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Changes in Mammary Histology and Transcriptome Profiles by Low-Dose Exposure to Environmental Phenols at Critical Windows of Development¹

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Abstract

Exposure to environmental chemicals has been linked to altered mammary development and cancer risk at high doses using animal models. Effects at low doses comparable to human exposure remain poorly understood, especially during critical developmental windows. We investigated the effects of two environmental phenols commonly used in personal care products – methyl paraben (MPB) and triclosan (TCS) – on the histology and transcriptome of normal mammary glands at low doses mimicking human exposure during critical windows of development. Sprague-Dawley rats were exposed during perinatal, prepubertal and pubertal windows, as well as from birth to lactation. Low-dose exposure to MPB and TCS induced measurable changes in both mammary histology (by Masson's Trichrome Stain) and transcriptome (by microarrays) in a window-specific fashion. Puberty represented a window of heightened sensitivity to MPB, with increased glandular tissue and changes of expression in 295 genes with significant enrichment in functions such as DNA replication and cell cycle regulation. Long-term exposure to TCS from birth to lactation was

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associated with increased adipose and reduced glandular and secretory tissue, with expression alterations in 993 genes enriched in pathways such as cholesterol synthesis and adipogenesis. Finally, enrichment analyses revealed that genes modified by MPB and TCS were over-represented in human breast cancer gene signatures, suggesting possible links with breast carcinogenesis. These findings highlight the issues of critical windows of susceptibility that may confer heightened sensitivity to environmental insults and implicate the potential health effects of these ubiquitous environmental chemicals in breast cancer.

Keywords

Paraben; Triclosan; Mammary Transcriptome and Histology; Breast Cancer; Windows of Susceptibility

1. INTRODUCTION

Animal studies have unequivocally demonstrated that environmental chemicals can alter mammary gland development and subsequent cancer risk, depending on timing of exposure (Rudel, et al. 2011). For example, prenatal exposure to bisphenol A (BPA) altered mammary gland morphology and gene expression signature indicative of transformation (Moral, et al. 2008). We previously showed that lactating mammary glands were more sensitive to chemical exposures compared to glands at later stages of development (Manservigi, et al. 2015). In the context of breast cancer, gestation, early childhood, puberty and pregnancy may represent ‘windows of susceptibility’ to environmental insults (Rudel, et al. 2011), as all of these developmental stages are characterized by profound changes in mammary gland structure and function (Macias and Hinck 2012) that may alter its susceptibility to cancer (Russo, et al. 2001).

The translation of findings on environmental effects from animal models into humans has been impeded by a number of limitations. First, doses tested in animals have usually been several orders of magnitude higher than human exposures (Rayner, et al. 2005; White, et al. 2007) when in fact environmental chemicals have been found to elicit adverse effects at low concentrations relevant to human exposures (Gioiosa, et al. 2015; Jacobsen, et al. 2012; Manservigi, et al. 2015; Welshons, et al. 2003). Indeed non-linear dose response relationships have been reported for several environmental chemicals (Welshons, et al. 2003). Furthermore, understanding of the mechanisms of action of these chemicals during normal mammary gland development has been largely limited because most animal studies employ carcinogen-induced models (Jenkins, et al. 2007; Lamartiniere, et al. 2011).

Sources of environmental chemicals, including phenols, are diverse and ubiquitous. We focus on two environmental phenols, methyl paraben (MPB) and triclosan (TCS), both of which are commonly added in personal care and household products. Parabens are a group of lipophilic compounds easily absorbed through skin and are widely used as antimicrobial preservatives in cosmetics, toiletries and pharmaceuticals (CIR 2008). Methyl paraben (MPB) is one of the most prevalent parabens detected in urine in the US population (Ye, et al. 2006). Triclosan (TCS) is commonly used as an antimicrobial agent in cosmetics, pharmaceuticals, toys and medical devices, with major exposure occurring via dermal and

oral routes (Yueh and Tukey 2016). Both chemicals have been detected in breast milk (Ye, et al. 2008) MPB was detected in human placenta (Towers, et al. 2015) and TCS was found to accumulate in rodent placenta (Feng, et al. 2016; Wang, et al. 2015). Moreover, both chemicals were detected in cord blood (Pycke, et al. 2014; Pycke, et al. 2015), indicating that MPB and TCS can enter the fetal environment through placental transfer, and also be transferred via breast milk. There are growing public health concerns with these chemicals because not only are their exposures prevalent in the general US population (CDC 2015), they have been found to be associated with adverse outcomes, for example, TCS with body mass index (Lankester, et al. 2013) and with earlier pubertal onset in girls (Wolff, et al. 2015).

Using the Sprague-Dawley (SD) rat model, we examined effects of MPB and TCS on histology and transcriptome profiles of normal (non-cancerous) mammary glands at doses mimicking human exposure. Animals were exposed across several key developmental stages including perinatal, prepubertal and pubertal windows as well as long-term exposures from birth to lactation. Results from our study clearly demonstrate that even at levels comparable to human exposure, environmental phenols can induce changes in mammary histology and gene expression in a window-specific fashion and these changes may have potential implications for breast cancer development.

2. MATERIALS AND METHODS

2.1. Test Chemicals

Methyl paraben (MPB; CAS # 99-76-3, lot # BCBG0852V, 99% purity) and triclosan (TCS; CAS # 3380-34-5, lot # 1412854V, 97% purity) were supplied in plastic containers (Sigma Aldrich, Italy). Compounds were dissolved in the vehicle olive oil (lot #111275, Montalbano Agricola Alimentare Toscana, Italy). All compounds were stored in the dark at room temperature (20°C). The solutions were prepared weekly on the basis of mean body weight of each group and were continuously stirred before and during the treatment. To minimize external contamination, the olive oil and chemicals were stored in glass containers and administered using 5 mL glass syringes. Biological samples were collected in polypropylene vials. Chemical analyses and stability testing have been described previously (Manservigi, et al. 2015).

2.2. Experimental Animals

Animal studies were carried out at Cesare Maltoni Cancer Research Centre/Ramazzini Institute (CMCRC/RI) (Bentivoglio, Italy) in accordance with the rules of Italian law for Animal Welfare (Decreto Legislativo 26, 2014), following the principles of Good Laboratory Practices and Standard Operating Procedures of the CMCRC/RI facility, which include authorization by the ethical committee. The experiment used female Sprague-Dawley (SD) rats which belong to the colony that has been used for over 40 years in the laboratory of the CMCRC/RI. There were no siblings in each treatment group and they were randomized so as to have minimal differences in body weight among them (standard deviation <10% of the average). Animals were housed in makrolon cages (41×25×15 cm) at 2 or 3 per cage, with a stainless steel wire top and a shallow layer of white fir shavings as bedding (Giuseppe

Bordignon, Reviso, Italy). All animals were kept in a single room at $23 \pm 3^{\circ}\text{C}$ and at 40–60% relative humidity. Lighting was artificial and the light/dark cycles were tended to be 12 hours each. All animals were given the same standard “Corticella” pellet diet (Piccioni Laboratory, Milan, Italy). Feed and tap water were available *ad libitum* and were both periodically analyzed to exclude biological and chemical contamination (mycotoxins, pesticides, arsenic, lead, mercury, selenium). F0 generation corresponds to breeders of the experimental animals (F1). The breeder animals were weighed weekly to determine treatment dose, starting from gestation for the perinatal group and during lactation for prepubertal, pubertal, and long-term treatment groups and the dose to be administered was calculated on the basis of the weekly weight. For the perinatal group, all the F1 pups were housed with their dams until sacrifice at postnatal day (PND) 21. F1 pups from all the other groups were housed with their dams until weaning (PND 28), then separated from the dam, identified by ear punch, weighed individually every week and dosed by gavage based on the weekly mean body weight of each group. After weaning, each litter contributed 1 female pup to the study. For animals in the long-term treatment group, water and food consumption, and body weight were recorded weekly for the first 13 weeks of the experiment and every other week thereafter.

2.3. Chemical Treatment

Female SD rats were treated with MPB or TCS at 0.105 and 0.05 (mg/kg/day), representing 1/10,000 and 1/1,000 no observed adverse effect levels (NOAEL) of these chemicals, respectively (Goldsmith LA 1983; Rodriguez and Sanchez 2010; U.S. EPA, Offices of Prevention, Pesticides, and Toxic Substances.). These low doses were previously determined to produce urinary metabolite concentrations comparable to those reported for the US population (Teitelbaum, et al. 2016). Control animals received olive oil. The number of rats used in experiments is shown in table 1. There were three short-term windows (perinatal, prepubertal, pubertal) and one long-term window of exposure from birth to lactation (Fig. 1). In the perinatal window, five animals were treated *in utero* daily with each test chemical or vehicle through oral gavage of their pregnant dams (F0), from gestation day (GD) 1 to GD 20; another five animals were treated daily from PND 1 to PND 20 through milk of lactating dams (F0) exposed by oral gavage; animals from these two groups (F1) were sacrificed at PND 21. Animals in the prepubertal window were exposed from PND 21 to PND 28 through the milk of daily gavaged lactating dams (F0). Subsequently, these rats were exposed by daily oral gavage from PND 28 (weaning) to PND 41 and sacrificed at PND 42. Animals in the pubertal window were treated daily by oral gavage from PND 42 to PND 62, and sacrificed at PND 63. F1 animals in the long-term treatment group were treated daily from PND 1 through milk of dams (F0) until weaning (PND 28) followed by oral gavage 3 times a week (Monday, Wednesday and Friday) until mating (PND 97), pregnancy, delivery of pups (F2) and lactation. F1 animals were sacrificed at the end of lactation at lactating day 28 (PND 146). Chemicals were administered by gastric intubation (gavage) starting with 0.5 mL/animal/dose from PND 28 to PND 63, and then with 1 mL/animal/dose until PND 146. All the experimental animals were euthanized via carbon dioxide inhalation and necropsy was immediately performed for collection of selected tissues. All animals belonging to the same developmental group were sacrificed on the same day and at random during the estrous cycle.

2.4.Histology

Mammary gland histology was performed at the CMCRC/RI, following the standard operating procedures of the laboratory. Number of experimental animals used is shown in table 1. In order to better identify base-line histology we also included results from untreated animals, i.e. age-matched female SD rats from the same breeding facility that were naïve from any chemical exposure, including the vehicle olive oil. Second axillary (right and left) mammary glands were dissected, placed on cardboard and fixed in alcohol 70% for 48 h for histopathological examination. Sections were stained by Masson's Trichrome method as previously described (Lendrum and McFarlane 1940). Weigert's iron hematoxylin stains the nuclei in black, Orange G stains erythrocytes and muscle fibers in red and phosphotungstic and phosphomolybdic acid with aniline blue stains collagen in blue (Bio Optica, Italy). Sections were manually examined by two pathologists in a blinded fashion. Scores for each tissue (glandular, adipose, connective and secretory) were assigned on a 4-point scale: 0= absent; 1= normal pattern, the tissue is consistent with the histological features of mammary gland during the corresponding stage of development; 2= increased; 3= increased and predominant. Statistical differences in distribution of scores between treatment and control were determined separately for each tissue component within each window using the Wilcoxon Rank-Sum test.

2.5.Transcriptome Profiling

The 5th left and right caudal mammary glands were collected in cryovials on ice and shipped at –80 °C to Icahn School of Medicine at Mount Sinai (New York, NY). Tissues were pulverized in liquid nitrogen and total RNA was extracted using Maxwell 16 LEV simplyRNA Blood kit (Promega, WI). RNA concentration was determined using Nanodrop (Thermo Scientific, MA) and RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, CA); samples with RNA Integrity Number ≥ 7 were used for microarrays. Table 1 indicates the number of animals in each group.

Transcriptomes were profiled by GeneChip Rat Gene 2.0 ST arrays (Affymetrix, CA) at the Yale Center for Genome Analysis (Yale School of Medicine, CT). Biotinylated cRNA were prepared according to the standard Affymetrix protocol from 250 ng total RNA (Manual Target Preparation for GeneChip® Whole Transcript (WT) Expression Arrays, Affymetrix 2015). Following fragmentation, 5.2 ug of cRNA were hybridized for 16 hr at 45°C on GeneChip Rat Gene 2.0 ST arrays. GeneChips were washed and stained in the Affymetrix Fluidics Station 450. Arrays were scanned using the GeneChip Scanner 3000. Quality control of .CEL files and pre-processing by robust multiarray average (RMA) method were done using expression console software (Affymetrix, CA). Batch effects were removed using ComBat package (Leek, et al. 2012) in RStudio (R 3.0.2). We applied a signal intensity filter to retain only those probesets with high and stable expression (signal value > 30th percentile in at least 1 experimental group). A variance-based filter was used to retain the top 50% of probesets with high interquartile range. Transcriptome results from short term windows – perinatal, prepubertal and pubertal – were pre-processed by treatment group (MPB and control, TCS and control). Since long-term MPB treatment (PND 1 to PND 146) did not show significant alterations in mammary histology, we generated transcriptome data for long-term TCS-exposed animals and corresponding controls only, and these data were pre-

processed separately from short term data. After normalization and filtering there were ~8000 genes in each of the three datasets. Differential gene expression analysis between treatment and control within each window was performed using linear models for microarray data (limma) package (Smyth 2004) in RStudio. A lenient false discovery rate (FDR) of 25% using Benjamini-Hochberg correction and a fold change of 1.5 fold was used to generate hypotheses about the effects of chemicals. Microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE72276 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72276>)

2.6. Enrichment Analysis

For functional enrichment analysis of differentially expressed genes we used gene ontology (GO) (Ashburner, et al. 2000) via DAVID Bioinformatics Resources 6.7 (Huang da, et al. 2009) and 'Canonical Pathways' as well as enrichment for transcription factors by 'Upstream Analysis' using Ingenuity Pathway Analysis (IPA; www.ingenuity.com). Fisher's exact test was used to assess significance of enrichment (FDR < 0.05 for GO, P < 0.05 for IPA). Enrichment results from GO were mapped into networks using EnrichmentMap (Merico, et al. 2010) in Cytoscape 3.2.0 (Shannon, et al. 2003).

We also used gene set enrichment analysis (GSEA) (Subramanian, et al. 2005) to test whether genes differentially modulated by MPB and TCS were also coordinately disrupted in human breast cancer. We defined MPB and TCS 'gene signatures' as genes differentially regulated by MPB and TCS respectively, at a FDR of 25% using Benjamini-Hochberg correction and a fold change of 1.5, as described above (section 2.5). These chemical gene signatures were split into 'up' and 'down' signatures to indicate direction of changes, resulting in four gene signatures: MPB up, MPB down, TCS up and TCS down. We tested for enrichment of MPB and TCS gene signatures in human breast cancer by using three independent human breast cancer microarray datasets – namely, the Cancer Genome Atlas (TCGA) (<http://cancergenome.nih.gov/>) (61 tumors and adjacent normal microarray data) as well as 2 other breast cancer microarray datasets from the gene expression omnibus (GEO) (Edgar, et al. 2002): GSE15852 (Pau Ni, et al. 2010) (43 tumors and adjacent normal tissues of a multi ethnic panel of Malaysian breast cancer patients profiled on affymetrix microarrays) and GSE14999 (Uva, et al. 2009) (68 breast tumors and 61 adjacent normal tissues purchased from SeraCare Life Sciences and profiled on Agilent microarrays).

GSEA ranks genes in the expression dataset (i.e., from TCGA or GEO) by comparing breast cancer to adjacent normal tissues and then calculates an enrichment score (ES) by walking down the ranked list of genes, increasing a running-sum statistic when a gene is in the chemical gene signature and decreasing it when it is not. The ES is the maximum deviation from zero encountered in walking the list and hence a measure of the degree to which the chemical gene signature (i.e., genes up- or down-regulated by MPB or TCS) is overrepresented at the top or bottom of the ranked list of genes in the expression dataset. In our study setting, a positive ES indicates that the chemical gene signature is enriched in breast cancer tissue, while a negative ES indicates enrichment in normal/adjacent tissue. The normalized ES (NES) accounts for differences in chemical gene signature size and in

correlations between the chemical gene signatures and the expression dataset. The FDR is the estimated probability that a chemical gene signature with a given NES represents a false positive finding, where $<25\%$ is considered appropriate when the number of samples is large (> 7) (Subramanian, et al. 2005). A detailed overview of GSEA is provided in Subramanian et al, 2005 (Subramanian, et al. 2005). We reasoned that if environmental chemical dysregulated genes were also found to be coordinately dysregulated in breast cancer, then the environmental chemical could be etiologically associated with breast cancer. A similar approach has been used previously with gene expression data to predict environmental chemicals associated with disease (Patel and Butte 2010).

2.7.Quantitative PCR (qPCR)

We selected genes for qPCR validation if they were among the top differentially expressed (FDR < 0.25 , fold change > 2) following exposure to MPB and TCS, belonged to key biological pathways in enrichment results from GO and/or IPA and were expressed at high levels ($\log_2$ levels > 7) in both treatment and control groups. Due to the large number of differentially expressed genes by TCS, we further restricted our selection to include genes representative of two key biological pathways disrupted by TCS: extracellular matrix (ECM) and *Ppar-gamma*. cDNA was synthesized from total RNA using iScript cDNA synthesis kit (Bio-Rad, CA). Gene-specific primers were designed using Primer-BLAST and synthesized by MWG Operon (Eurofins Genomics, KY) (Table S3). Forty cycles of PCR amplification were performed as follows: denature at 95°C for 15 sec, anneal at 55°C for 20 sec, extend at 72°C for 30 sec. Assays were performed in triplicate using iQ SYBR Green Supermix (Bio-Rad, CA) on ABI PRISM 7900HT sequence detection system (Applied Biosystems). Data were normalized using expression values of ribosomal protein S11 (*Rps11*) and alpha-tubulin (*Tuba1a*) as reference genes. Statistical differences between treatment and control were determined using t-test.

3. RESULTS

3.1.Environmental phenol exposure induces histological changes in mammary glands

Mammary gland sections were stained with Masson's Trichrome to assess the collagen distribution in the stroma and to determine the relative proportion of each tissue type (glandular, adipose, connective and secretory) at each developmental stage of the gland in control and treatment groups (Fig. 2). The staining scores are summarized in Table 2. Mammary glands from perinatal to pubertal windows had a score of 0 for secretory tissue in all animals examined; hence only the secretory tissue component of long-term (birth to lactation) treated animals is shown.

Distinct histological features were observed at each developmental stage in control mammary glands. In the perinatal window (Fig. 2A–B), the parenchyma was rudimentary and the stromal compartment was filled with adipocytes. Each branch was composed of a single layer of epithelial cells surrounding a central lumen. Prepubertal mammary glands (Fig. 2E–F) had scattered ducts lined by a single or double layer of cuboidal epithelium, which became multilayered to form the terminal end buds (TEBs) comprised of three to six layers of epithelial cells. High rates of cell proliferation have been shown to be associated

with the increased number of cells in TEBs (Hinck and Silberstein 2005). In pubertal glands (Fig. 2I–J) increased side branching and formation of alveolar buds was apparent and lobules of compound branched alveolar glands were separated by dense interlobular connective tissue and fat. In figure 2M–N, the alveoli expanded to fill the gland; as lactation was established at this stage, the fat in the adipocytes was metabolized and mobilization of fat from adipocytes to epithelial cells of the alveoli occurred (Neville 1999). Concurrently there was a reduction in the amount of intra- and interlobular connective tissue.

MPB-treated animals in the perinatal window had significantly decreased amounts of adipose tissue and increased expansion of the ductal tree within the fat pad (Fig. 2C, Table 2). Prepubertal MPB treatment was also associated with a significant reduction in adipose tissue and more abundant glandular tissue (Fig. 2G, Table 2). Pubertal MPB-treated animals had significantly elevated amounts of glandular tissue compared to control, visible as a higher degree of branching relative to the total gland area (Fig. 2K, Table 2). Long-term MPB-treatment from birth to lactation did not result in significant histological changes compared to age-matched controls (Fig. 2O, Table 2).

Animals treated with TCS during perinatal window presented similar histology as age-matched controls (Fig. 2D, Table 2). Prepubertal TCS animals had a significant reduction in adipose tissue and more abundant glandular tissue (Fig. 2H, Table 2). Pubertal TCS animals had significantly elevated amounts of glandular tissue compared to control, visible as a higher degree of branching relative to the total area of the gland (Fig. 2L, Table 2). In long-term TCS-exposed animals from birth to lactation, the stroma increased around the clusters of collapsed alveoli. There was a marked reduction in the size and amount of the lobular structures resulting in reduced amount of glandular and secretory tissue and increased amount of adipose tissue; these changes were not statistically significant ($P = 0.059$), probably due to small sample size (Fig. 2P, Table 2).

3.2. Environmental phenol exposure induces transcriptome changes in mammary glands

MPB treatment led to significant gene expression changes compared to controls; however these changes were observed in the pubertal window only with 295 differentially expressed genes (Fig. 3A). Genes down-regulated by MPB (157 genes) were enriched for ‘immune response’ (GO, FDR = $2.61E-04$) and ‘interferon signaling’ (IPA canonical pathways, $P = 1.97E-03$). Interferon regulatory factor 7 (*Irf7*), a member of the interferon regulatory transcription factor family, was found to be a common transcription factor for many genes down-regulated by MPB (IPA upstream analysis, $P = 2.4E-15$). Up-regulated genes (138 genes) were enriched for functions related to DNA replication and cell cycle regulation, for example, ‘DNA replication’ (GO, FDR = $2.48E-12$) (Fig. 3B) and ‘cell cycle control of chromosomal replication’ (IPA canonical pathways, $P = 2.29E-13$). IPA upstream analysis found E2F transcription factor 1 (*E2f1*), a crucial cell cycle regulator (Muller and Helin 2000), to be a common transcription factor for many genes up-regulated by MPB ($P = 2.6E-21$). Top up-regulated genes by MPB included minichromosome maintenance complex genes (*Mcm2*, *Mcm6*, *Mcm5*), whose products play essential roles in regulating DNA replication and cell proliferation (Tye 1999). Some of the top differentially expressed genes

by MPB (Fig. 3C, Table S1) were chosen for validation by qPCR and direction of gene expression changes was largely consistent with microarray results (Fig. S1A–B).

TCS treatment during short-term windows yielded subtle changes in mammary transcriptome with 11, 1 and 5 differentially expressed genes in perinatal, prepubertal, and pubertal windows, respectively. Long-term TCS treatment from birth to lactation (PND 146) resulted in profound changes with 993 differentially expressed genes (Fig. 4A). Down-regulated genes (397 genes) were enriched for functions related to steroid metabolism including ‘lipid biosynthetic process’ (GO, FDR = 9.08E-05) (Fig. 4B) and ‘super-pathway of cholesterol biosynthesis’ (IPA canonical pathways, $P = 3.11\text{E-}12$). Sterol regulatory element binding transcription factor 2 (*Srebf2*), a critical regulator of cholesterol homeostasis (Yang, et al. 2002), was found to be a common transcription factor for many genes down-regulated by TCS (IPA upstream analysis, $P = 1.52\text{E-}08$). Top down-regulated genes by TCS included two genes which encode rate-limiting enzymes in cholesterol biosynthesis – 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*) and squalene epoxidase (*Sqle*) (Goldstein and Brown 1990; Hidaka, et al. 1990). Up-regulated genes by TCS exposure from birth to lactation (596 genes) were enriched for multiple biological functions including ‘extracellular matrix organization’ (GO, FDR = 1.07E-04) and ‘response to wounding’ (GO, FDR = 0.001) (Fig. 4C) where genes such as matrix metalloproteinases (*Mmp2*, *Mmp3*, *Mmp11*, *Mmp27*) and collagens (*Colla1*, *Colla2*, *Col6a6*) were involved. IPA analysis of up-regulated genes by TCS showed enrichment of the canonical pathway ‘adipogenesis’ ($P = 3.75\text{E-}05$), where *Ppar-gamma*, a master regulator of adipocyte differentiation (Ahmadian, et al. 2013), and several members of the *Ppar-gamma* pathway such as adiponectin (*Adipoq*) and fatty acid binding protein (*Fabp3*), were involved. IPA upstream analysis found CCAAT/enhancer binding protein beta (*Cebpb*), an important regulator of adipogenesis (Cao, et al. 1991), to be a common transcription factor for many genes up-regulated by TCS ($P = 3.04\text{E-}13$). Some of the top significant genes that were representative of key biological functions disrupted by TCS exposure (Fig. 4D, Table S2) were validated by q-PCR (Fig. S1B–C).

3.3.Environmental phenol gene signatures are enriched in breast cancer

In order to examine whether mammary transcriptome changes induced by MPB during the pubertal window and by TCS during lactation were relevant to molecular changes in human breast cancer, we used gene set enrichment analysis (GSEA) to test whether MPB and TCS ‘gene signatures’ – genes differentially expressed at FDR < 0.25 and fold change 1.5 – were enriched among genes differentially expressed in breast cancer compared to adjacent tissues. We used the TCGA breast cancer microarray dataset containing 61 tumors and paired adjacent tissues as well as two other human breast cancer and paired adjacent microarray datasets from GEO (details in Materials and Methods). A similar approach has been used previously whereby gene expression data was used to predict environmental chemicals associated with disease (Patel and Butte 2010). Our result with TCGA showed that genes up-regulated by MPB in our study (‘MPB up’ signature, 138 genes) were enriched with genes that were up-regulated in breast cancers compared to adjacent normal tissues. Genes down-regulated by MPB (‘MPB down’ signature, 157 genes) were enriched with those genes that were coordinately down-regulated in breast cancers (Fig. 5A).

Interestingly, the opposite result was observed for TCS; genes up-regulated by TCS ('TCS up' signature, 596 genes), were uniformly down-regulated in breast cancers and genes down-regulated by TCS ('TCS down' signature, 397 genes), were found to be up-regulated in breast cancers compared to adjacent tissues (Fig. 5B). We also confirmed these findings with two other independent breast cancer datasets from GEO (Fig. S2). In summary, based on these results we hypothesize that the two environmental phenols, MPB and TCS, may have opposing effects on breast cancer pathogenesis with MPB possibly being pro-carcinogenic and TCS possibly being anti-carcinogenic. Further studies will be needed to confirm this hypothesis.

4. DISCUSSION

In this study we observed that human-level exposure to environmental phenols has the capacity to impact mammary histology as well as transcriptome profiles. Developing tissues, including that of the mammary gland, can be particularly sensitive to environmental chemical insults (Rudel, et al. 2011). Several common environmental chemicals have been shown to adversely impact mammary glands at doses below those typically used in toxicological studies (Osborne, et al. 2015). A unique aspect of our study is the use of low doses that are biologically relevant to human exposure. These doses (in mg/kg/day, MPB: 0.105, TCS: 0.05) were 10,000 to 1,000 orders of magnitude lower than NOAEL, respectively (Goldsmith LA 1983; Rodriguez and Sanchez 2010; U.S. EPA, Offices of Prevention, Pesticides, and Toxic Substances.); in our comprehensive dose calibration study we demonstrated that these doses resulted in urinary metabolite levels comparable to those reported for the US population (Teitelbaum, et al. 2016). Another unique aspect is that instead of tumor transcriptome, the study focused on normal tissues in which cancer-induced genomic changes were minimized allowing environmental-induced signals to be detected even at such low doses, enabling better understanding of underlying mechanisms of these common environmental chemicals.

We demonstrated the existence of windows of susceptibility to environmental phenols. Specifically, we noted measurable changes in the mammary gland histology of MPB-treated animals at the pubertal window and of TCS-treated animals from birth to lactation; these changes also corresponded with measurable alterations in mammary transcriptome profiles. In order to better elucidate the molecular mechanisms underlying changes in histology and how these changes may be linked with breast cancer, we performed enrichment analyses for both biological functions as well as for breast cancer, respectively. Our results clearly demonstrate that genes modulated by environmental phenols were not only enriched for functions relevant to mammary development at specific windows of susceptibility – for example cell cycle regulation during puberty (Howlin, et al. 2006) and adipogenesis during the lactation-involution cycle (Pai and Horseman 2011) – but these chemical signatures were also enriched with breast cancer. Many of the changes we report here, including altered mammary gland development, cell cycle regulation and steroidogenesis have all been identified as critical endpoints relevant to breast cancer etiology (Schwarzman, et al. 2015). Even though we observed changes in some histological features of perinatal and prepubertal treated animals, these were not associated with substantial transcriptome changes. Early life exposures to environmental chemicals have been shown to have adverse effects later in life

(Macon and Fenton 2013). Since animals in this study were sacrificed at the end of their exposure window, we did not assess later life consequences after the end of exposure; instead we exposed animals from birth to lactation to investigate long-term effects.

During puberty, the mammary epithelium undergoes extensive growth and forms TEBs, which are highly proliferative, undifferentiated structures that display enhanced sensitivity to environmental insults (Macias and Hinck 2012). MPB-treated animals at the pubertal window had reduced expression of genes involved in immune signaling pathways. Similar results were reported for other common environmental phenols such as BPA and phthalates and these gene disruptions were suggested to be associated with increased cancer susceptibility (Moral, et al. 2008; Moral, et al. 2011). Trichrome staining revealed increased glandular tissue in pubertal MPB-treated animals. Transcriptome results from these animals revealed increased expression of genes involved in DNA replication and cell cycle regulation such as *Mcm2*, *Mcm5* and *Mcm6* and also showed *E2f1* to be an upstream regulator of many MPB up-regulated genes, suggesting increased proliferative activity induced by pubertal MPB treatment (Muller and Helin 2000; Tye 1999). Enhanced growth in breast epithelial and cancer cells were observed upon exposure to MPB at concentrations relevant to those measured in human breast tissue (Charles and Darbre 2013; Khanna and Darbre 2013). Increased glandular tissue and gene changes suggesting increased proliferation associated with MPB exposure in our study may indicate accelerated mammary development, which in turn has been associated with higher mammary cancer risk (Fenton, et al. 2012).

The lactating mammary gland is filled with lobuloalveoli, differentiated structures capable of milk secretion (Macias and Hinck 2012), that have been found to modify breast cancer risk (Russo and Russo 2011). We observed overt disruption in the mammary histology of animals exposed to TCS from birth to lactation, with corresponding alterations in gene expression profiles. In breast cancer cells, TCS has been shown to inhibit fatty acid synthase (Liu, et al. 2002). In our data, many genes in the steroid/cholesterol biosynthesis pathway were down-regulated by TCS, notably two rate-limiting enzymes, *Hmgcr* and *Sqle* (Goldstein and Brown 1990; Hidaka, et al. 1990); Additionally *Srebf2*, a key transcription factor involved in cholesterol synthesis (Yang, et al. 2002), was found to be a common regulator for several genes down-regulated by TCS. These results were consistent with reduced cholesterol biosynthesis. Cholesterol is a significant component of secreted milk (Clarenburg and Chaikoff 1966), hence reduced levels may suggest impaired milk production. The steroid biosynthesis pathway has been shown to be a target for several environmental chemicals (Sanderson 2006). Many genes belonging to lipid biosynthesis were also down-regulated by long-term TCS. Lipid synthesis genes remain elevated throughout lactation with decline only during post-lactational involution (Rudolph, et al. 2003). Our observation of lower secretory and glandular tissue components in the mammary gland coupled with reduced cholesterol and lipid synthesis genes suggests lower milk synthesis in mammary glands of long-term TCS exposed animals. We previously showed by whole-mount preparations that lactating TCS-treated animals had mammary glands containing collapsed alveoli with reduced milk content and decreased expression of several lactation genes (Manservigi, et al. 2015). *Ppar-gamma*, a master regulator of adipogenesis highly expressed in adipose tissue (Ahmadian, et al. 2013), and several genes belonging to the *Ppar-gamma* pathway, were among genes up-regulated by long-term TCS exposure.

Concurrently *Cebpb*, another important regulator of adipogenesis (Cao, et al. 1991) that is intimately linked with *Ppar-gamma* (Rosen, et al. 2002) was found to be a transcription factor for several TCS up-regulated genes. These results were consistent with increased adipose tissue content observed in these animals by histology analysis. Other environmental phenols including BPA have been shown to activate *Ppar-gamma* and induce adipogenesis in cell cultures (Biemann, et al. 2014). During mammary gland involution, apoptosis of the secretory epithelium and replacement by adipocytes occurs (Watson 2006), where activation of wound healing, ECM and vascular remodeling and adipogenesis are involved (Pai and Horseman 2011). Genes with roles in these processes such as matrix metalloproteinases and collagens were among the top up-regulated genes by TCS exposure, suggesting a switch from lactation into involution in these animals (O'Brien, et al. 2010). Exposure to environmental chemicals has been hypothesized to contribute to impaired milk secretion and composition (Neville and Walsh 1995). Our findings lend support to this by suggesting that long-term TCS exposure from birth may be associated with earlier inhibition of milk secretion and altered histology in lactation-stage mammary glands. We previously observed that TCS treatment from birth to lactation was associated with increased pup mortality, with no significant changes in litter size or weight gain, suggesting that impaired gland development and lactation could be associated with reduced offspring survival (Manservigi, et al. 2015).

One surprising finding was the distinctive and even opposing effects of MPB and TCS, both common environmental phenols. Not only did the two chemicals affect different exposure windows, a modest overlap of 46 differentially expressed genes resulted in unique biological functions being enriched with MPB and TCS. While gene signatures of both chemicals were significantly associated with breast cancer, directions of association were opposite – MPB led to changes in genes that are positively correlated with breast cancer, while TCS treatment led to changes in genes that are negatively correlated with breast cancer. It would also be interesting to examine if these genes modified by MPB and TCS in normal mammary glands are enriched in breast cancer adjacent tissues compared to normal breast tissue, given that tumor-adjacent tissues also harbor important genetic information (Troester, et al. 2016), from which environmental signals could possibly be detected. There is a paucity of studies linking parabens and triclosan to breast cancer in human populations; our enrichment analysis results help shed light on such possible associations.

We acknowledge some limitations of our study. One is the limited sample size per experimental group. As a result, a lenient FDR cutoff of 25% to report genes differentially expressed by chemicals was used. However, we used an additional criterion of fold change 1.5 together with the FDR to lend confidence to the results. Another limitation is the lack of a ‘disease’ outcome, such as mammary cancer in our animal experiment. The study was designed to focus on environmental influences on normal mammary development; rats were sacrificed at the end of each treatment window (except for *in utero*-treated rats in the perinatal window, which were sacrificed at PND 21), thus no spontaneous tumors developed. We feel strongly that it is important to examine environmental influences in normal developing mammary glands because tumor tissues harbor gross genomic abnormalities (aneuploidy, gene deletion or duplication, etc.) thus limiting our ability to detect environmental signals that are likely to be subtle. A long-term life-course study allowing

spontaneous mammary tumors to occur would be warranted. Nevertheless, we were able to show by enrichment analyses that our environmental phenol modified genes were enriched with genes differentially expressed in breast cancer, thereby linking our findings in animals with breast carcinogenesis in human populations. The route of exposure in our study was by oral gavage while human exposure to these chemicals is likely to involve multiple routes of exposure, not only oral, but also through the skin or by inhalation (CIR 2008; Yueh and Tukey 2016). Nevertheless, we tried to resolve this issue by calibrating the exposure dose to achieve similar urinary biomarker concentrations between rats and humans. Lastly, mammary glands were collected at random phases of the estrous cycle, which could contribute to some variability within animals belonging to the same group; however we tried to minimize within-group variability by sacrificing animals at the same age and on the same day in the morning. Nevertheless, the uncontrolled estrous cycle is more likely to increase false negative results due to increased within group variations.

Conclusions

Using an animal model designed to mimic human exposure to common environmental phenols, we clearly demonstrate that paraben and triclosan resulted in observable changes in mammary histology and transcriptome that are dependent upon the timing of exposure. Our findings highlight the issues of critical windows of susceptibility that may confer heightened sensitivity to environmental insults and implicate the potential health effects of these ubiquitous environmental chemicals in breast cancer.

Experimental animal studies

Animal studies were carried out at the Cesare Maltoni Cancer Research Centre/Ramazzini Institute (CMCRC/RI) (Bentivoglio, Italy) in accordance with the rules of Italian law for Animal Welfare (Decreto Legislativo 26, 2014), following the principles of Good Laboratory Practices and Standard Operating Procedures of the CMCRC/RI facility, which include authorization by the ethical committee.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Human-level exposure to environmental phenols modifies mammary transcriptome
- Human-level exposure to environmental phenols induces changes in mammary histology
- Exposure-induced changes in mammary glands are developmental window specific
- Chemical-modified genes are enriched with human breast cancer gene signatures

Figure 1. Study design

Female Sprague-Dawley rats were treated by oral gavage with low doses of MPB or TCS with olive oil as control. Treatment was for three short-term windows (perinatal, prepubertal, pubertal) and one long-term window (birth to lactation). In the perinatal window, one group received treatment *in utero* and another group received treatment during early postnatal development. Animals were sacrificed at the end of each treatment window.

Figure 2. Mammary gland histology with Masson's Trichrome staining (10× original magnification)

The second axillary mammary glands were dissected from female Sprague-Dawley rats. Untreated animals refers to age-matched animals of the RI breeding facility and 'experimental controls' refers to vehicle (olive oil)-treated animals. A–B, D. The stromal compartment was predominant and filled with adipocytes. C. Decreased adipose tissue; ductal budding was visible (arrow). E–F. Ductal growth indicated by terminal end buds (TEBs) (asterisk); adipose tissue was predominant. G–H. Decreased adipose tissue; more abundant branching and TEBs. I–J. Branching and formation of alveolar buds; arrow indicates the dense stroma surrounding ducts. K–L. Mammary glands are arranged into lobules of compound branched alveolar glands, separated by dense interlobular connective tissue and fat. M. The secretory alveolar structures filled the fat pad; arrow indicates secretion of milk lipid droplets in the luminal spaces. N–O. Complete development of lobular structures, filling of the mammary fat pad, and secretion of milk fat droplets observed. P. Marked reduction in the size and amount of the lobular structures, with increased adiposity around the clusters of collapsed alveoli.

Figure 3. Pubertal MPB exposure induced transcriptome changes

A. Volcano plot highlighting significantly differentially expressed genes. B. Gene ontology (GO) categories enriched by 138 up-regulated genes. Width of edges is proportional to the number of shared genes between GO categories. C. Heatmap showing top up- (red) and down-regulated (blue) genes by MPB (FDR < 0.25, fold change > 2) and associated biological functions.

Figure 4. TCS exposure from birth to lactation induced transcriptome changes

A. Volcano plot highlighting significantly differentially expressed genes. Enriched GO categories of B. 397 down-regulated and C. 596 up-regulated genes. Width of edges is proportional to the number of shared genes between GO categories. D. Heatmap showing top up- (red) and down-regulated (blue) genes by TCS (FDR < 0.25, fold change > 2) and associated biological functions.

Figure 5. GSEA of environmental phenol gene signatures with breast cancer versus adjacent normal tissues from TCGA

Shown here are enrichment plots of TCGA dataset with A. MPB and B. TCS gene signatures where 'UP' and 'DOWN' indicate genes up- and down-regulated by MPB and TCS, respectively. Green: Profile of the enrichment score (ES), the maximum deviation from 0. A positive ES indicates that the chemical gene signature is enriched in breast cancer tissue, while a negative ES indicates enrichment in normal/adjacent tissue. The normalized ES (NES) accounts for differences in gene signature size and in correlations between the gene signatures and the expression dataset. FDR is a measure of the proportion of false positives corresponding to each NES. Black vertical lines: positions of genes in the chemical gene signature that lie along the ranked breast cancer versus adjacent normal dataset from TCGA.

Table 1
Number of female Sprague-Dawley (SD) rats used in the study

Number of animals receiving treatment by oral gavage with MPB or TCS or vehicle control (olive oil) in each treatment window; (number of animals in histological analyses using Masson's Trichrome staining, number of animals in transcriptome analyses using microarrays). A grand total of 69 animals were used in this study. Age-matched female SD rats from the same RI breeding facility ('untreated animals') were also evaluated by histology; these animals are not shown in this table.

	Control	MPB	TCS	Total <i>n</i> in Windows
Perinatal	10 (9, 10)	10 (9, 10)	10 (10, 9)	30 (28, 29)
Prepubertal	5 (5, 5)	5 (5, 5)	5 (4, 3)	15 (14, 13)
Pubertal	5 (5, 5)	5 (4, 5)	5 (5, 3)	15 (14, 13)
Lactation	3 (3, 3)	3 (3, 0)	3 (3, 3)	9 (9, 6)
Total <i>n</i> in control and treatment groups	23 (22, 23)	23 (21, 20)	23 (22, 18)	69 (65, 61)

Table 2

Masson's Trichrome stain scores for various mammary tissue components (glandular, adipose, connective, secretory) examined in each treatment window. Numbers shown indicate the number of animals achieving a score from 0 to 3 for each tissue component examined (0= absent; 1= normal pattern, the tissue is consistent with the histological features of mammary gland during the corresponding stage of development; 2= increased; 3= increased and predominant). Mammary glands from perinatal to pubertal windows had a score of 0 for secretory tissue in all animals examined; hence only the secretory tissue component of long-term treated animals is shown.

Treatment Window	Tissue component	Compound	Trichrome staining evaluation scores				P value
			0	1	2	3	
Perinatal, PND 21	Glandular	Control	7	2	0	0	Reference
		MPB	5	4	0	0	0.359
		TCS	8	2	0	0	0.954
	Adipose	Control	0	0	0	9	Reference
		MPB	0	8	1	0	*** ⁷ E-05
		TCS	0	2	1	7	0.094
	Connective	Control	0	5	4	0	Reference
		MPB	0	4	5	0	0.341
		TCS	0	9	1	0	0.221
Prepubertal, PND 42	Glandular	Control	1	2	2	0	Reference
		MPB	0	3	2	0	0.817
		TCS	0	0	4	0	0.107
	Adipose	Control	0	0	4	1	Reference
		MPB	0	5	0	0	** ⁰ .0006
		TCS	0	4	0	0	* ⁰ .011
	Connective	Control	0	1	4	0	Reference
		MPB	0	3	2	0	0.27
		TCS	0	3	1	0	0.157
Pubertal, PND 63	Glandular	Control	1	4	0	0	Reference
		MPB	0	0	4	0	* ⁰ .011
		TCS	0	1	4	0	* ⁰ .021
	Adipose	Control	0	5	0	0	Reference

Treatment Window	Tissue component	Compound	Trichrome staining evaluation scores				P value
			0	1	2	3	
Lactation, PND 146	Connective	MPB	0	4	0	0	na
		TCS	0	5	0	0	na
		Control	0	1	4	0	Reference
		MPB	0	0	4	0	0.502
	Glandular	TCS	0	0	5	0	0.424
		Control	0	0	0	3	Reference
		MPB	0	0	0	3	na
		TCS	0	1	2	0	0.059
	Adipose	Control	0	3	0	0	Reference
		MPB	1	2	0	0	0.505
		TCS	0	0	1	2	0.059
		Control	0	1	2	0	Reference
	Connective	MPB	0	3	0	0	0.188
		TCS	0	0	2	1	0.302
		Control	0	0	0	3	Reference
	Secretory*	MPB	0	0	0	3	na
		TCS	0	2	1	0	0.059

Statistical difference in distribution of scores between MPB or TCS treatment and control (olive oil) as reference was determined for each tissue component within each window using Wilcoxon Rank-Sum test:

* indicates $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$.

* The different scores between glandular tissue and secretory activity (as in the case of TCS-exposed mammary gland) was due to the observation that not all the alveoli milk-filled, which is observed as a violet stain with white circles of milk fat visible in Masson's Trichrome stained sections (Fig. 2 M, arrow). Even if the alveoli are expanded to fill the gland (glandular score), the milk-secreting lobules are not always differentiated (secretory score).