to ensure death and tumor location was recorded. For each animal, remaining mammary tissue from the 4th inguinal gland was collected along with the tumor, as well as the 3rd pectoral pair of mammary glands, which were comprised of wildtype, non-transplanted epithelium.

Pectoral and remnants of inguinal mammary glands were spread on glass slides (Thermo Fisher Scientific, Waltham, MA), and fixed overnight in 10% neutral-buffered formalin (Thermo Fisher Scientific, Waltham, MA) for whole mount analysis. Tumor tissue collected for histopathological analysis was fixed overnight in 10% neutral buffered formalin before being washed, dehydrated, and embedded in paraffin.
Whole mounted mammary gland processing and morphometric evaluation

Whole mounted mammary glands were processed through a graded series of alcohols followed by two washes of toluene to remove fat. They were then rehydrated through a series of alcohols and stained overnight with carmine. Finally, the samples were dehydrated through a series of alcohols and xylene and preserved in individual k-pax heat-sealed pouches (Fisher Scientific) containing methyl salicylate (Acros Organics, Morris Plains, NJ).

Whole mounted mammary glands were imaged with a Zeiss AxioImager dissection microscope (Carl Zeiss Microscopy, Jena, Germany) and a Zeiss high-resolution color camera, to verify a successful outgrowth of the $Tp53^{-/-}$ transplants. All outgrowths were examined for possible tumors and other gross abnormalities. When observed, aberrant tissues were excised from the whole mounted gland, embedded in paraffin, and sectioned for histopathological analysis.

For analysis of mammary gland morphology in the unmodified gland, whole-mounted pectoral mammary glands were imaged at 30x magnification, and the optical density was measured in both glands using ZEN software (Carl Zeiss Microscopy); optical density is inversely correlated with the amount of mammary epithelium present. Whole-mounted glands were also evaluated for the presence of intraductal hyperplasias [observed as ducts with a beaded appearance (Vandenberg et al., 2008a)] and hyperplastic alveolar buds. Each gland was assigned an “abnormality” score based on the presence and extent of these abnormalities in the unmodified glands.
**Tissue embedding, sectioning and histological staining.**

Fixed mammary glands were washed in 1x phosphate buffered saline, dehydrated through a series of alcohols and embedded with paraffin (Leica Biosystems, Richmond, IL) under vacuum. Paraffin blocks were cut into five micrometer sections on a rotary microtome (Fisher Scientific) and mounted on positively charged slides (Fisher Scientific) for histological and immunohistochemistry evaluation.

For histological evaluations, slides were deparaffinized with xylene and a series of alcohols, stained with Harris' hematoxylin and eosin (H&E) (Fisher Scientific) or toluidine blue, then dehydrated, and mounted with permanent mounting media (Fisher Scientific). Digital images were collected using a Zeiss Axio Observer.Z1 inverted microscope, a 40x objective, and a high-resolution color camera (Carl Zeiss Microscopy).

**Histopathological Analyses**

**Tumor type and Grade**

H&E-stained tumor sections were evaluated by a trained pathologist. The tumor type and grade were determined according to the consensus criteria from the Annapolis expert pathologists meeting (Cardiff et al., 2000). In brief, tumors were classified as carcinoma, carcinosarcoma, or sarcoma based on their tissue origins. Morphological descriptors (e.g., acinar, cribriform, papillary, solid, and squamous) were used when appropriate. While both carcinoma and adenocarcinoma represent a type of neoplasm originating from epithelium and glandular epithelium with proven malignant behavior, adenocarcinoma contains both glandular and squamous types of differentiation. Tumors described as carcinosarcoma are comprised of two tissue
type components, epithelial and mesenchymal, with malignant behavior. Lastly, some lesions were described as *mammary intraepithelial neoplasia* (MIN), e.g., lesions with intraluminal epithelial proliferation with cytologic atypia including carcinoma *in situ*.

Tumor grade was assigned based upon histological and cytological criteria, mitotic rate and necrosis. Low-grade carcinomas are characterized by the presence of well-organized glandular patterns without significant pleomorphism, such as acinar carcinoma, which is composed of small clusters of tumor cells forming glands with small lumens. High-grade carcinomas have less differentiation, more solid growth, more nuclear pleomorphism and a higher mitotic rate. Solid carcinomas, adenocarcinomas, and carcinosarcomas were scored as grade 1, 2, 3, or 4. MIN were graded as “low” or “high” grade and for the purpose of statistical analyses were attributed grade 1 or 4, respectively.

**Necrosis and Angiogenesis**

H&E-stained tumor sections were qualitatively assessed for the presence and degree of necrosis by two independent investigators. Each sample was graded as “no necrosis”, “low degree”, “medium”, or “high degree” of necrosis. The degree of angiogenesis was similarly scored in H&E-stained tumor sections by two independent investigators. Because all samples had at least some blood vessels present, scoring categories included “low degree”, “medium”, and “high degree” of angiogenesis. For both measures, when samples were heterogeneous for measures of necrosis or angiogenesis, the highest degree was selected as indicative for the sample.
Lymphocyte presence in the tumor and peri-tumor stroma

H&E-stained tumor sections and stroma surrounding the tumor were qualitatively assessed for the presence and degree of tumor-infiltrating lymphocytes (TILs) (Salgado et al., 2015). Within the tumor, TILs were scored as “absent”, “low numbered”, “moderate numbered” or “high numbered”. In the stromal regions surrounding the tumor, the presence of lymphocytes in the stroma (sTILs) were scored as “low”, “moderate” or “high”. Scoring was performed by two independent observers; if no consensus could be reached a third observer was consulted.

Quantification of mast cells

To quantify the distribution of mast cells in and around tumors, the total number of mast cells were counted within the tumor and in the stroma surrounding the tumor in Toluidine blue-stained sections. Mast cell distribution in the tumor and stroma was reported as number of mast cells per area.

Immunohistochemistry

Immunohistochemical analyses of four markers were evaluated using standard methods and commercial antibodies including rabbit anti-ERα (EMD Millipore, Cat# 06-935, Temecula, CA); rabbit anti-Ki67 (Fisher Scientific, Cat# RM-9106-S1), a marker of proliferation; rabbit anti-progesterone receptor (PR; Abcam, Cat# ab131486, Cambridge, MA); and rabbit anti-F4/80 (Cell Signaling Technology, Cat#70076), a cell surface antigen expressed on macrophages. Briefly, sections were deparaffinized, hydrated through a series of alcohols, microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval, and treated with hydrogen peroxide to quench endogenous peroxidases. Non-specific binding was blocked with 1% milk protein in 5% normal goat serum (Cell Signaling Technology, Danvers, MA).

Sections were incubated with primary antibodies (typically diluted 1:500 or
1:1000 in blocking solution) at 4°C for 14-16 h. They were then washed and incubated with secondary antibody (goat anti-rabbit, Abcam, Cat# ab64256) followed by streptavidin peroxidase complex (Abcam, Cat# ab64269). Diaminobenzidine chromogen (Abcam, Cat# ab64238) was used to visualize reactions. Sections were counterstained with hematoxylin (Fisher Scientific). Each immunohistochemical run included a negative control in which the primary antibody was replaced with 5% normal goat serum.

Expression of ERα and PR were evaluated according to guidelines by the American Society of Clinical Oncology (Allison et al., 2020). In brief, samples with less than 1% of ERα- or PR-positive cells were interpreted as ER or PR negative while samples with >1% tumor nuclei expressing the respective hormone receptor were interpreted as positive. Note that all positive samples met the criteria for “Low Positive” because none expressed ER or PR in greater than 10% of nuclei.

We used a nuclear proliferation marker, Ki67, to quantify the fraction of tumor cells in mitosis. Ki67 tags cells in various stages of mitosis except for G0, therefore its expression identifies active proliferation. Samples were viewed at 20x magnification and representative images were taken by the ZEN imaging software using a Zeiss AxioObserver.Z1 inverted microscope and a high-resolution color camera (Carl Zeiss Microscopy). Both positive and negative cells were counted, and the percent of positive cells was calculated for each tumor.

**Analysis of macrophages using TissueQuant**

To quantify F4/80 positivity in the tumor samples, representative images were processed using ImageJ/FIJI and TissueQuant software. TissueQuant compares different color shades within an image to a DAB-stained reference color and calculates a color score based on the normalized hue, saturation, and color intensity of each pixel.
Before batch processing all images in TissueQuant, the DAB stain was isolated using predetermined regions of interest with the Colour Deconvolution ImageJ/FIJ1 plugin to minimize nonspecific background staining. These DAB-filtered images were then used to calculate the overall concentration of positive staining and density of positively stained areas using TissueQuant.

Statistical analysis

Prior to conducting statistical analyses, we evaluated whether the mouse should be treated as the statistical unit, or the mammary gland (considering each mouse was given two distinct Tp53-/- epithelial transplants). In other words, we considered the issue of the intra-correlation among mammary glands from the same animal and the associated increase in probability of type I error of our statistical tests of group differences. We recognize that appropriately defined mixed model methods for correlated data would have ensured that our hypothesis tests were based on the correct “effective” sample size (i.e. less than 72), provided all model assumptions are met. Our rationale in performing the simpler analyses (i.e. analyses that ignored the correlation structure) was to balance the increased probability of type I error with the associated limitations of fitting complex mixed models (e.g., modeling the correlations structure in data that is unbalanced) in modest data settings. Therefore, in ancillary analyses, we estimated the correlations in tumor characteristics between left and right mammary tumors in the same animal. None were statistically significant and data visualizations revealed no patterns of association (data not shown). Therefore, we are confident that where statistically significant group differences are reported, they are unlikely to be spurious due to type I error.
Next, prior to analyses, we applied selected exclusions: A) n=9 transplanted mammary glands showed no epithelial outgrowth. B) n=3 mammary glands could not be collected due to technical problems. And C) n=7 mammary glands were excluded because transplanted tissues exhibited ductal epithelia free from any observable tumor growth at 30x magnification (using the Zeiss AxioImager dissection microscope). These seven samples were excluded from all analyses. Together, these exclusions yielded available data on 42 mice representing 72 correlated observations in the mammary tissues.

We used GraphPad (Prism version 9.1.1) and R (2021) in all statistical analyses. Our analyses utilized the mammary as the unit of analysis and assumed independence. Group differences in tumor latency were assessed using Kaplan-Meier estimation of time to tumor and the log rank test. Variations in discrete variable measures of tumor characteristics (type, grade, level of angiogenesis, level of necrosis) across groups were assessed using the Fisher Exact test. Variations in continuous variable measures of tumor characteristics (macrophage infiltration score, LIST) were assessed using the Student t-test or the Wilcoxon Rank Sum Test as appropriate, depending on the validity of the assumption of normality. We report achieved levels of significance and did not adjust for multiple comparisons.

For all evaluations, a p-value of less than 0.05 was considered statistically significant. All continuous data are graphed as mean ± standard error. Categorical data are graphed as percentages of each treatment group assigned to each category. All analyses were conducted by experimenters blinded to treatment group.
8.4 Results

8.4.1 Exposure to Oxybenzone during Pregnancy and Lactation has a Modest Effect on Tumor Latency in a Tp53−/− mouse model

Tumor latency data were collected throughout 42 weeks of surveillance and evaluated using survival analysis. As was anticipated for this tumorigenic model (Jerry et al., 2000), 38 of 42 mice developed a tumor on at least one side; a total of 65 tumors were observed in 84 mammary glands. When evaluating each mammary gland separately, a Kaplan-Meier survival curve indicates that the latency to develop a tumor was shifted to the left in the oxybenzone-treated group compared to the control group, although the median time to develop tumors in all mice was not significantly different between treatment groups (Kaplan-Meier, Chi Square, 1 degree of freedom, p = 0.5, FIGURE 8.2A). When only mammary glands that developed tumors were included in the analyses, the median time-to-tumor detection was longer in controls (294 days) compared to the oxybenzone-treated group (265 days, Shapiro-Wilk normality test, p<0.001; Kruskal Wallis rank sum test, p=0.079, FIGURE 8.2B). These results suggest that in the genetically susceptible Tp53−/− mouse model, oxybenzone exposure modestly accelerates mammary tumor development.
8.4.2 Oxybenzone exposure alters the extent of tumor vascularization, but not tumor type, tumor grade, or necrosis score

Tumors from both control and oxybenzone-treated females were characterized as solid carcinomas, adenocarcinomas, carcinosarcomas, MIN in situ, or sarcoma/angiosarcomas. There were no statistically significant effects on the type of tumors observed in the two treatment groups (Chi Square = 3.85, p=0.43; Fisher’s exact test, p=0.43, FIGURES 8.3A,B). There was also no effect of treatment on the mammary tumor grade (Chi Square = 3.94, p=0.27; Fisher’s exact test, p=0.35, FIGURES 8.3C,D). Most tumors were considered high grade. Collectively, these data support the conclusion that there was no effect of oxybenzone exposure during pregnancy and lactation on tumor type or tumor grade in the Tp53+/- model.
We next evaluated vascularization and necrotic foci, a morphological marker of deficient vascularization and subsequent tissue hypoxia. In both treatment groups, the majority of the tumors exhibited some degree of necrosis and quite naturally, all tumors were vascularized. Tumors from oxybenzone-exposed females were more likely to be given a score of highly vascularized compared to tumors from control females (Chi-square = 6.778, p value=0.03; Fisher’s exact test, p =0.04, FIGURES 8.4A,B). More than half of the tumors from control animals had no or low necrosis (scores of 0 or 1, 54.3%) whereas no or low necrosis was only observed in 26.7% of tumors from oxybenzone-treated animals. However, there was no statistically

Figure 8.3: Tumor Type & Grade.
A) Representative images of four tumor types. B) Tumor type distribution among control vs oxybenzone-treated mammary glands. C) Representative images of the tumor grades. D) Tumor grade distribution among control vs oxybenzone-treated mammary glands.
significant effect of oxybenzone on necrosis scores (Chi-square = 6.59, p value = 0.16, FIGURES 8,4C,D).

**Figure 8.4: Tumor severity.** A) Angiogenesis in tumor tissue (Chi-square = 6.778, p value=0.03; Fisher’s exact test, p =0.04). B) Representative image of tumor vascularity in the tumor (arrows pointing at blood vessels). C) Necrosis in tumor tissue. D) Representative image of necrotic tissue (the center of necrosis encircled) E) Proliferation in the tumor. F) Ki67-positive cells in the tumor tissue (arrows).
8.4.3 Oxybenzone exposure during pregnancy and lactation does not alter tumor cell proliferation in a Tp53 -/- mouse model

We used Ki67, a marker of proliferation that typically correlates well with tumor grade in solid neoplasia (Weidner, Moore, & Vartanian, 1994), to further characterize the tumor samples. There was no effect of oxybenzone treatment on the fraction of tumor cells that were positive for Ki67 (Two sample t-test, p value = 0.57; FIGURES 8.4E,F). As expected, and there was a strong correlation between the Ki67 index and the histological tumor grade across the treatment groups (data not shown).

8.4.4 Oxybenzone treatment is associated with a lower macrophage infiltration score in tumors, but no effect on mast cells or tumor-infiltrating lymphocytes

Tumor-associated macrophages are believed to play a role in suppressing anti-tumor immune responses and the presence of macrophages in tumors is associated with tumor promotion via tumor cell invasion, angiogenesis and metastasis (Qian & Pollard, 2010; Su et al., 2014). Therefore, their presence in the tumor microenvironment is considered to be a marker of poor prognosis in human breast cancer (Allavena, Sica, Solinas, Porta, & Mantovani, 2008). After evaluating the F4/80-stained tumor tissues, we found that oxybenzone exposure significantly decreased macrophage tumor infiltration compared to parous controls (Two-sample t-test, p value = 0.0001, FIGURES 8.5A,B).

Mast cells also play an important role in the tumor immune microenvironment, and are associated with angiogenesis, tumor cell proliferation and pro-growth signaling compounds in experimental models (Davio et al., 1994; Ranieri et al., 2009). Moreover, mast cells express ER and therefore respond to ER agonists; they
also associate with proliferating terminal end buds and promote ductal elongation in the mammary gland (Jing et al., 2012). The role of mast cells in breast cancer, however, remains unresolved (Aponte-Lopez, Fuentes-Panana, Cortes-Munoz, & Munoz-Cruz, 2018). We observed no effect of oxybenzone on the number of mast cells either in the tumor (mean 1.92 per mm\(^2\) in controls versus 1.25 per mm\(^2\) in oxybenzone-exposed females; Two Sample t-test, p=0.51) or in the stroma surrounding the tumor (mean 7.85 per mm\(^2\) in controls versus 9.72 per mm\(^2\) in oxybenzone-treated females; Two sample t-test, p = 0.34, see FIGURES 8.5C,D,E).

Likewise, the role of cell-mediated cytotoxic T-lymphocytes in the breast tumor remains equivocal in regard to tumor prognosis; its prognostic value greatly depends on tumor type and phenotype (Inwald et al., 2015; Stanton & Disis, 2016).
We observed no effect of oxybenzone treatment on the score for either TILs (Chi-Square = 0.44, p=0.93; Fisher’s exact test, p=0.92) or sTILs (Chi-Square = 1.34, p=0.51; Fisher’s exact test, p=0.59, FIGURES 8.5F,G).

8.4.5 Oxybenzone induces a non-significant decrease in ER expression in mammary tumors in the Tp53−/− mouse model

ER and PR expression in mammary tumors is highly relevant in the clinical approach to treatment because only patients with sufficient ER expression in breast tumor cells will benefit from common endocrine therapies like tamoxifen (Davies et al., 2011). When ERα expression status was evaluated, almost half as many oxybenzone-exposed tumors were considered ER-positive compared to controls (26.1% and 46.7%, respectively, FIGURE 8.6A) although this difference was not statistically significant (Chi-Square = 1.55, p=0.21; Fisher’s exact test, p = 0.16). The fraction of PR-positive tumors was not affected by oxybenzone treatment (Chi-Square = 0, p=1; Fisher’s exact test, p =1). Low ER and PR positivity across

![Graph A: Expression of Receptors. A) Estrogen receptor α expression in the tumor tissue; positive vs negative. B) Progesterone receptor expression in the tumor tissue; positive vs negative.](image)

Figure 8.6: Expression of Receptors. A) Estrogen receptor α expression in the tumor tissue; positive vs negative. B) Progesterone receptor expression in the tumor tissue; positive vs negative.
treatment groups was not surprising because the *Tp53* null mouse model commonly produces triple negative mammary tumors (Yan et al., 2010).

8.4.6 Oxybenzone exposure during pregnancy and lactation induces permanent changes in mammary gland morphology

Our prior studies of oxybenzone revealed that exposures during pregnancy and lactation alter mammary gland morphology five weeks after exposures have ceased, once involution is completed (LaPlante et al., 2018). To evaluate the long-term effects of exposure to oxybenzone on mammary gland morphology in parous females, ductal density was evaluated in the wildtype pectoral glands. Optical density, a measure that is inversely correlated with ductal complexity, was significantly lower in glands collected from controls compared to oxybenzone-treated

![Figure 8.7: Pectoral mammary gland (wild type) analyses. A) Optical density of the mammary epithelium (unpaired t-test, p = 0.0026). B) Abnormality score for hyperplasia, alveolar or beaded ducts in the mammary epithelium. C) Mammary epithelium abnormalities during involution: beaded ducts – white arrow tips, alveolar end buds – black arrows (magnification 47x).](image)
mice (unpaired t-test, p = 0.0026, FIGURE 8.7A). These effects are consistent with a permanent disruption to the morphology of the post-involution mammary gland.

Finally, whole-mounted wildtype pectoral glands were evaluated for the presence of two gross abnormalities: hyperplastic alveolar structures (FIGURE 8.7B) and intraductal hyperplasias, which have an appearance of beaded ducts in the whole-mount (FIGURE 8.7C). When accounting for the severity of abnormal structures in the pectoral glands, there was no difference based on oxybenzone treatment (Unpaired T-test, p=0.09, FIGURE 8.7B).

8.5 Discussion

Genetic susceptibility to breast cancer is a well-established concept wherein the germline or somatically mutated genes have been identified as having high, moderate, or low penetrance. The high-risk genes include BRCA1, responsible for about 45% familial breast neoplasia (Easton, Bishop, Ford, & Crockford, 1993), BRCA2, TP53, and several rare germline mutations in genes encoding various enzymes (Evans, 2016). TP53 is altered in over 50% of breast cancers, making it the gene most closely related to cancer initiation and promotion (Ozaki & Nakagawara, 2011), and BALB/c mice with transplanted Tp53−/− mammary epithelium are highly susceptible to tumor formation (Jerry et al., 2000).

In the real-life setting, genetic risk factors are naturally combined with risks due to exogenous agents. Studies examining genetic and environmental interactions remain relatively rare, but there is some evidence that genetically susceptible animals and humans – either individuals or populations - are more sensitive to tumorigenic environmental agents. For example, BRCA1 mutant mice displayed a
hypersensitivity to the estrogenic chemical bisphenol A, with enhanced proliferation in mammary epithelial cells and more severe epithelial hyperplasias observed in the mammary ductal epithelium after exposures (L. P. Jones et al., 2010). Further, women with genetic breast cancer risk are met with a doubling of their risk of developing the disease if they have detectable levels of polycyclic aromatic hydrocarbons in their plasma compared to women without such genetic predisposition but similar exposures (Shen et al., 2017).

There are now several experimental rodent models that are genetically susceptible to breast cancer that can be used to investigate the effects of environmental pollutants. We combined the genetically susceptible Tp53 -/- mouse model with exposure to a ubiquitous environmental contaminant, oxybenzone, a chemical which we previously reported alters proliferation in the mammary gland (LaPlante et al., 2018), and induces both DNA double strand breaks and R-loop formation (Majhi et al., 2020). We found that exposure to oxybenzone during pregnancy and lactation had modest effects on tumor latency in this tumor-prone model. At approximately 7-8 months (220 days) of age, the survival curves for the parous controls and the oxybenzone-exposed females diverged, revealing a shorter latency to develop tumors in the treated group (FIGURE 8.2). The mammary gland is especially sensitive to environmental chemicals during life periods marked with profound structural and functional changes, i.e., in utero development, puberty, and/or pregnancy/lactation (Macon & Fenton, 2013). Alterations that occur during these vulnerable life stages can lead to pathologies that manifest later in life (Heindel & Vandenberg, 2015; Terry, 2020). Although parity is thought to provide protection against mammary cancer in the long term, only a small number of rodent studies have evaluated the impact of environmental agents on the gland during pregnancy.
on that protection (LaPlante et al., 2018; LaPlante, Catanese, Bansal, & Vandenberg, 2017; Vorderstrasse, Fenton, Bohn, Cundiff, & Lawrence, 2004).

Post-partum remodeling of the mammary gland seems to progress normally in mice with two defective $p53$ alleles (M. Li, Hu, Heermeier, Hennighausen, & Furth, 1996), but prior studies also suggest no protective effect of parity in mice lacking $Tp53$ in the mammary gland (Medina & Kittrell, 2003). Our study design investigated if oxybenzone could provide protection, or conversely enhance susceptibility to mammary cancers in mice following transplantation of $Tp53^{-/-}$ mammary epithelium. Our results were more consistent with the latter possibility. However, there is no evidence that oxybenzone can initiate tumorigenesis in the absence of a genetically susceptible epithelium. In the $Tp53^{+/+}$ mammary tissue (e.g., the non-transplanted pectoral glands), oxybenzone exposure during pregnancy & lactation permanently altered mammary gland morphology (FIGURE 8.7A), but with no effect on the severity of epithelial lesions (alveolar or intraductal hyperplasias; FIGURE 8.7B). Carcinomas were never observed in these tissues.

In humans, the majority of mammary tumors originate in the epithelium – from the lobules in 10-15% of cases, and the ducts connecting the lobules to the nipple in 75% of invasive breast cancers (C. I. Li, Anderson, Daling, & Moe, 2003). To understand whether oxybenzone alters other aspects of tumor biology, we next assessed each tumor for type and grade, guided by the consensus on murine mammary pathology reached among veterinary pathologists during the NIH “Annapolis meeting” (Cardiff et al., 2000). In our evaluation of features of the tumors, we found no effect of oxybenzone on tumor grade or tumor type. Other features associated with tumor severity include tumor necrosis, angiogenesis, and proliferation of the tumor cells. Necrosis worsens the patient’s prognosis due to the
consequences of excessive cell proliferation and limited apoptosis (Richards, Mohammed, Qayyum, Horgan, & McMillan, 2011). Loss of *Tp53* typically disrupts apoptosis or ferroptosis (Donehower, 1996; L. Jiang et al., 2015), producing necrotic loci that are scattered throughout the tumor tissue, which can contribute to a less favorable prognosis. Loss of *Tp53* has also been documented to promote neovascularization (Donehower, 1996); the functional product of *Tp53* inhibits angiogenesis via activating collagen α1 type IV and by releasing its product, collagen-derived antiangiogenic factor, which decreases tumor growth in the mouse model (Assadian et al., 2012). Although there was no effect of oxybenzone on necrosis scores, tumors from oxybenzone-exposed females may have a worse prognosis given the greater incidence of “high degree” of vascularization compared to controls (Figure 8.4A). Future studies should evaluate the effect of oxybenzone on this pathway. Lastly, we evaluated the prevalence of proliferating cells in the tumor because sustained proliferation of tumor cells is one of the established hallmarks of cancer (Hanahan & Weinberg, 2011). Although we previously observed increased epithelial proliferation in post-involuting mammary glands following exposure to oxybenzone (LaPlante et al., 2018), proliferation did not differ between tumors collected from our two treatment groups.

The surrounding extraepithelial environment including the cells of the immune system plays a critical part in tumorigenesis (Chollet-Hinton et al., 2018; J. B. Kim, Stein, & O’Hare, 2005) and in chemical carcinogen-induced breast neoplasia (Maffini et al., 2004). The dual character of macrophages underscores their possible role in either promoting carcinogenesis via involvement in angiogenesis and immunosuppression (C. E. Lewis & Pollard, 2006) or protection against carcinogenesis via removal of damaged cells (Gasser, Orsulic, Brown, & Raulet,
Macrophages are also critical to the normal postpartum remodelling of mammary ducts and repopulation of the gland with adipocytes (O'Brien et al., 2012). Here, we found a decrease in the number of macrophages observed in oxybenzone-treated tumors [FIGURE 8.5A]. Other studies have reported that macrophage infiltration is typically positively correlated with angiogenesis (Schwertfeger et al., 2006) or neovasculogenesis (Bingle, Brown, & Lewis, 2002), and thus the presence of macrophages may be linked to poor prognosis (Yamaguchi, Wyckoff, & Condeelis, 2005). Tumor-associated macrophages are drawn to and accumulate in the zone of tissue hypoxia (C. Lewis & Murdoch, 2005). However, in our samples, the degree of necrosis was not correlated to the macrophage score (Pearson coefficient $r = -0.22$, $R^2 = 0.05$, $p$ value = 0.114, data not shown). Because we have not observed any significant differences in the degree of tumor necrosis between our treatment groups, the varying degree of angiogenesis we observed may occur independently of chemotactic signals produced by hypoxic cells that attract macrophages.

Many environmental pollutants function as xenoestrogens and as such bind primarily to the ERs, but some of these chemicals also affect metabolic and immune responses via binding to the pluripotent aryl hydroxyl receptor (AhR), pregnane X receptor (PXR), or peroxisome proliferation-activated receptor $\alpha$ (PPAR$\alpha$); others act by activating CYP enzymes (Rodgers et al., 2018). While the mechanism of action of oxybenzone in the mammary gland is poorly understood, in vitro studies using yeast and mammalian cell cultures have demonstrated that it is an ER agonist, ER antagonist, and AR antagonist (Watanabe et al., 2015, Kunz and Fent, 2006). Furthermore, oxybenzone and benzophenone 8, one of its metabolites, have been evaluated for binding to PPAR$\gamma$ and are suggested to have an obesogenic potential.
akin to quintessential obesogens like organotins (Heindel & Blumberg, 2019; Shin et al., 2020). Thus, it is plausible that oxybenzone and its metabolites alter hormonal pathways in the mammary gland, potentially promoting carcinogenesis. In human breast cancer interventions, positive expression of receptors is associated with more positive responses to hormonal interventions such as tamoxifen (Weinberg et al., 2005). We observed that oxybenzone decreased the percentage of tumors that were considered ER-positive, although this effect was not statistically significant (FIGURE 8.6A). If similar results were observed in humans, they would be consistent with poorer responses to common clinical treatments.

Rodents have proven particularly helpful in investigations that aim to determine how genetic susceptibility interacts with environmental features including responsiveness to endocrine disrupting chemicals (Schaffer et al., 2013; Shull, Dennison, Chack, & Trentham-Dietz, 2018). For example, genetic linkage studies have revealed 9 quantitative trait loci affecting mammary tumor incidence or latency after exposure to ER agonists, and 20 quantitative trait loci affecting aspects of mammary ductal growth and branching after ER agonist treatments (Jerry et al., 2018). Further, genetically susceptible or resistant rat strains respond differently to exogenous estrogen and progestins (Shull et al., 2018). Such studies in rodents also revealed that mutations in more than 160 genes are responsible for increasing lifetime risk of developing breast tumors, equal to about 6.4% of all invasive breast cancer cases. Several genes (e.g., CHEK2, PALB2, and ATM) are known to pose a moderate risk (relative risk between 2 and 2.37 compared to individuals without mutations in these genes) (Evans, 2016) and a small number of high penetrance genes are associated with a much greater lifetime risk of breast cancer (Campeau, Foulkes, & Tischkowitz, 2008). What is clear is that these genetic features do not
sufficiently explain the risk of breast cancer women are facing today (SEER, 2021b). Our work with oxybenzone advances the hypothesis that environmental chemicals can act on a genetically susceptible background to decrease tumor latency and influence tumor severity. Importantly, in human populations, the manifestation of this disease has increased for women with familial risk, as carriers of high-penetrance genes born before 1940 had the cumulative breast cancer incidence at the age of 50 years nearly three times lower than female carriers of the same high-risk genes born after 1940, when environmental chemical burden increased (King et al., 2003).

All animal models of human diseases have strengths and limitations. Unlike tumor-resistant C57Bl/6 or B6CF1 mice, BALB/c mice were shown to be sensitive to radiation-induced mammary cancer (Ullrich et al., 1996) although wild-type BALB/c mice did not develop any tumors over 80 weeks of observation (Kuperwasser et al., 2000). On the other hand, BALB/c mice with global loss of Tp53 have a median survival of only 15.4 weeks; these mice develop mostly sarcomas and lymphomas, whereas about 50% of Tp53+/- mice develop tumors within 54 weeks of surveillance. Consequently, epithelial cells transplanted from Tp53-/- tumors into a wild-type mammary gland were shown to produce tumors in 75% of the tissues (Kuperwasser et al., 2000), similar to what we observed in our study (73.9% of our tissue developed malignant epithelium-related tumors over 52 weeks of surveillance; see FIGURE 8.2). This is an important limitation of our study, because the high penetrance of tumors with a median latency of 294 days in the control group means that it was statistically challenging to observe a significant change in tumor latency. Future experiments may benefit from using alternative tumor induction. Chemically induced neoplasia using DMBA or N-methyl nitrosourea are well-established models in the rodent producing sufficient rates of tumors with a penetrance that can be
modified by altering the carcinogen dose (Barros et al., 2004; Murray, Ucci, Maffini, Sonnenschein, & Soto, 2009; I. H. Russo & Russo, 1996).

8.6 Conclusions

Combining an environmental challenge with genetic susceptibility of the individual offers an important and relatively novel perspective on breast cancer. Given the abundance of exogenous agents with the potential to induce carcinogenesis (Smith et al., 2020), much more needs to be done to understand how gene-environment interactions might contribute to diseases including breast cancer. Here, we combined a mutation in Tp53 in transplanted epithelium in the mammary gland of BALB/c mice with an exposure to a common environmental pollutant and endocrine disruptor during pregnancy and lactation. We observed modest changes in tumor latency and alterations to tumor characteristics in oxybenzone-treated females that may be consistent with a worse clinical prognosis. Furthermore, we have confirmed that exposures to oxybenzone during pregnancy and lactation produce permanent alterations in the morphology of the mammary gland.
CHAPTER 9

TOWARDS A PARADIGM SHIFT IN ENVIRONMENTAL HEALTH DECISION-MAKING:

A CASE STUDY OF OXYBENZONE

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Manuscript accepted for publication as:

9.1 Abstract

Background: Technological advancements make lives safer and more convenient. Unfortunately, many of these advances come with costs to susceptible individuals and public health, the environment, and other species and ecosystems. Synthetic chemicals in consumer products represent a quintessential example of the complexity of both the benefits and burdens of modern living. How we navigate this complexity is a matter of a society’s values and corresponding principles.

Objectives: We aimed to develop a series of ethical principles to guide decision-making within the landscape of environmental health, and then apply these principles to a specific environmental chemical, oxybenzone. Oxybenzone is a widely used ultraviolet (UV) filter added to personal care products and other consumer goods to prevent UV damage, but potentially poses harm to humans, wildlife, and ecosystems. It provides an excellent example of a chemical that is widely used for the alleged purpose of protecting human health and product safety,
but with *costs* to human health and the environment that are often ignored by stakeholders.

**Discussion:** We propose six ethical principles to guide environmental health decision-making: principles of sustainability, beneficence, non-maleficence, justice, community, and precautionary substitution. We apply these principles to the case of oxybenzone to demonstrate the complex but imperative decision-making required if we are to address the limits of the biosphere’s regenerative rates. We conclude that both ethical and practical considerations should be included in decisions about the commercial, pervasive application of synthetic compounds and that the current flawed practice of cost-benefit analysis be recognized for what it is: a technocratic approach to support corporate interests.

**Key words:** sunscreen, endocrine disruptor, skin cancer, benzophenone 3, intergenerational, externality, melanoma, conflicting interests

### 9.2 Introduction

Thousands of synthetic chemicals (mostly inadequately tested) are currently on the market (Z. Y. Wang, Walker, Muir, & Nagatani-Yoshida, 2020). Some of these compounds are intended to increase human safety, while others enable medical interventions, and still others provide human conveniences. For better or worse, technological and chemical advances of the last century have improved and eased individual and public well-being. Yet, these advances do not come without a cost to the environment, other species and, somewhat paradoxically and with unintended consequences, to human health. For example, phthalates added to flexible tubing (an important feature of medical equipment) also interfere with male reproduction (Pallotti et al., 2020). The conflict between the benefits of safety or convenience
versus the unexpected cost to human health is, however, only one of the many controversial facets of decision-making pertaining to environmental chemicals.

Environmental health decision-making also extends to the identification of economically and environmentally sustainable solutions, exercises precaution and prevents “plausible threats”, avoids quick fixes involving regrettable substitutions, addresses disproportionate impacts of environmental burdens on communities, and considers individual rights and obligations including the rights of individuals to know (or not know) about their environmental exposures (C. L. Soskolne, 1997). Here, we propose six principles of environmental health that can assist in environmental health decision-making relative to environmental chemicals (FIGURE 1).

**Figure 9.1: Six proposed principles of environmental health ethics.**

Ethical principles of sustainability, beneficence, non-maleficence, justice, community, and precautionary substitution provide a framework that can be used to evaluate environmental chemicals. This framework allows for decision-making about synthetic compounds beyond the traditional cost-benefit analysis.
9.3 Introduction to Oxybenzone

To illustrate the framework of the proposed principles, we evaluated oxybenzone, a synthetic UV filter used in sunscreens and other personal care products (Figure 3). Oxybenzone it is also added to many consumer products including cardboard inks, plastic packaging, fabrics, and furniture finishes to protect these commodities from UV-induced fading or damage (Agency, 2015). As a result of its popularity, oxybenzone is now among the most widespread environmental pollutants routinely detected in fish and avian tissues, plants and microorganisms (Balmer et al., 2005; Molins-Delgado et al., 2018; T. Zhang et al., 2013). Collectively, in humans, all sources of exposure contribute to detectable urinary concentrations in over 98% of the U.S. population (Han et al., 2016); it has also been measured in blood, amniotic fluid, cord blood, semen and breast milk (Buck Louis et al., 2015; Krause, Frederiksen, Sundberg, Jorgensen, Jensen, Norgaard, Jorgensen, Ertberg, Petersen, et al., 2018).

Recent randomized control trials revealed that oxybenzone reaches and exceeds the US FDA’s threshold of concern (0.5 ng/ml in blood) two hours after sunscreen application; these concentrations remained above the threshold for 23 hours in all participants, and for three weeks in 96% of participants (Matta et al., 2020; Matta et al., 2019). Based on these new findings, the U.S. FDA recently requested additional safety studies on oxybenzone and other sunscreen ingredients (FDA, 2019a).

The effects of oxybenzone in vivo are broad: it is toxic to cyanobacteria (Mao et al., 2017; Zhong et al., 2019), green algae (Mao et al., 2017; Sieratowicz, Kaiser, Behr, Oetken, & Oehlmann, 2011) and coral (Downs et al., 2016). In rodents,
oxybenzone alters the development of the mammary gland, alters the weight of the liver, kidney, and reproductive organs, decreases the number of spermatocytes in males (LaPlante et al., 2018; K. Matouskova et al., 2019; Nakagawa & Suzuki, 2002; Nakamura et al., 2015) and induces DNA damage in the mammary epithelium (Majhi et al., 2020). In fish, oxybenzone interferes with reproduction (Coronado et al., 2008; S. Kim, Jung, Kho, & Choi, 2014).

There is also evidence that oxybenzone is an endocrine disrupting chemical. In vitro screening tests have revealed that it is an estrogen receptor (ER) agonist and antagonist (Balazs et al., 2016; Kunz & Fent, 2006a; Suzuki et al., 2005) and that some oxybenzone metabolites have greater estrogenic activity than the parent compound (Morohoshi et al., 2005; Watanabe et al., 2015). Oxybenzone is also an androgen receptor (AR) antagonist (Balazs et al., 2016; Suzuki et al., 2005). Consistent with AR antagonist activity, oxybenzone induces shortened anogenital

![Figure 9.2: An overview of the oxybenzone case study.](image)

Owing to the production and manufacture of products containing oxybenzone, it is released into the environment, contributing to ubiquitous human exposures. Exposures are associated with a range of hazards to wildlife, laboratory animals and human health.
distance in rats (Nakamura et al., 2015) and mice (K. Matouskova et al., 2019) following perinatal exposure (FIGURE 9.2).

9.4 Principles

“It is essential my Son in order that you may go through this Life with comfort to yourself and usefulness to your fellow creatures that you should form and adopt certain principles for the Government of your own conduct and temper—unless you have such rules and principles there will be numberless occasions on which you will have no guide for your Government but your Passions…”

J. Q. Adams (Adams, 1811)

Although principles originate from virtues conceived by the Ancient Greeks around 5th Century BCE if not earlier, the modern method of principlism in human health emerged during the late 20th Century - initially as a response to atrocities of the first half of the century (e.g., human experimentation by the Nazi regime, the Tuskegee syphilis study, and others), later as reflecting emerging environmentalist movements (D. B. Resnik, 2012). In the field of ethics, principlism is considered a practical framework for “people making real-world decision[s]” (Hain, 2016). Our everyday personal, societal, and professional conduct is guided by commonly accepted ethical principles: regardless of our religiosity, we shalt not murder or steal; democratic societies are ruled by consensus of “the people” rather than the will of an individual; and guilds and professional organizations declare their ethics guidelines and standards of practice.

Modern clinical medicine and related scientific disciplines have codified four guiding principles: non-maleficence (often translated as “do no harm”), beneficence, justice, and respect for autonomy (Beauchamp, 2019). However, the values and principles for public health or environmental health cannot be directly transferred
from the field of medicine owing to differences in the type of interventions in public health vs. clinical practice (e.g., indirect and preventive vs. direct and curative), type of professionals involved (e.g., a diverse group vs. specialized), and the ultimate focus of interventions (e.g., to protect populations including unspecified individuals vs. a single well-known patient) (L. M. Lee, 2012). Unlike medical ethics, public health ethics frameworks have not yet fixed upon a set of universally accepted principles; however, several foundational values, such as transparency, reduction of inequities, and solidarity run through many proposed frameworks. On the global end, a comprehensive document of sixteen principles, the Earth Charter, lays an ethical foundation for acting based on respect and justice, nonviolence and democracy, and ecological integrity (ECI, 2000; Schröder-Bäck & Vincenten, 2018; Colin Lionel Soskolne et al., 2008).

Environmental health ethics (not to be confused with environmental ethics, which does not focus principally on the health of people) represents an approach where human health stands paramount in the context of and connection with the environment, non-human species, and possibly, future human generations. This may seem to present a tension between eco-centric and anthropo-centric approaches, but such dissention is anticipated and welcomed because applied ethics arises from epistemic uncertainty and works to address tensions. In environmental health ethics, an immediate focus on human health is extended towards non-human species, ecosystems and the biosphere, as well as the responsibilities of the human species that rises from its rights. Scholars of environmental health ethics have only begun discussing the guiding principles of the field (D. B. Resnik, 2012). In this context, the thousands of newly synthetized chemicals and the ubiquitous environmental
pollution that is inevitably reaching human tissues have provided challenges to
ethical decision-making and ethical actions.

9.4.1 Principle of Sustainability

This we know: the earth does not belong to man; man belongs to the earth. All things are connected like the blood that unites all Man did not weave the web of life; he is merely a strand in it. What he does to the web, he does to himself.

Sealth, Chief Seattle

Stewardship is broadly defined as a call to “take good care of natural resources” and implies additional subsidiary principles such as “protect species and biodiversity” and “avoid destruction of habitats and ecosystems” [p.73 (D. B. Resnik, 2012)]. That humans are not external to ecosystems and their biodiversity has been recognized by the global community (UN, 1992; Whitmee et al., 2015). Nonetheless, responsible stewardship is still on occasion seen as binary— one may protect either nature or civilization - but like many issues presented as an either/or choice, this dichotomy is false. In fact, economists have repeatedly demonstrated that “the viability of business itself depends on the resources of healthy ecosystems” (Chouinard, Ellison, & Ridgeway, 2011).

Acting as responsible stewards leads to a “practice of sustainable uses of biological resources” (D. B. Resnik, 2012). The Principle of Sustainability thus implies a balance of using - but not overusing; harvesting - but not overharvesting.

Prior to the Anthropocene epoch, industrial chemicals would not present a threat because their volumes did not exceed the rate of individual and environmental biotransformation (S. L. Lewis & Maslin, 2015). In the case of oxybenzone, this is no longer true. First synthesized in the mid-20th century, oxybenzone quickly gained
dominance among sunscreen products and equally rapidly began polluting coastal waters when washed off the skin of beachgoers. The levels of oxybenzone measured in coastal waters harboring coral reefs are no longer sufficiently diluted to avoid harm to these species (Downs et al., 2016; Mitchelmore et al., 2019). The demand for sunburn-free midday beachgoing, combined with climatic and oceanographic conditions produce the present situation in which oxybenzone threatens the survival of aqueous species and ecosystems (Wijgerde et al., 2020).

Although the chemical is readily metabolized in human tissues and 93% of the compound is transformed in the marine environment within 120 days of its introduction, oxybenzone is routinely detected in nearly all human samples, in water and soil, consistent with continuous pollution sources (Wolfson, Porter, Villani, Simon, & Young, 2019).

Although people have changed landscapes for the sake of utility, beauty, or leisure for most of human history, for millennia these changes played by nature’s rules simply because the members of our species were “few, humble, and weak” (Kohak, 2000). In recent history, however, technological and chemical discoveries have led to advances in which the rules of nature could be dismissed or ignored. Humans devised methods to defeat infectious diseases, to bypass infertility, and to stretch the limits of materials on water-repellency, flammability, and flexibility. With these changes, our civilization seemed invincible. Yet, in spite of these advances we have reached several “walls” where the limits of demography, physics and biology matter again. No longer can the biosphere transform all synthetic substances into harmless metabolites – there are tens of thousands of newly synthesized chemical compounds in the environment, many of them produced in large volumes, some of them highly stable and persistent in the environment (Z. Y. Wang et al., 2020). After
times when the elements have had extreme impacts on civilizations, and times when civilization has had extreme impacts on the environment, it becomes necessary to execute responsible and sustainable stewardship.

Implied in the Principle of Sustainability is the matter of future generations. Given that coral reefs that form over thousands of years have shrunk by 20 to 80% over the past two generations, we are already past the turning point of our grandchildren experiencing the vast reefs as we did (Regan, 2020). Some have argued that obligations to hypothetical future individuals is a paradox: how can “actions that make things worse for no one [in particular] be wrong?” (Roberts, 2009). The “non-identity case” solution most relevant to environmental health suggests replacing the hypothetical person of the future with an entire community potentially harmed by today’s action because “we have a moral duty to promote the overall well-being of future generations, even if we do not have moral duties to any particular future person” [p.74 (D. B. Resnik, 2012)]. The Principle of Sustainability weighs the impact of today’s production and use of anthropogenic chemicals along with the wellbeing of current and future humans, non-human species and the biosphere. Oxybenzone fails this test (Figure 9.3).

9.4.2 Principle of Beneficence

On one level, modern sunscreen isn’t so far from smearing yourself with clays, minerals, or a mixture of sand and oil like the ancient Egyptians or Greeks did. But on another level, modern sunscreens are some mind-bending magico-chemical spellwork. Our species should be patting ourselves on the back right now. But does our little magic trick actually work? George Zaidan, (Zaidan, 2020)
In the clinical setting, the *Principle of Beneficence* can be easily translated as actions that benefit the patient. Experimentally, chemical sunscreens have been clearly demonstrated to provide protection against UV-induced damage, and on the level of populations they were pledged to decrease skin cancer incidence (Lowe, 2006; Urbach et al., 1976). Therefore, the *Principle of Beneficence* raises a question: do chemical sunscreens actually offer a means of skin cancer prevention to at-risk populations?

The incidence of malignant keratinocyte carcinomas, i.e., squamous and basal skin cancers – previously referred to as non-melanoma skin cancers (Karimkhani, Boyers, Dellavalle, & Weinstock, 2015) – has increased by 14% between 2006 and 2012 with >3 million U.S. residents affected in 2012 (Rogers, Weinstock, Feldman, & Coldiron, 2015a). Melanoma cases are reported to cancer registries thus their statistics and trends are more precise: in 1975, the National Cancer Institute (NCI) recorded 7.9 cases of melanoma per 100,000 US residents; in 1995 the rate doubled to 16.5, and the latest data from 2018 report 22.4 melanoma cases per 100,000 residents (NCI, 2021).

Although their convenience is rarely disputed, the efficacy of sunscreens remains ambiguous. Whether sunscreens modify the risk of sunburn, melanoma and keratinocyte cancers have been studied predominantly using human cohorts, and the body of evidence provides a heterogenous and contradictory picture. In 2001, the International Agency for Research on Cancer published a review of case-control studies and concluded that the evidence for sunscreen providing protection from melanoma and basal cell carcinoma was “inadequate” while “limited” evidence for preventive effects was found for cases of squamous cell carcinoma (IARC, 2001). A meta-analysis of 11 human cohorts found no effect of sunscreen use on sunburn
prevention (Huncharek & Kupelnick, 2002). On the other hand, a French collaborative report concluded that “topical use of sunscreens reduces the risk of sunburn in humans” yet “no conclusion can be drawn about the cancer-preventive activity of topical use of sunscreens” (AFSSE, 2005). A more recent meta-analysis included epidemiological studies beyond case-control designs, and carefully assessed the quality of evidence of sunscreen-melanoma associations; yet even this review finds the evidence inconclusive (Rueegg et al., 2019). What is more, sunscreens seem to have no effect on melanoma risk in dark-skinned populations (Adamson, 2019).

Only one study to date shows a protective effect of sunscreens (containing avobenzone and octinoxate) in an Australian randomized controlled trial (Green, Williams, Logan, & Strutton, 2011). However, individuals assigned to the “control” group were not restricted in their use of sunscreen, and the study was burdened with a number of biases, including selection and funding bias, and detected only half of the melanoma cases that were expected given incidence of the disease in the Australian population (NCCI, 2019).

In spite of the paucity of efficacy evidence, humans mass-produce and promote sunscreens, and encourage an everyday, one-size-fits-all use. Since the early 1970s, guidelines of the American Association for Dermatology caution users to apply sunscreen on the “entire body before [a person] dress[es] for the day” and repeat applications throughout the day (AADA, 2020); the goal for children and infants older than 6 months is “to protect all parts of the skin exposed to the sun by using a variety of techniques, including sunscreen” (Paller et al., 2011). For many, everyday use of broad-spectrum sunscreens has become a matter of fact (Svarc, 2015), but humans’ behavior sometimes works contradictory to the best public-
health intentions. For example, there is evidence that application of sunscreen may increase sun exposure and a false sense of sun safety (Autier, Boniol, & Dore, 2007) by “extend[ing] the duration of intentional sun exposure, such as sunbathing… increas[ing] the risk for cutaneous melanoma” (AFSSE, 2005). Lastly, sunscreen’s efficacy is subverted if users apply “however much feels right” (Zaidan, 2020), i.e. less than the recommended amount of 1 ounce per application, or if a person fails to re-apply the product every two hours (Petersen, Datta, Philipson, & Wulf, 2013).

The Principle of Beneficence calls for convincing evidence and significant benefits to public health. The above arguments undermine the alleged beneficence of sunscreens, and oxybenzone specifically, in the prevention of skin cancer.

9.4.3 Principle of Non-maleficence

“Animal studies have raised concerns about endocrine disruption and reproductive issues. But animals are not people, [Dr. Henry] Lim [a dermatologist] says. And despite decades of sunscreen use, there has been no population-wide signal that rates of infertility, birth defects or other health problems are higher in people who use more sunscreen or in places where people apply more of it” (Sohn, 2019).

“Primum non nocere” (first, do no harm) obligates health care professionals to abstain from inflicting harm to a patient if s/he cannot benefit from the care provided. The Principle of Non-maleficence builds on the argument of beneficence, and asks “is it safe for me”? Sunscreens are regulated by the FDA and tested for sun protection efficacy. Yet, testing the 17 over-the-counter UV filters for safety (e.g., hazard identification) was previously not seen as necessary; the sunscreen ingredients, including oxybenzone, were considered by FDA to be “generally recognized as safe and effective” because it was assumed that their concentrations would not exceed a
threshold of 0.5 ng/mL in systemic circulation after dermal application (FDA, 2019b). Now that this assumption has been shown to be wrong (Matta et al., 2020; Matta et al., 2019), the FDA has called for more data and possibly stricter regulation (FDA, 2019b).

Beyond concerns about exposure, research published in peer-reviewed journals paints a more complex story of possible health effects (e.g., hazards). An increasing number of epidemiological studies have evaluated the effects of oxybenzone on human health outcomes. Although many of these studies are limited by their design (e.g., cross-sectional studies), there is evidence that oxybenzone and its metabolites can affect human health, including effects on reproduction (time to pregnancy, risk of endometriosis, sperm quality, measures of infertility) (Arya et al., 2020; Kunisue et al., 2012; Peinado et al., 2020; Rehfeld, Dissing, & Skakkebaek, 2016); birth outcomes (Philippat et al., 2012; Tang et al., 2013); neurobehavioral outcomes in the offspring (Guo et al., 2020); and health of other organs, e.g., the thyroid gland (S. Kim et al., 2017; Schmutzler et al., 2007). Furthermore, oxybenzone was identified as an allergen in 1-3% of the population (Heurung, Raju, & Warshaw, 2014) and while allergic reactions to sunscreens affect only a small proportion of the population, oxybenzone is a common photoallergic agent (Subiabre-Ferrer et al., 2019). In addition to the weight of epidemiological cues, as described in more detail above, mammalian toxicological studies similarly point to oxybenzone’s effects on the structure and function of tissues including endocrine organs (Ghazipura et al., 2017).

Regardless, some dermatologists continue to promote chemical sunscreens as the ultimate means of sun safety and call for epidemiological studies to detect a “population-wide signal” for an effect of sunscreen use on human health before even
entertaining the possibility that oxybenzone might cause harm. Unfortunately, such
“population-wide” evidence will be incredibly challenging to collect. Epidemiology
studies that attempt to examine such relationships often fail due to the ecological
fallacy (i.e., drawing conclusions about individuals based on evidence collected for a
group, in this case, concluding that oxybenzone is safe for individuals based on a
failure to obtain a “population-wide signal” of harm). Furthermore, it is almost
impossible to find a population of individuals without exposure to oxybenzone to
serve as an “unexposed control group”; in 2011, the chemical was detected in the
urine of 98% of non-pregnant women and 100% of pregnant women (Woodruff et al.,
2011). Even individuals that report “never” using sunscreen have detectable levels in
their urine, indicating that exposures come from a wide variety of sources (Zamoiski
et al., 2015). Of course, if such a population-wide signal of harm were identified, that
would represent a massive public health failure; the possibility of such catastrophic
outcomes creates an even stronger case for precaution (C. L. Soskolne, 2004).

In spite of these limitations, an increasing number of human studies suggest
associations between oxybenzone and harm. These studies examine exposures and
health outcomes at the level of individuals, finding “signals” of harm that have
unfortunately been dismissed by some in the dermatology community because the
studies do not examine the population in entirety. These findings should not be
ignored in a quest for a “perfect” human cohort. With the evidence that is currently
available, the principle of “first do no harm” disqualifies oxybenzone due to its
maleficence.
9.4.4 Principle of Justice

*Hawaii is definitely on the cutting edge by banning these dangerous chemicals in sunscreens.*

Hawaii State Senator Mike Gabbard

The *Principle of Justice* in environmental health demands that individuals and communities share “justly and equally both burdens and benefits” and that “societies follow fair procedures” in making and implementing policy decisions concerning the environment (D. B. Resnik, 2012). In the Rawlsian approach of justice – where justice is consistent with fairness (Rawls, 1971) – and in the extension of Rawls’s principles to health care, unequal health burdens are seen as unjust when social determinants of health such as education or job opportunities are also unjustly distributed (Daniels, 2008).

There are many benefits of coral reefs to humans, including tourist spending, food sources, coastal protection and populations that live near these locations can enjoy them (UNEP, 2004). Yet those unique ecosystems are subjected to unprecedented worldwide losses. Oxybenzone is one of several contributors to coral bleaching, brought to reefs indirectly from residential and municipal wastewaters or washed off the skin of swimmers and beachgoers. Beach tourism has become ever more popular; for example, the annual number of visitors to the State of Hawaii – whose islands are surrounded by coral reefs - increased from 6.7 million to 10.4 million between 1999 and 2019 (DBEDT, 2000; HTA, 2019). Coastal pollution with oxybenzone results in an unequal distribution of an environmental burden. After Hawaiian residents realized the distributive injustice of this burden, they exercised their right to procedural justice. In 2018 the State Senate voted to ban sunscreen products containing oxybenzone and octinoxate from being distributed or sold in Hawaii, taking effect in January 2021 (Senate, 2018).
The Hawaii case study provides an example of a specific population acting meaningfully against environmental pollution. Yet, a situation where governing legislators rule confidently and effectively against a polluting agent is an exception rather than a norm. Typical stories of disproportionate pollution in communities commonly end – if resolved at all – in cumbersome repressive legal actions and public budgets paying for the consequences of the pollution (EPA, 2021).

Ideally, distributional injustice is prevented. If it cannot be prevented, the disadvantaged community should have the means to decide on the burden experienced through procedural justice or to be compensated for the unequal and unjust distribution. Concerted efforts are needed to reduce the unjust, distributional burden of pollution on the global scale.

Oxybenzone provides one additional angle to the Principle of Justice: this chemical has been detected in the bodies of individuals across the globe, regardless of their personal decisions or reported use of chemical sunscreens (Zamoiski et al., 2015). Above all, neonates are born having already been exposed to chemical sunscreens (Woodruff et al., 2011). Polluting our descendants raises the question of intergenerational justice, a topic described above.

9.4.5 Principle of Community

“The innocent greed of the affluent may easily be the most threatening ecological time bomb.”

E. Kohak (Kohak, 2000)

The Principle of Autonomy – defined as “acting on the basis of choices guided by values and principles that one accepts as one’s own” (Jameton & Frumkin, 2010) – is the paramount guiding principle in medical practice and bioethical theory. But public health practice typically applies a number of restrictions on this
fundamental principle because in challenges posed by public health, the “interests of the community” override individual choices if personal preferences pose a risk to health or safety of the community (Holland, 2015). Examples of what we have dubbed the *Principle of Community*, where the health of the community is seen as a vital priority, include restrictions on smoking in public or shared spaces due to the risks of secondhand smoke (Jarvie & Malone, 2008), required vaccination for the sake of herd immunity (Luyten, 2011), or mask mandates in the case of a pandemic caused by a respiratory virus. Environmental health practice builds on similar premises of community kinship, where the focus is on the interests of environment and human health even when the benefits to the community conflict with individual preferences. The concept of the ‘tragedy of the commons’ relates to this point, because the benefits received by the individual (or a specific industry) can detract from the health of the whole (P. S. Dasgupta & Ehrlich, 2013).

To illustrate the environmental health conflict, Kohak [p.9 (Kohak, 2000)] uses the example of a retired clergyman whose hobby is to fly surplus fighter jets, arguing that it is his *right* to engage in the activity, even while he acknowledges the detrimental environmental impact of fighter jet engines. Kohak frames such reasoning as an “innocent greed of the affluent” and asserts that similar forms of argumentation are not uncommon in human-environment conflicts (Kohak, 2000). The “innocent greed” argument represents one of many hypothetical explanations for individual choices to use sunscreens containing oxybenzone despite their adverse effects on the environment. Users may think, “It is my right to use this sunscreen, even if it contributes to environmental harm.” For those reasons, human-environment conflicts are often addressed with regulatory action to restrict autonomous decisions.
Although the “innocent greed” argument is certainly important to consider, lack of awareness reflects another, more likely, concern because the general public is not typically expected to judge the risks associated with the use of synthetic chemicals or exposure to environmental pollutants. Instead, experienced professionals – scientists, public health professionals, and regulators – are tasked with using expert judgement to tackle the complex problems of environmental and human risk assessment. In other words, regulatory agencies and their employees are tasked to prevent the necessity of the consumers’ dilemma.

Unfortunately, autonomous choices cannot always be respected when environmental health is at stake or when the impacts of autonomous choice are potentially disastrous (C. L. Soskolne, 2004). Such choices should not even be expected when addressing issues that are as complex and complicated as the case of chemical safety evaluations. While legislative regulations are not without risk [e.g., what has been described as the “slippery slope of regulations (D. Resnik, 2010)], the case of oxybenzone shows that that under the Principle of Community, some groups may need to override autonomous (individual) decisions to ward off the magnitude of environmental, societal and/or health costs that are attributed to the collective impact of individual acts.

9.4.6 The Principle of Precautionary Substitution

The term ‘precautionary principle’ can be traced to the German word Vorsorgeprinzip. An alternative translation of this word might be the foresight or ‘forecaring’ principle—emphasizing anticipatory, forward-looking action rather than reactive impeding of progress. 

Tickner, Kriebel & Wright (Tickner, Kriebel, & Wright, 2003)
The Principle of Precautionary Substitution cautions against replacement of harmful chemicals if such a replacement introduces another equally or more harmful chemical. In recent decades, a number of everyday or industrial chemicals were disfavored and substituted with different chemicals that were later found to be harmful. For example, lead arsenate, an insecticide comprised of two heavy metals, was used on deciduous trees (including many apple orchards) (EPA, 2004) until it was replaced by the infamous DDT. The impact of Rachel Carson’s Silent Spring brought the use of DDT in U.S. agriculture to an end and organophosphate pesticides quickly replaced organochlorines. Yet, with time, many of these were also found to be toxic and were later banned from residential use (although malathion is still heavily used for mosquito control and in agriculture) (Blus, 2003; Harnly, McLaughlin, Bradman, Anderson, & Gunier, 2005). Then came the neonicotinoids, insecticides now described as “bee neurotoxins”; three of them are now “severely restricted” in the EU and only three are permitted in Canada (EC, 2018; PMRA, 2020). The newest pest control inventions target genetic information, e.g. RNAi (RNA intervention) pesticides, but whether the “blissful enthusiasm that accompanies every new advance in modern technology and medicine” (Colborn, 2004) will lead to a similar disillusionment with these technologies remains to be seen.

Precaution pertaining to oxybenzone requires us to examine two opposing sides: the concern about not using chemical sunscreens which is perceived as increasing the risk of skin cancer (Diffey, 2016), and concerns about using chemical sunscreens, which increases environmental pollution and adverse health effects in human and non-human species. Both these facets are rarely examined together and often fail to address the relevant issues, e.g., a frank accounting of the effectiveness
of sunscreens in skin cancer prevention, as well as the full magnitude of understanding the many ways chemical sunscreens impact life on the planet. The former issue was addressed above in the *Principle of Beneficence* and we turn to the latter in this section.

Extensive evidence collected in wildlife and from experimental studies suggests that oxybenzone harms a wide range of species including bacteria, algae, plants, fish and mammals (Figure 9.2). When the impact of oxybenzone on coral reefs galvanized concerns and induced a negative public response, attention shifted towards UV protecting alternatives. Novel technological-chemical solutions (e.g., variants of chemical sunscreens including mineral-based sunblock improved with nanotechnologies), and emerging chemical solutions (e.g., benzotriazoles, a newer family of UV stabilizing chemicals added to consumer products) represent a marketing opportunity, but they also raise red flags around human health and sustainability. For example, titanium dioxide nanoparticles penetrate human skin in certain formulations and emerging evidence points to their potential toxicity (Bokobza et al., 2021; Musial, Krakowiak, Mlynarczyk, Goslinski, & Stanisz, 2020).

Similarly, UV-328, a benzotriazole UV stabilizer added to many plastics is proposed to be added to the Stockholm Convention’s list of persistent organic pollutants (UNEP, 2020) and other benzotriazoles such as HDBB and UV-324 induce toxicity in aquatic species and act as endocrine disruptors in both fish and mammals (IPEN, 2020).

To address the issue of regrettable replacements, several distinct actions have been proposed. First, it is possible to regulate chemicals by *groups or classes*. An international effort on “Substances depleting the Ozone Layer” – The Montreal Protocol – provides an example of successful regulation of a chemical class based
on the type of harm inflicted by chemicals (Velders, Andersen, Daniel, Fahey, & McFarland, 2007). Alternatively, chemicals could be classified and regulated based on their intended purpose. The concept of “essential” vs. “non-essential” use suggested for classification of per- and polyfluoroalkyl substances (PFAS) would enable regulators to identify and phase-out the most non-essential uses (Cousins et al., 2019). Second, the price of a product producing unintended harms could more fully reflect those detrimental costs known as externalities. Economists specialized in sustainability (i.e., the discipline of Ecological Economics) can apply various tools for internalizing costs associated with disposal and pollution and can quantify the benefits obtained by industries utilizing natural ecosystems (P. Dasgupta, 2021). Accountability of the markets for using and overusing natural resources differs from recent economic theories (and practice) but a society in which individuals and public budgets cover health and societal costs associated with profit-driven polluting producers is not sustainable on the scale we have achieved today.

9.5 CONCLUSIONS

Environmental pollution with thousands of inadequately tested synthetic chemicals is among the many urgent threats that challenge human health and environmental sustainability. While the Paris Agreement takes action against greenhouse gas emissions, a similar international vision on anthropogenic pollution has yet to be formed.

We have offered six principles that are relevant for environmental health decision-making (Figure 9.1); these principles illustrate the complexity of the problem of environmental chemical pollution and should be considered when searching for solutions. A reliance on principles provides guidance to evaluate compounds that
present a patchwork of risks and benefits in both societal and scientific contexts.

Thus, the case of oxybenzone lends an excellent opportunity to apply these six principles of environmental health to a specific chemical that is used in consumer products to meet a specific need (protection from UV-induced damage), but with costs to human and environmental health that have been largely unexplored (Figure 9.3). With the framework of principlism, it becomes clear that even for a chemical with relatively moderate stakes (compared to the wide spectrum of highly persistent

Figure 9.3: Evaluating oxybenzone based on the proposed six principles. Oxybenzone as a UV-absorbing filter in sunscreens, products, and packaging induces short- and long-term adverse effects on both humans and environment. Further, heavy coastal pollution stands as unequal and unjust. Systemic regulation on regional and global levels, tiered according to essentiality of use will affect autonomous decisions. On the other hand, alternatives to oxybenzone exist as: 1. non-technical solutions (preferred, when possible), 2. safer chemical UV filters (where necessary), and 3. inorganic UV-filters (to be used with caution).
and highly accumulative compounds that have been released to the environment, action is required to address the limits of the biosphere’s regenerative rates.

Declarations

**Ethical Approval and Consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of supporting data**

Not applicable

**Competing interests**

LNV has received travel reimbursement from Universities, Governments, NGOs and Industry, to speak about endocrine-disrupting chemicals. Her EDC-related work has been supported by US federal agencies, the University of Massachusetts Amherst, and NGOs including the Cornell Douglas Foundation, JPB Foundation and the Great Neck Breast Cancer Coalition. She is a paid scientific advisor to SUDOC LLC. KM has no conflicts to disclose.

**Funding**

The authors acknowledge support from the National Institute of Environmental Health Sciences of the National Institutes of Health (Award Number U01ES026140). The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the University of Massachusetts.

**Authors’ contributions**
LNV and KM conceptualized this project. KM wrote the first draft of the paper and created the figures. LNV provided critical edits to the manuscript. LNV and KM both agree to publish the paper.

Acknowledgements

The authors gratefully acknowledge members of the Vandenberg lab for helpful conversations on this topic. We also thank Dr. Dan Goldstein for feedback on an early draft of this manuscript. This work was funded through support from the National Institute of Environmental Health Sciences of the National Institutes of Health (Award Number U01ES026140). The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the University of Massachusetts.

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KM is a PhD Candidate at the University of Massachusetts Amherst. She works in the laboratory of LNV in the School of Public Health & Health Sciences, Department of Environmental Health Sciences. Both authors have been working to study the effects of oxybenzone and other endocrine disrupting chemicals on the health, function, and disease of the mammary gland.
Oxybenzone has been produced and released into the environment for several decades yet only recently the chemical has attracted the attention of the public and regulatory bodies. Over this same period of time, the chemical has been integrated into numerous consumer products, ensuring ubiquitous exposures, even of individuals that avoid using dermal oxybenzone-based sunscreens. Thus, like many other emerging pollutants, oxybenzone poses numerous problems; compared to pharmaceuticals like DES, which were purposefully administered to pregnant women, oxybenzone is challenging to study in human populations due to a lack of an unexposed population, and exposures that extend across the lifecourse.

Like many other environmental pollutants, as it is currently used, oxybenzone does not represent a classic toxicant or teratogen in the traditional sense. By this, what we mean is that it is not overtly killing or maiming individuals that are routinely exposed to it via use of personal care products, consumption of contaminated food, ingestion of dust and water, or dermal exposure to fabrics and upholsteries. This has led some regulator to ask the question “where are the bodies?” [This question was asked by a member of an FDA panel for evaluating chemicals in the early days of EDC research, to suggest that without a link to mortality, chemicals need not be considered harmful (Tom Zoeller, personal communication)]. Indeed, typical everyday exposures to oxybenzone are not deadly, but the research to date suggests that this chemical does contribute to adverse health outcomes in humans and wildlife. Yet some of the most relevant effects that have been observed (e.g., disruption to the morphology of the mammary gland) in controlled experimental
models are not considered adverse by the risk assessment and risk management decision-making processes (Cardona & Rudel, 2020). Furthermore, most wildlife species and adverse outcomes in their habitats are viewed as “invalid” due to use of “non-standardized test species” or “lack of ecological relevance” (Burns, Csiszar, Roush, & Davies, 2021), and the doses relevant for environmental exposures are often too low to be tested under laboratory conditions used in traditional guideline studies (vom Saal & Hughes, 2005; Vom Saal & Vandenberg, 2021). All of these factors, and others, have created a Catch-22 in the evaluation of oxybenzone and similar environmental chemicals: associations observed in epidemiology studies are dismissed because the exposures are uncontrolled, yet results from controlled studies in laboratory animals are dismissed because the effects seen in animals are not considered relevant for human health.

The results presented in this dissertation offer additional evidence that the mammary gland is a target of oxybenzone exposure. In females exposed to oxybenzone during pregnancy and lactation, prior studies in mice revealed increased proliferation in the mammary epithelium and decreased epithelial density, as well as increased expression of Esr1 (the gene encoding ERα) in the uterus of oxybenzone-exposed animals compared to parous controls (LaPlante et al., 2018). Additional mechanistic studies determined that oxybenzone induces DNA damage and R-loops in three cells lines: T47D, MCF7, and 76N-Tert-ESR1 + dox, and in the mammary epithelial cells of ovariectomized BALB/c mice (Majhi et al., 2020). These studies led us to ask if exposures to oxybenzone during pregnancy and lactation could alter susceptibility to carcinogenesis. Because the wildtype mouse is not prone to mammary tumors, to address this question, we relied on a genetically susceptible mouse model which is known to develop mammary tumors. Using this model, we
found that the mammary tumors in animals treated with oxybenzone have lower infiltration of macrophages, increased level of angiogenesis, and modestly shorter tumor latency. Based on these results, we concluded that oxybenzone treatment during pregnancy and lactation moderately decreases time to mammary neoplasia and induces tumor characteristics consistent with worse tumor prognosis.

We also conducted several studies to evaluate the effects of oxybenzone on the mammary gland of females exposed to the chemical during perinatal development (i.e., the offspring of the mothers exposed during pregnancy and lactation). This series of studies evaluated whether oxybenzone altered the morphology of the mammary gland at several stages of development, whether it altered proliferation or expression of hormone receptors in the epithelial cells, whether there were disruptions to several components of the mammary stroma, and whether it would potentiate (or attenuate) the effects of pubertal exposures to estrogens. We found alterations in the epithelium including a larger area of the mammary tree and lower rates of epithelial cell proliferation at PND21, greater expression of ERα at PND26, greater ductal extension within the fat pad and complexity measured as the tree branching, and disruption to progesterone receptors at one of the doses tested (212 µg/kg/day) at puberty; in adulthood, the oxybenzone-exposed animals differed from controls with decreased epithelial volume fraction, lower expression of ERα, and greater numbers of alveolar buds. The stromal changes included a thicker periductal stroma at puberty and a larger size of adipocytes in adulthood. These results provide the first evidence, to our knowledge, that oxybenzone affects the development of the mammary gland with measurable disruptions to both the epithelial and stromal compartments.
Although our experiments cannot determine if the effects of oxybenzone on the mammary gland are due to actions via a specific hormone receptor, they also add to the evidence that this chemical is an endocrine disruptor. A 2020 publication proposed ten key characteristics of endocrine disrupting chemicals (La Merrill et al., 2020) and our studies are consistent with several of these key characteristics including alterations to the expression of hormone receptors and alterations to the fate of cells in hormone-sensitive organs. Combined with earlier publications indicating that oxybenzone is an ER agonist and an ER and AR antagonist (Kunz & Fent, 2006b; Schlecht, Klammer, Jarry, & Wuttke, 2004; Watanabe et al., 2015), there are now at least four key characteristics of an endocrine disruptor displayed by this chemical.

Finally, in two of our studies, we evaluated the effects of perinatal exposures to oxybenzone on the male mouse mammary gland. The male mammary gland is relatively understudied, and ongoing work is revealing important aspects of its normal development in addition to identifying effects of environmental chemicals on this sensitive tissue. Our results suggested that early life exposures to oxybenzone alter the morphology of the male mammary gland, with decreased size of the ductal tree and reduced complexity in the right mammary gland. Interestingly, results from our follow-up study indicate that perinatal exposures to oxybenzone alter the response of the male mammary gland to an estrogen challenge at the onset of puberty. Although much work still needs to be done to fully characterize the effects of oxybenzone and other environmental pollutants on this organ, our results provide strong evidence that it is affected by exposures during early development.

In our mouse studies (described in Chapters 4, 5, 6, and 8), and ethics decision-making manuscript (Chapter 9), we mark the ways to go forward in
evaluating adverse effect in the mammary gland, and describe the values and principle-based decision making processes, respectively. Moving forward, based on the data from our lab and others, laboratory research on oxybenzone may revolve around three aspects: 1. Oxybenzone as a possible obesogen, 2. The effects of oxybenzone in other models of mammary carcinogenesis; and 3. Obesity propelled by oxybenzone leading to tumorigenesis.

**Oxybenzone – a suspected obesogen?**

Our data suggest that developmental exposures to oxybenzone increase the size of the white adipocytes in the mammary gland (which encompasses the subcutaneous fat pad) in adulthood (Chapter 5, FIGURE 5.3). In controls, the size of adipocyte decreases from nearly 1700 to 1218 µm$^2$ between puberty and adulthood. In response to early exposure to oxybenzone, adult adipocytes not only reverse the direction and grew larger, but they also grew larger by almost twice as much (from 1218 to 2023 µm$^2$ in the 30µg/kg/day group). Moreover, adult females exposed to 3000 µg/kg/day and 212µg/kg/day of oxybenzone in utero and during the perinatal period had significantly increased body weight at puberty and in adulthood respectively (Chapter 4, FIGURE 4.1).

The hypothesis that endocrine disrupting chemicals could play a part in metabolism and namely obesity was proposed by Blumberg & Grün (Grun & Blumberg, 2006), and they coined the term obesogens for chemicals that promote obesity in humans and animals. According to the obesogen hypothesis, which was largely based on the effects of tributyltin, a model obesogen, these chemicals act by altering adipocytes directly and indirectly in a number of ways including increasing the number of adipocytes and storage of triglycerides, changing basal metabolism.
and energy balance in favor of storage of calories, and modifying food intake through effects on other organs (Heindel et al., 2017). Importantly, the obesogen hypothesis builds on Barker’s theory of the developmental origins of adult diseases, and therefore emphasizes a "set point" or phenotypic plasticity in the fetus. Applied to the issue of metabolism, fetuses that experience malnutrition in utero are born smaller, but will be "programmed" to consume excess calories in expectation of scarcity in later life.

Future research of oxybenzone should evaluate whether or not this chemical is truly an obesogen. Such studies ought to begin with well-established cell-culture models screening for elementary signs of metabolic disruption. A recent international interlaboratory study used the 3R2-L1 pre-adipocyte model that has proven highly consistent for triglyceride accumulation induced by several EDCs (Kassotis et al., 2021). Pre-adipocyte cultures are also essential for establishing the storage of oxybenzone and its release from the adipose tissue, as well as its effects on adipocyte differentiation from pre-adipocytes (Kassotis et al., 2021; Sun et al., 2021).

Both cell cultures and animal models would enable evaluating the mechanisms that may lead to disruption of the adipose tissue in oxybenzone-treated animals. While my analyses did not detect significant differences in relative mRNA expression of PPARγ, adiponectin, or fatty acid synthase (FIGURE 5.3), studies using human bone marrow-derived mesenchymal stem cells demonstrated that both oxybenzone and its metabolite benzophenone 8 increases the size and number of lipid droplets in differentiated adipocytes similar to two phthalates (BBP and DEHP), and tributyltin; this study also determined that oxybenzone significantly increased the mRNA levels of PPARγ (but not PPARα and PPARβ, adiponectin or fatty acid binding protein 4) while directly binding PPARγ (Shin et al., 2020).
Based on this prior study and our evaluations in mice, oxybenzone is a suspected metabolism disrupting chemical. While there is a genetic component to obesity (Fawcett & Barroso, 2010), FIGURE 10.1. summarizes also the non-Mendelian aspect of obesogenesis and the potential roles of oxybenzone in pathogenic features such as adipocyte differentiation, proliferation and apoptosis, and disruption of obesity and other metabolism related hormones [e.g., the balance between insulin and glucagon or leptin, as demonstrated for BPA (Angle et al., 2013)]. In humans, parental obesity begets offspring obesity via persistent lifestyle habits (Gray, Hernandez Alava, Kelly, & Campbell, 2018) but emerging hypotheses also point to possible epigenetic transgenerational inheritance of obesity due to

**Figure 10.1: Hallmarks of obesogenesis for oxybenzone.**
maternal diet and possibly also due to exposures to environmental contaminants (Chamorro-Garcia et al., 2013; Lillycrop, 2011). Additional studies are also needed to investigate whether adipose tissue has "windows of sensitivity" to oxybenzone and other metabolism disrupting chemicals, and if, so how long these windows last as well as the span between an exposure and effect. The in utero "set point" seems the most likely window of vulnerability, however as Heindel & Blumberg emphasize, these windows may differ by chemical (Heindel & Blumberg, 2019).

**Oxybenzone – a mammary carcinogen?**

Several studies over the past decade have indicated oxybenzone’s potential to co-induce tumorigenic conditions in the mammary gland. Increased proliferation in the post-involuting epithelium and increased volume fraction of the epithelial compartment, along with an increased ratio of ERα positive cells, and DNA damage as well as R-loop formation suggest oxybenzone-induced disruptions to the mammary epithelium consistent with risk factors for mammary cancer. Furthermore, the increased angiogenesis and altered number of macrophages in the mammary tumors we observed in oxybenzone-treated animals, combined with increased motility of breast cancer cells in *in vitro* models, are suggestive of a worse tumor prognosis in oxybenzone-exposed individuals. Smith et al. proposed ten key characteristics of human carcinogens, some of which are overlapping with the up-to-date findings about oxybenzone [FIGURE 10.2] (Smith et al., 2020). Like the key characteristics of endocrine disrupting chemicals, the goal is not to “check all the boxes” to determine if a chemical meets the criteria for a carcinogen. Rather, strong evidence for even one of those key characteristics is sufficient to determine that a chemical is a carcinogen (or an endocrine disruptor). The case for oxybenzone in
Carcinogenesis could be made stronger by further investigating the important emerging hallmarks of cancer including oxidative stress and tumor-associated inflammation, and epigenetic markers of tumorigenesis (Hanahan & Weinberg, 2011). Oxidative stress exemplifies the interconnectivity of the individual hallmarks. Tissue oxidative stress may lead to damage to the DNA, as well as chronic inflammation, increased proliferation and evasion of apoptosis, and angiogenesis (Hayes, Dinkova-Kostova, & Tew, 2020). Epigenetic changes by estrogens and ER agonists include binding to the EREs of the HOX antisense intergenic RNA (HOTAIR) promoters and thereby silencing genes in normal cell development (Bhan et al., 2014; Mozdarani, Ezzatizadeh, & Rahbar Parvaneh, 2020).

Figure 10.2: Hallmarks of Carcinogenesis of Oxybenzone in the Mammary Gland.
In addition to additional studies evaluating these key characteristics of carcinogens, a stronger case for oxybenzone in the process of carcinogenesis would include studies utilizing other models of tumor induction. In Chapter 8, we propose that future studies might utilize mammary carcinogens such as DMBA or NMU, which could be titered to allow evaluations of whether oxybenzone enhances or prevent tumor development at different stages of development. Other ongoing studies in the Schneider lab are evaluating how oxybenzone alters features of metastasis in a mouse model. Collectively, these studies will shed greater light on the role of different cell types (including immune cells) in the response of the mammary gland to oxybenzone.

Oxybenzone – Acting at the intersection of adipose and mammary cancer

Finally, two studies have been published recently that create a link between the two features discussed above: adipose tissue and carcinogenesis. Bokobza et al. considered the adipose tissue a source of chronic exposure to EDCs that can be dissolved, and thus stored, in the fat. Oxybenzone has a partition coefficient (log\text{K}_{\text{ow}}) of 3.45, therefore it is moderately distributed to the body lipids. Obesogenic EDCs capable of accumulating in the fat often create a vicious cycle: while inducing lipogenesis, they also accumulate in the fat and their chronic stimulation of adipocytes releases obesogenic cytokines that further stimulate adipocyte differentiation and triglyceride accumulation that further accumulate obesogenic EDCs such as PFOA or TBT (Bokobza et al., 2021). While oxybenzone has been detected in various bodily samples including the breast tissue, additional evidence is needed to determine the extent that it is stored in body fat.
The second manuscript revealed the results of a dietary experiment combined with an exposure to oxybenzone in the mouse model. It showed that a low-fat diet may provide a degree of protection against mammary tumors in the presence of oxybenzone. On the contrary, even short-term exposure to a high-fat diet during puberty allows oxybenzone to alter some of the key tumor characteristics including tumor latency and tumor type (Kariagina et al., 2020). These findings open up some concerning aspects of the effects of high-fat diet and obesity, both of which have been on the rise worldwide.

Beyond scientific issues

Oxybenzone, like other environmental chemicals and many endocrine disruptors, has implications that reach beyond research to quantify levels of exposures and their effects. As a compound produced in volumes up to 500,000 lbs/year in the U.S. alone (U.S.EPA, 2017), with data suggesting that much of its production has been shifted overseas, oxybenzone is an important chemical in the marketplace. Four industries use the majority of the oxybenzone that is produced annually: the paint industry, floor and wall covering manufacturers, producers of rubber and plastic products, and the industry for perfumes, personal care products and toiletries (SPIN, 2021). The large magnitude of oxybenzone production implies that an a posteriori characterization of its hazards, and assessment of its exposures and the possible risks that the chemical may pose to the human health and environment is needed; it is already too late for a truly public health protective action, considering its widespread use, its contamination of the environment, and its detection in human bodies.
Effects of oxybenzone in traditional toxicity tests elicit decision-making and risk-management decisions issued by respective regulatory state, national or supranational agencies including the U.S. EPA, the U.S. FDA, or the European Commission in the U.S. and EU, respectively. Therefore, apart from being a scientific issue, endocrine disrupting chemicals and their regulation are a political issue. The large magnitude of oxybenzone production also naturally invites the attention of manufacturers of oxybenzone-based products to contribute to political and scientific perspectives. Chemical manufacturing industries perform their own research and create ties to decision-makers in both the regulatory and scientific tiers.

For cosmetics containing oxybenzone, manufacturers in the U.S. rely on the Personal Care Products Council (PCPC) to “cultivat[e] a strong working relationship on behalf of the industry with [….] National Academies” and to “engage with FDA regarding the implementation of [over-the-counter] drug reform” as “PCPC created technical teams for work streams dedicated to supporting eight key sunscreen ingredients” (PCPC, 2021). While theoretically, the regulatory process is open to commentaries and suggestions from all stakeholders, both historically and practically industry’s active involvement in the regulatory process represents a conflict of interest. As an example, the petroleum industry exists “to explore for, find, develop, and sell petroleum resources, and by doing so to make a profit and return value to shareholders” [p.65 (Oreskes, 2019)]. Industries have developed a large number of strategies to conceal these types of conflicts of interest; industries use these tactics extensively (Goldberg & Vandenberg, 2021; Michaels, 2020). One of the latest efforts regarding oxybenzone has been publication of a collaborative paper “critically re-analyzing” the hazard data on oxybenzone while reaching conclusions that
downplay the earlier concerns (Mitchelmore, Burns, Conway, Heyes, & Davies, 2021).

Lastly, oxybenzone and UV protection of the skin is a *medical* issue in the most complex sense. Medical practitioners are rarely versed in environmental aspects of health beyond the patient’s lifestyle choices and occupational risks. As we argue throughout this dissertation, oxybenzone exposure influences multiple aspects of health, including the larger environment of an individual. Therefore, communicating the risks and benefits of various types of skin protection must become a routine practice for medical professionals.

In conclusion, the case of oxybenzone reflects on multiple aspects of human relationships towards their individual choices and the larger environment, on values prevailing in communities, and on regulatory decision-making when facing complex range of variables. Our research has contributed with a small yet not insignificant share to the enormous mosaic of research on endocrine disrupting chemicals, the mammary gland, and environmental health.
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