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THE BREAST CANCER PROMOTING EFFECTS OF BISPHENOL A (BPA) AND ITS ANALOGUES BPF AND BPS ARE ASSOCIATED WITH ABERRATIONS IN TELOMERE/TELOMERASE AND DNA METHYLOME-WIDE LINKED MECHANISMS

by
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A dissertation
submitted in partial fulfillment of the requirements
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AN ABSTRACT OF THE DISSERTATION OF

Zainab Ibrahim Awada for Doctor of Philosophy
Major: Biomedical Sciences-Pharmacology and Toxicology

Title: The breast cancer promoting effects of bisphenol A (BPA) and its analogues BPF and BPS are associated with aberrations in telomere/telomerase and DNA methylome-wide linked mechanisms

Background: Bisphenol A (BPA), an estrogen-like endocrine disruptor used in plastic production, has been associated with development and promotion of breast cancer in multiple animal and cell culture studies, so plastic manufacturers shifted towards the less-studied analogues, BPF and BPS. Telomerase overexpression and DNA methylation aberrations are known to play essential roles in breast cancer development and/or progression. Hence, studying the cancer promoting effects and associated telomere/telomerase- and DNA methylation-linked mechanisms of BPA analogues is timely, particularly in comparison to BPA.

Methods: We conducted a dual epidemiological-cell culture approach to test the cancer promoting effects of the three bisphenols and their associated telomere/telomerase and DNA methylation effects. In the epidemiological part, we built on two recruited cohorts: 1) 482 non-breast cancer individuals for whom urinary BPA levels were already measured and peripheral blood samples were stored at -80 ^{0}C and 2) 84 breast cancer patients for whom we had access to peripheral blood and tumor and normal adjacent tissue samples. RTL and *LINE-1* methylation were measured in both cohorts using quantitative polymerase chain reaction (qPCR) and bisulfite pyrosequencing, respectively. In the cell culture part, we assessed the metabolic activity, viability, cell cycle and migration of breast cancer cells (estrogen receptor-positive (ER-positive): MCF-7 and ER-negative: MDA-MB-231) treated with BPF and BPS ± ER inhibitor (ERI) in comparison to BPA ± ERI. RNA expression, activity of telomerase and DNA (de)methylation enzymes, relative telomere length (RTL) and *LINE-1* methylation were quantified. DNA methylome-wide analysis was also evaluated in bisphenol-treated MCF-7 cells, and compared to clinical data on 595 ER-positive breast cancer tissues relative to 124 normal adjacent tissues from the Cancer Genome Atlas (TCGA) database.

Results: In the epidemiological part, we observed that higher urinary BPA levels adjusted for urinary creatinine were associated with shorter RTL in females, and were not associated

with *LINE-1* methylation in the peripheral blood of non-breast cancer individuals. Shorter RTL was also observed in peripheral blood and cancer tissues of breast cancer patients when compared to their normal counterparts. *LINE-1* was hypermethylated in peripheral blood of breast cancer patients when compared to non-breast cancer individuals, yet no change was observed in breast cancer tissues relative to normal adjacent tissues. In the cell culture part, the three bisphenols caused ER-dependent increase in proliferation and migration of MCF-7 but not in MDA-MB-231 cells, with BPS being 10 times less potent than BPA and BPF. In MCF-7 cells, these cancer promoting effects were associated with an ER-dependent increase in expression and activity of telomerase, without affecting RTL and DNA (de)methylation enzyme activity. The three bisphenols induced differential DNA methylation alterations at several genomic clusters of or single CpG sites, with the majority of these being ER-dependent. At equipotent concentrations, BPA had the strongest effect on the DNA methylome, followed by BPS then BPF. No pathways were enriched for BPF while BPA- and BPS-induced methylome alterations were enriched in focal adhesion, cGMP-PKG, and cancer pathways, which were also dysregulated in ER-positive breast cancer patients from the TCGA database.

Conclusions: Our results went a step further in elucidating the telomere- and DNA methylation-linked mechanisms of BPF and BPS in comparison to BPA, the contribution of ER pathway in these mechanisms, and their overlap with aberrations occurring in breast cancer patients. We conclude that the three bisphenols induce cancer promoting effects potentially through shortening of RTL, overexpression of telomerase and aberrations in genome-wide DNA methylation that are associated with breast cancer. Further investigation is required to enhance our understanding of the safety of the three bisphenols.

CONTENTS

ILLUSTRATIONS

Figure Page

TABLES

Table Page

LIST OF ABBREVIATIONS

75th P	75th percentile
95th P	95th percentile
ALT	Alternative lengthening of telomeres
AR	Androgen receptors
AVPV	Anteroventral periventricular nucleus
BMI	Body mass index
BPA	Biphenol A
BPF	Bisphenol F
BPS	Bisphenol S
BSA	Bovine serum albumin
BW	Body weight
CD	Cluster of differentiation
Cf	Cell-free
cGMP	cyclic guanosine monophosphate
CHAPS	3[(cholamidopropyl)-dimethyl-ammonium]-1-propanesulfonate
ChIP	Chromatin immunoprecipitation
CI	Confidence interval
CV	Clonal variant
CYP	Cytochrome P450
DES	Diethylstilbestrol
DKK3	Dickkopf WNT signaling pathway inhibitor 3
DMBA	Dimethylbenzanthracene
DMEM	Dubecco's Modified Eagle's Medium
DMP	Differentially methylated probe
DMR	Differentially methylated region
DMSO	Dimethylsulfoxide
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
DOK7	Docking protein 7
EDC	Endocrine disrupting compound
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Agency
ELISA	Enzyme-linked immunosorbent assay,
ER	Estrogen receptor

ERE	Estrogen response element
ERI	Estrogen receptor inhibitor
ERR-ɣ	Estrogen-related receptor gamma
ESI	Electrospray triple quadrupole
ESR1	Estrogen receptor 1 gene
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FDR	False discovery rate
FLD	Fluorescence detector
GC-(ECNI)	Gas chromatography-electron capture negative ion
GD	Gestation day
GM	Geometric mean
HMEC	Human mammary epithelial cells
HPLC-MS	High-performance liquid chromatography tandem mass spectrometry
HPOA	Hypothalamic-pituitary-ovarian axis
*h*PXR	*human* pregnane X receptor
HRBEC	Human high-risk donor breast epithelial cells
*h*TERT	*human* telomere reverse transcriptase
IARC	International Agency for Research on Cancer
IDC	Infiltrating ductal carcinoma
IGF2R	Insulin-like growth factor 2 receptor
IP	Immunoprecipitation
IRB	Institutional Review Board
ITIH5	Inter-alpha-trypsin inhibitor heavy chain family, member 5
KCC2	Potassium chloride cotransporter 2
KIF1A	Kinesin family member 1A
LC	Liquid chromatography
LINE-1	*Long interspersed nuclear element-1*
LOD	Limit of detection
MBDCap-seq	Methyl-binding domain capture sequencing
MBP	4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene
mC	Methylcytosine
MIRA	Methylated CpG island recovery assay
MMTV	Mouse mammary tumor virus
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Not available
NAC	No amplification control
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of health

NMU	*N*-nitroso-*N*-methylurea
NOAEL	No-adverse-effect level
NTC	No template control
OD	Optical density
OR	Odds ratio
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PEG3	Paternally-expressed gene 3
PKG	Protein kinase G
PND	Post-natal day
PR	Progesterone receptor
qPCR	Quantitative polymerase chain reaction
RASSF1A	Ras association domain family member 1
RLM	Robust linear model
ROI	Region of interest
RTL	Relative telomere length
RT-PCR	Reverse transcription-polymerase chain reaction
SC	Subcutaneous
SCG	Single copy gene
SD	Standard deviation
SEM	Standard error of the mean
SHBG	Sex hormone binding globulin
SVA	Surrogate variate analysis
TCGA	The Cancer Genome Atlas
TET	Ten–eleven translocation
TR	Telomerase RNA
TRF	Telomere repeat-binding factor
TRAP	Telomeric repeat amplification protocol
UDP-UGTs	Uridine 5´-diphospho-glucuronosyltransferases
US	United States
USEPA	US Environmental Protection Agency
UTR	Untranslated region
WC	Waist circumference
WHO	World Health Organization
Δß	Delta beta

DEDICATION

To my father and mother who always support me,

and to my grandparents who would have been proud.

CHAPTER I

INTRODUCTION

A. Breast Cancer

1. Breast cancer definition

Breast cancer is a cancer that stems from the mammary gland and is characterized by multiple histopathological types and molecular subtypes (Waks & Winer, 2019). The most common histopathological type is the invasive ductal carcinoma that starts from the breast ducts which physiologically serve the role of carrying milk from the breast lobules (milk glands) to the nipples. The second most common type is the invasive lobular carcinoma which originates from the lobules of the breast (Waks & Winer, 2019). Breast cancer occurs in both males and females, though it is very rare in males (Rudlowski, 2008).

2. Breast cancer epidemiology

According to the most recent estimates from the Global Cancer Observatory, breast cancer is the most common and fatal cancer type among females globally (IARC, 2019). Currently, around 2 million females suffer from breast cancer worldwide, and this number is expected to increase by 970,000 in 2040. Lebanon is among the top 10 countries in the world in age-

standardized breast cancer incidence rate among females (97.6 in 100,000), and has the highest age-standardized breast cancer mortality rate in females (25.3 per 100,000) (IARC, https://gco.iarc.fr/today/home Last accessed: Jan-2019). According to a study by Lakkis *et al.*, median age for breast cancer onset in Lebanon was 52.5 years, a decade earlier than in western countries (Lakkis, Adib, Osman, Musharafieh, & Hamadeh, 2010).

3. Breast cancer risk factors

Heritable factors are responsible for 5-27% of breast cancer risk; however a substantial risk could be contributed by environmental and lifestyle factors which are mostly unknown (Macon & Fenton, 2013). Established risk factors of breast cancer include, besides genetic factors, sex, age, early menstruation, late menopause, late age of first pregnancy, estrogen, obesity, and alcohol consumption (Horn & Vatten, 2017; Jeronimo, Freitas, & Weller, 2017; Lakkis et al., 2010; Sun et al., 2017). Although tobacco smoking contains known carcinogens, its association with breast cancer risk remains controversial (Kispert & McHowat, 2017). Cigarette smoking could be linked to breast cancer through inducing DNA damage and alterations in DNA methylation and expression levels of genes which play a pivotal role in breast cancer progression. One of these genes is *CYP1A1* which has been reported to be hypomethylated and overexpressed in cigarette smokers (K. W. Lee & Pausova, 2013; Rodriguez & Potter, 2013). However, alcohol consumption increases breast cancer risk potentially through elevating estrogen-related hormones in blood and inducing estrogen signaling pathways (Sun et al., 2017). Importantly, many of the breast cancer risk factors such as age, obesity and alcohol intake were shown to be associated with epigenetic

and genetic aberrations such as in DNA methylation (Awada et al., 2019; Christensen et al., 2010; Flanagan et al., 2015; Hannum et al., 2013) and telomere length (Pavanello et al., 2011; Zgheib et al., 2018) respectively.

4. Endocrine disrupting compounds (EDC) and breast cancer

Endocrine disrupting compounds (EDC) are defined by the US Environmental Protection Agency as "exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes". Rodent and cell culture based studies showed that some EDCs such as diethylstilbestrol (DES), phthalates and bisphenol A (BPA) induce breast carcinogenesis either directly or indirectly by increasing the sensitivity of the breast tissue to other carcinogens. These EDCs mimic estrogen in structure, so they bind to estrogen receptors (ER), among others, and interfere with estrogen signaling and disrupt endocrine pathways. Several of breast cancer risk factors (parity, age of menstruation, age of menopause) are estrogen-dependent processes that may also be influenced by exposure to EDCs (Macon & Fenton, 2013). Noteworthy, EDCs may influence tumorigenesis through genomic mechanisms involving ER-dependent regulation of transcription of target genes, as well as non-genomic mechanisms *via* ER-mediated activation of other signaling pathways such as PI3K-pAkt, MAPK-pErk and GPER-pErk, and epigenetic mechanisms by aberrations in DNA methylation, miRNA and histone structure (Gore et al., 2015).

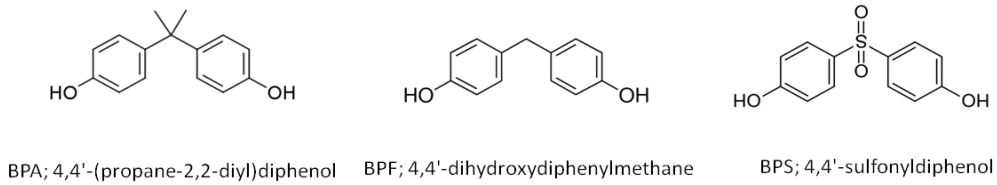
B. BPA and its analogues BPF and BPS

BPA is a monomer of polycarbonate plastics and resins that are used in the production of several consumer products including food cans, thermal receipts, water pipes, toys, medical equipment and electronics. A number of compounds that are structurally similar to BPA, such as bisphenol F (BPF) and bisphenol S (BPS) are collectively termed as bisphenol analogues, and have been more recently used in the synthesis of polycarbonate plastics and resins. BPF is used in the production of lacquers, water pipes, dental sealants, and food containers. BPS is extensively used in the production of food cans and thermal receipts. Bisphenols have been found to be detectable in several environmental compartments such as indoor dust, water and sediments, and in a wide variety of consumer and food products (D. Chen et al., 2016).

1. Chemical structure of BPA, BPF and BPS

BPA, BPF and BPS are structurally similar compounds (**Figure 1**). BPA (bis(4-hydroxyphenyl) propane) is an organic compound made of two benzene rings, two methyl groups and two hydroxide groups (C15H6O2) (Apau, Acheampong, & Adua, 2018). Similarly to BPA, BPF (4,4'-dihydroxydiphenylmethane) has two benzene rings and two hydroxyl groups, but it lacks the two methyl moieties making it more polar than BPA. BPS (4,4'-Sulfonyldiphenol) shares two benzene rings and two hydroxyl groups with BPA but instead of the carbon bonded to the two methyl moieties, BPS has a sulfonyl group that bridges the two benzene rings. Hence, BPS is the most polar amongst the three bisphenols.

Figure 1. Chemical structures of bisphenol A (BPA), bisphenol F (BPF) and bisphenol S (BPS)



BPA; 4,4'-(propane-2,2-diyl)diphenol BPF; 4,4'-dihydroxydiphenylmethane BPS; 4,4'-sulfonyldiphenol

2. Pharmacokinetics of BPA, BPF and BPS

Humans are exposed to BPA mainly through oral intake of contaminated food and water and to a lesser extent through transdermal absorption or inhalation of airborne particles. After oral intake, BPA is completely and rapidly absorbed from the gastrointestinal tract (Volkel, Colnot, Csanady, Filser, & Dekant, 2002). This is rapidly followed by extensive first-pass metabolism in the liver and gut by uridine 5´-diphospho (UDP)-glucuronosyltransferases (UGTs) into the biologically inert BPA glucuronide, and to a lesser extent by sulfotransferases in the liver to BPA sulfate. BPA and its conjugates are released into the blood stream and rapidly eliminated in the urine (Nachman, Hartle, Lees, & Groopman, 2014). Importantly, BPA was more recently reported to be metabolized into a biologically active metabolite, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), which has 1000 times higher potency to ERs than BPA (Baker & Chandsawangbhuwana, 2012). BPA can also be metabolized by several CYP2C enzymes as shown in yeast Saccharomyces cerevisiae and Escherichia Coli, suggesting that CYP2C enzymes in the human liver are involved in its metabolism. Besides, BPA was shown to competitively inhibit the hydroxylation of progesterone by cytochrome P450 (CYP) 2C17, although it was not metabolized by this enzyme. This suggests that it may interfere with the metabolism of

this hormone in the body (Niwa et al., 2001). In the plasma, BPA binds to albumin, yet with low affinity, and it also binds to sex hormone binding globulin (SHBG) (Teeguarden, Waechter, Clewell, Covington, & Barton, 2005).

Oral administration of 100 µg/kg body weight BPA to men and women resulted in a maximum total BPA (conjugated and unconjugated) of 390ng/ml at 1.1 hrs. Unconjugated BPA started to appear in blood after 5 to 20 min of administration and reached maximum concentration of 1.5 ng/ml at 1.3 hrs. Most participants eliminated almost all of BPA (> 90%) as metabolites in the urine within 24hrs (Thayer et al., 2015). Although BPA has a short half-life of 5-6 hrs in adults (Volkel et al., 2002), it is believed that it maintains a detectable and steady serum level because of its continuous exposure.

The pharmacokinetics of BPF and BPS are less clear, but animal and cell culture models revealed that the biotransformation of BPF and BPS is similar to that of BPA (Apau et al., 2018). In addition to its conjugation into BPF glucuronide and sulfate, BPF is transformed to several hydroxylated and non-polar metabolites (N. Cabaton et al., 2008; Dumont et al., 2011). As for BPS, cell culture studies reported that its hydroxylation by CYP3A4 and CYP2C9 is the major phase I biotransformation reaction. However, the resulting hydroxylated derivative is still active (weak estrogen agonist), and has antagonistic activity against thyroid hormone receptor. Similarly to BPA and BPF, BPS is transformed to BPS glucuronide that is an inactive metabolite; hence, this was reported to be the major BPS metabolic and detoxification pathway (Skledar et al., 2016; Y. Song, Xie, & Cai, 2017).

3. Human exposure to BPA

It is believed that human exposure to BPA mainly occurs through ingestion, as high temperature and acidic or basic conditions may lead to hydrolysis of polymers and eventually leaching of bisphenols into food or drinks. Exposure can also occur through dermal contact with thermal receipt papers or inhalation of contaminated airborne dust (Kundakovic & Champagne, 2011; Mileva, Baker, Konkle, & Bielajew, 2014). BPA exposure is thus considered a major health concern due to its extensive use and remarkable environmental exposure.

BPA has been detected in serum, fetal plasma, placental tissue, amniotic fluid, urine, saliva and breast milk samples from individuals of different populations (Kelley, Ngounou Wetie, & Darie, 2015). Several studies measured non-occupational BPA in blood samples (plasma or serum) of human individuals, and levels ranged from below the limit of detection (LOD) to 22 ng/ml (<LOD – 96nM) (**Table 1**). In one study with 37 pregnant women between weeks 32 and 41 of gestation, BPA was detected in maternal plasma, fetal plasma and placental tissues, and its levels in placental tissues ranged from 1.0 to 104.9 ng/g with median being 12.7 ng/g (Schonfelder et al., 2002).

Since BPA is rapidly conjugated before urinary elimination, urine is considered the best matrix for epidemiological studies investigating human exposure to BPA (A. M. Calafat et al., 2013). Hence, more epidemiological studies measured total BPA (free and conjugated) in urine than in blood samples. As such, large biomonitoring studies revealed that greater than 90% of the US (A.M. Calafat, Ye, Wong, Reidy, & Needham, 2008) and Canadian participants (Bushnik et al., 2010) had detectable urinary BPA concentrations of $\geq$

0.1ng/ml. Similarly, total BPA (free and conjugated) was detectable in more than 90% of a random sample of 653 mother children pairs from six European member states, and concentrations reached 455.62 ng/ml in mothers and 821.9 ng/ml in children. Interestingly, BPA levels in mothers were correlated with those in their children indicating common dietary/environmental determinants of BPA levels (Covaci et al., 2015). Besides, BPA and its conjugates were detected in urine samples of different populations in Southeast Asia (Arakawa et al., 2004; Matsumoto et al., 2003; Ouchi & Watanabe, 2002; M. Yang et al., 2003). For instance, BPA glucuronide was detected in urine samples from forty-eight female Japanese students at concentrations ranging from 0.2-19.1 ng/ml (Ouchi & Watanabe, 2002). In addition, biological monitoring studies in seventy-three Korean individuals observed a geometric mean of urinary BPA of 9.54ng/ml (M. Yang et al., 2003). Moreover, total BPA (free and conjugated) was detectable in more than 90% of 296 urine samples derived from participants belonging to seven Asian countries: Kuwait, Korea, India, China, Vietnam, Malaysia, and Japan, with median BPA levels of 3.05, 2.17, 1.71, 1.18, 1.10, 1.06, and 0.95 ng/ml respectively (Z. Zhang et al., 2011).

It is worth to note that occupational BPA levels in BPA manufacturers were around 10-100 times higher than non-occupational levels (Heinala, Ylinen, Tuomi, Santonen, & Porras, 2017; Ribeiro, Ladeira, & Viegas, 2017; Xiao, Wang, Cai, He, & Zhou, 2009). As such, one study in China reported a median BPA level of 101.94 ng/ml in blood samples from 20 BPA exposed workers (Heinala et al., 2017). Another study in Finland reported a median BPA level of 130-250 ng/ml in post-shift urine samples from manufacturers of coating

material, and the highest BPA levels ranged from 1000 to 1500 ng/ml in their study (Xiao et al., 2009).

In Lebanon, Mouneimneh *et al.* measured non-occupational BPA levels in urine samples from 501 adults and observed detectable BPA levels (>LOD of 0.1ng/ml) in 89% of participants, whereby mean BPA ± SD was 3.67 ± 4.75 ng/ml and mean creatinine-adjusted BPA ± SD was 2.90 ± 4.79 μg/g (Mouneimne et al., 2017).

4. Human exposure to BPA analogues: BPF and BPS

Compared to BPA, fewer studies measured the levels of BPF and BPS in human samples. The majority of these studies focused on measuring BPF and BPS levels in urine samples (Lehmler, Liu, Gadogbe, & Bao, 2018; Liao et al., 2012; Philips et al., 2018; Ye et al., 2015; T. Zhang et al., 2016; X. Zhou, Kramer, Calafat, & Ye, 2014), and only one study measured BPS levels in blood samples from human individuals (Thayer et al., 2016). In one study measuring BPA, BPF and BPS in urine samples collected at eight time points between 2000 and 2014 from 616 US adults, the detection frequency of BPF and BPS was lower than that of BPA, whereby BPF was detected in 42 to 88%, BPS in 19 to 74%, while BPA in 74 to 99% of the samples depending on the sampling year. The 95th percentiles of BPF were comparable and at some time points even higher than those of BPA and ranged from 0.7 to 11.7 ng/ml (3.5nM to 58nM) over the eight sampling time points. Median levels of BPF ranged from <LOD to 0.4ng/ml (<LOD to 2nM) (Ye et al., 2015), and these levels were roughly similar to those reported in other populations (**Table1**).

BPS levels in human samples were first investigated in urine samples from USA and seven Asian countries, whereby they were detectable in 81% of the urine samples with a mean of 0.168 ng/ml and a maximum of 21 ng/ml. These levels were found to be 10 folds less than that of BPA, except in Japanese samples where BPS levels were higher with a mean of 1.18ng/ml (Liao et al., 2012). BPS levels were also high in urine samples of people residing in Jeddah and Saudi Arabia, whereby mean BPS levels were higher than those of BPA and BPF (Asimakopoulos et al., 2016). The three bisphenols were also detected in urine samples from a population in China (Y. Yang, Guan, Yin, Shao, & Li, 2014). So far, there are no data on BPF and BPS levels in the Lebanese population.

Only one study measured BPS in serum samples of cashiers handling different forms of thermal receipt papers before and after their shift. Interestingly, more individuals had detectable BPS levels in serum after handling of BPS-containing thermal receipts (13 out of 32) when compared to results before handling of BPS thermal receipts (5 out of 32) with LOD being 0.01 ng/ml (3.9nM) (Thayer et al., 2016). No investigators have yet measured BPF levels in serum. In addition, there are no data on BPF and BPS serum levels in the Lebanese.

It is noteworthy that occupational levels of BPS were 10 times higher than non-occupational levels; for instance, a recent study reported that median BPS levels in urine samples were 2.53 ng/ml in manufacturers and 0.67 ng/ml in controls (Ndaw et al., 2018). However, there are no data on occupational BPF levels in literature.

Table 1. BPA, BPF and BPS levels in human blood and urine from different populations

EDC	Reference	Sample	N	Description of participants	Country	Measurement method	LOD (ng/ml)	Levels (ng/ml)	Max. (ng/ml)
BPA	Mouneimneh 2017 (Mouneimne et al., 2017)	Urine	501	Lebanese adults	Lebanon	HPLC-MS	0.1	Mean (SD): 3.67 ± 4.75 μg/L	59.72
	Savastano 2015 (Savastano et al., 2015)	Plasma	76	Caucasian adult men	Italy	Competitive ELISA protocol	0.025	Mean (SD): 1.04 (0.77)	3.3
	Sprague 2013 (Sprague et al., 2013)	Serum	264	Postmenopausal women	US	HPLC-MS	0.24	Median: 0.55	8.77
	Zhou 2013 (Q. Zhou et al., 2013)	Serum	290	137 exposed to BPA and 153 unexposed	China	HPLC/FLD	0.39	median (exposed): 3.198 median (unexposed): 0.276	NA
	Cobellis 2009 (Cobellis, Colacurci, Trabucco, Carpentiero, & Grumetto, 2009)	Serum	69	11 healthy and 58 endometriotic women	Italy	HPLC/FLD	0.18	Mean (SD): 2.91 (1.74)	7.12
	Padmanabhan 2008 (Padmanabhan et al., 2008)	Maternal plasma	40	Pregnant women	US	HPLC-MS	0.5	Mean (SEM): 5.9 (0.94)	22.3
	Kuroda 2003 (Kuroda et al., 2003)	Maternal serum, umbilical	9	Pregnant women	Japan	HPLC/FLD	0.04	Maternal serum: 0.46 (0.20) and umbilical cord	7.0

	cord serum						serum: 0.62 (0.13)	
Schönfelder 2002 (Schonfelder et al., 2002)	Maternal plasma, fetal plasma, placental tissue	37	Caucasian women	Germany	GC-MS	0.1	Mean (SD) 4.4 (3.9) (maternal plasma), 2.9 (2.5) (fetal plasma), 11.2 (9.1) ng/g (placental tissue)	Maternal plasma: 18.9, fetal plasma: 0.2, placental tissue: 1.0ng/g
Sajiki 1999 (Sajiki, Takahashi, & Yonekubo, 1999)	Serum	21	12 Women and 9 men	Japan	HPLC-MS	0.1 for HPLC-MS	Mean (SD): 0.33 (0.54) (women), 0.59 (0.21) (men)	Women: 1.6, men: 1.0
Lehmler *et al.* 2018 (Lehmler et al., 2018)	Urine	1808	Adults	US	HPLC-MS/MS	0.1	median: 1.24	2.49 (75^{th} P)
		868	Children	US	HPLC-MS/MS	0.1	median: 1.25	2.42 (75^{th} P)
Philips *et al.* 2018 (Philips et al., 2018)	Urine	1396	Pregnant women	The Netherlands	HPLC-MS/MS	0.03 - 0.18	median: 1.66	3.56 (75^{th} P)
Zhang *et al.* 2016 (T. Zhang et al., 2016)	Urine	116	Men (66), Women (50)	China	ESI-MS/MS	0.05	Median: 3.00	27.6
Covaci *et al.* 2015 (Covaci et al., 2015)	Urine	674	Women	Europe[#]	LC-MS/MS and GC-(ECNI)MS	0.11 - 1	GM (95%CI): 1.78 (1.62–1.94)	455.62
		674	Children	Europe[#]	LC-MS/MS and GC-	0.11 - 1	GM (95%CI): 1.97 (1.81 - 2.15)	821.90

						(ECNI)MS			
	Ye *et al.* 2015 (Ye et al., 2015)	Urine	616	Adults (males and females)	US	HPLC-MS/MS	0.1	median: 1.075	6.79 (95^{th} P)
	Zhou *et al.* 2014 (X. Zhou et al., 2014)	Urine	100	Adults (males and females)	US	HPLC-MS/MS	0.06	median: 0.72	37.7 (95^{th} P)
	Zhang *et al.* 2011 (Z. Zhang et al., 2011)	Urine	296	Men (153), women (143)	Asian countries*	HPLC-MS/MS	0.1	GM (95% CI): 1.20 (1.06 - 1.50)	30.1
	Calafat *et al.* 2008 (A.M. Calafat et al., 2008)	Urine	2517	Adults and children ≥ 6 years	US	HPLC-MS/MS	0.4	median: 2.7	149
BPF	Lehmler *et al.* 2018 (Lehmler et al., 2018)	Urine	1808	Adults	US	HPLC-MS/MS	0.2	median 0.35	1.11 (75^{th} P)
			868	Children	US	HPLC-MS/MS	0.2	median: 0.32	0.99 (75^{th} P)
	Philips *et al.* 2018 (Philips et al., 2018)	Urine	1396	Pregnant women	The Netherlands	HPLC-MS/MS	0.03 - 0.18	median: 0.57	1.29 (75^{th} P
	Zhang *et al.* 2016 (T. Zhang et al., 2016)	Urine	116	Men (66), Women (50)	China	ESI-MS/MS	0.12	median: 0.365	8.68
	Ye *et al.* 2015 (Ye et al., 2015)	Urine	616	Adults (males and females)	US	HPLC-MS/MS	0.1	median: 0.28	6.15 (95^{th} P)
	Zhou *et al.* 2014 (X. Zhou et al., 2014)	Urine	100	Adults (males and females)	US	HPLC-MS/MS	0.06	median: 0.08	212
BPS	Thayer *et al.* 2015 (Thayer et al., 2016)	Serum	77	Cashiers	USA	LC-MS/MS	0.01	Mean (SD): 0.037 (0.026)	0.082

	Lehmler *et al.* 2018 (Lehmler et al., 2018)	Urine	1808	Adults	US	HPLC-MS/MS	0.1	median: 0.37	0.88 (75^{th} P)
	Philips *et al.* 2018 (Philips et al., 2018)	Urine	1396	Pregnant women	Netherlands	HPLC-MS/MS	0.03 - 0.18	median: 0.36	1.08 (75^{th} P)
	Zhang *et al.* 2016 (T. Zhang et al., 2016)	Urine	116	Men (66), Women (50)	China	ESI-MS/MS	0.12	median: 0.364	1.38
	Ye *et al.* 2015 (Ye et al., 2015)	Urine	616	Adults (males and females)	US	HPLC-MS/MS	0.1	median: 0.14	1.11 (95^{th} P)
	Zhou *et al.* 2014 (X. Zhou et al., 2014)	Urine	100	Adults (males and females)	US	HPLC-MS/MS	0.06	median: 0.13	12.3 (95^{th} P)
	Liao *et al.* 2012 (Liao et al., 2012)	Urine	31	males and females (2-84 yrs)	USA	HPLC-MS/MS	0.02	Mean (SD): 1.12 (3.74)	21.0
			89	males and females (2-84 yrs)	China	HPLC-MS/MS	0.02	Mean (SD): 0.525 (0.62)	3.16
			38	males and females (2-84 yrs)	India	HPLC-MS/MS	0.02	Mean (SD): 0.171 (0.239)	0.881
			36	males and females (2-84 yrs)	Japan	HPLC-MS/MS	0.02	Mean (SD): 2.27 (2.57)	9.57
			33	males and females (2-84 yrs)	Korea	HPLC-MS/MS	0.02	Mean (SD): 0.099 (0.34)	1.98
			30	males and females (2-84	Kuwait	HPLC-MS/MS	0.02	Mean (SD): 0.785 (2.18)	12.1

				yrs)					
			29	males and females (2-84 yrs)	Malaysia	HPLC-MS/MS	0.02	Mean (SD): 0.128 (0.17)	0.922
			29	males and females (2-84 yrs)	Vietnam	HPLC-MS/MS	0.02	Mean (SD): 0.198 (0.164)	0.932

EDC: endocrine disrupting compound, HPLC-MS/MS: high-performance liquid chromatography tandem mass spectrometry, ELISA: enzyme-linked immunosorbent assay, US: United States, GC-(ECNI): gas chromatography-electron capture negative ion, LC: liquid chromatography, ESI: electrospray triple quadrupole, FLD: fluorescence detector, 75^{th} P: 75^{th} percentile, 95^{th} P: 95^{th} percentile.

[#]Europe: six countries (Belgium, Denmark, Luxembourg, Slovenia, Spain and Sweden), *Seven Asian countries: China, Vietnam, Malaysia, India, Kuwait, Japan, Korea.

5. Mechanism of action of BPA

According to E-screen assay (assay for screening of estrogenic activity), BPA can induce the proliferation of MCF-7 cells (ER-positive breast cancer cells); hence, proving that it possesses estrogenic activity. However, this assay, although considered sensitive for detection of estrogenic activity of compounds, is based solely on the ability of compounds to induce proliferation of MCF cells and is not specific (Soto et al., 1995). For instance, mitogens that induce proliferation without interacting with ERs may be falsely detected as estrogenic compounds by this assay. Nevertheless, the estrogenic activity of BPA was confirmed in additional assays such as by competitive binding assays and in transient gene expression assays (using human embryonal kidney 293 cells transfected with both ER cDNA and estrogen-dependent luciferase reporter plasmids). In the competitive binding assay, BPA was found to bind to both subunits of ERs, ER1 and ER2, with an affinity of approximately 1,000 to 10,000 folds lower than that of estradiol (Kuiper et al., 1998). In the transient gene expression assay, BPA was able to induce the transcriptional activity of ER at concentrations between 100-1000nM (Kuiper et al., 1998).

The low affinity of BPA to nuclear ER does not translate into negligible biological activity, since it was observed that BPA could induce estrogen-like effects that are equal to or even stronger than that of estradiol (H. Gao et al., 2015).This is attributable to the mechanisms other than the genomic pathways that are mediated by BPA.

Concerning the genomic mechanism, similar to estrogen, BPA binds to the nuclear ER and induces the formation of transcriptionally active ER dimers that regulate gene expression

through the classical genomic pathway which involves binding of the activated ER dimer to estrogen response elements (ERE) in promoters of target genes. However, ER dimers can also regulate gene expression in an ERE-independent mechanism through protein-protein interaction with DNA-binding transcriptional factors (Bjornstrom & Sjoberg, 2005; Fujimoto & Kitamura, 2004).

As for the non-genomic mechanisms, this occurs through binding to plasma membrane ER or GPR30 and inducing signaling pathways such as PI3K-pAkt, MAPK-pErk and GPER-pErk (Gore et al., 2015). In contrast to the genomic mechanisms that are slower, non-genomic mechanisms are rapid and can be induced at very low levels of BPA. For instance, 1pg of BPA stimulated calcium influx within 30 sec, and prolactin release within 1 min in rat pituitary tumor cells. Besides, 0.1nM of BPA stimulated the influx of calcium within 1.5 min in MCF-7 cells, and the EC50 of BPA (0.11nM) was similar to that of estradiol (0.15nM). In mouse Leydig cells, 1nM BPA increased the expression of Nur77 gene potentially through phosphorylating MAPK that occurs within 5-10 min of administration of BPA. Using breast cancer cells that lack ERs, BPA was reported to stimulate GPER/EGFR/ERK pathway. When compared to estradiol, BPA interacts differently with ER1, binds to a domain of ERs distinct from the estradiol binding site (H. Gao et al., 2015), and recruits different transcriptional co-regulators in different cells contingent on the available ER subtype (Welshons, Nagel, & vom Saal, 2006). In addition, BPA binds to ER-related receptor gamma (ERR-ɣ) that has been found in significant amount in the placenta, which may explain BPA-induced developmental effects (H. Gao et al., 2015).

Beside estrogen-like effects, BPA mediates anti-androgen effects through binding to

androgen receptors (AR) and forming AR/BPA complexes that prevent the binding of endogenous androgens to AR. Moreover, BPA is a weak agonist to human pregnane X receptor (*h*PXR) (Molina-Molina et al., 2013). It also binds to the thyroid hormone receptors and inhibits the gene transcriptional effects of the thyroid hormone (H. Gao et al., 2015; Moriyama et al., 2002).

6. Mechanism of action of BPA analogues: BPF and BPS

As for BPF and BPS, some cell culture studies have been performed to detect their mechanism of action, the majority of which focused on their estrogenic activity. Similarly to BPA, BPF and BPS possess estrogenic and antiandrogenic activity (Molina-Molina et al., 2013; Rochester & Bolden, 2015; Rosenmai et al., 2014). A systematic review compared the estrogenic genomic activities mediated by BPA, BPF and BPS and reported that the mean estrogenic potency ± SD of BPF relative to BPA is 1.07 ± 1.2, while that of BPS relative to BPA is 0.32 ± 0.28, indicating that the estrogenic potency of BPF is comparable to that of BPA, whereas BPS is ~ 10 folds less potent than BPA and BPF (Rochester & Bolden, 2015). Noteworthy, this is not the case for non-genomic effects of BPS, whereby the potency of BPS in non-genomic signaling was similar to that of BPA. For instance, similarly to BPA, short exposure to picomolar to femtomolar concentrations of BPS (10^{-14} M) stimulated plasma membrane ER and activated MAPK signaling pathways in rat pituitary cells. This concentration of BPS also increased caspase 8 activity which was not altered by BPA. Noteworthy, 10^{-15} M of BPA or BPS activated the ERK pathway more than 10^{-9} M estradiol in rat pituitary cells indicating that the potency of BPA and BPS in non-genomic signaling is sometimes stronger than that of estradiol (Vinas & Watson, 2013).

Notably, the non-genomic mechanism of BPF is not elucidated well in the literature. Furthermore, BPF and BPS were found to bind to the thyroid hormone receptor but had lower potency than that of BPA. In the absence of thyroid hormone, they act as agonists and induce gene transcription, but in the presence of the hormone, they act as agonists or antagonists depending on the concentration (Y. F. Zhang et al., 2018).

7. BPA-associated health outcome

Numerous preclinical and clinical studies reported that BPA exposure is associated with several adverse health outcomes on the developmental, reproductive, endocrine, cardiovascular, and nervous systems (Bonefeld-Jorgensen, Long, Hofmeister, & Vinggaard, 2007; Crain et al., 2007; Lang et al., 2008; Melzer, Rice, Lewis, Henley, & Galloway, 2010; Richter et al., 2007; vom Saal et al., 2007). Moreover, animal studies reported a link between BPA and carcinogenesis in estrogen-responsive tissues such as the mammary gland and several reproductive organs (Seachrist et al., 2016).

a. BPA and developmental effects

Fetuses during gestation and infants during lactation periods are the most vulnerable to the effects of BPA exposure. BPA has been detected in placental tissues, fetal liver tissues, fetal serum and breast milk (Kelley et al., 2015). In a human study, prenatal exposure to BPA (measured in maternal urine samples at around 16 weeks of gestation) was associated with hyperactivity and aggressive behaviors in the children at 2 years of age; particularly in

females (Braun et al., 2009). Moreover, prenatal exposure to BPA was associated with decreased lung function and more incidence of persistent wheezes among children (Spanier et al., 2014). Prenatal BPA exposure in humans was also associated with chromosomal abnormalities; for instance, one study reported that serum BPA levels in women with fetuses of abnormal karyotypes were significantly higher than BPA levels in women with fetuses of normal karyotype (Yamada et al., 2002).

Data from animal studies indicate that unconjugated BPA diffuses through the placenta into the fetus, and absence of the primary phase II BPA metabolizing enzyme, UDP-glucuronosyltransferase in fetuses and newly born neonates results in BPA accumulation, and thereby developmental adverse events (Kelley et al., 2015). Gestational and/or lactational exposure to BPA in animals resulted in several adverse health outcomes at doses below the no-adverse-effect level (NOAEL) (5 mg/kg/day) and even below the human safe reference dose (50 μg/kg/day).These were mostly related to changes in estrogen responsive tissues (breast, uterus, ovaries) and endocrine organs (prostate), such as alterations in puberty time and estrous cycles, development of prostatic neoplasias, changes in mammary gland development which included development of precancerous mammary lesions and intraductal hyperplasias in adulthood, changes in the uterus and ovaries, changes in body weight, and altered glucose homeostasis. Other adverse events included alterations in brain development with secondary behavioral changes including increased aggressiveness, reduced differences in sexually dimorphic behaviors, changes in cognitive behaviors, and increased susceptibility to drug addiction (Rubin, 2011).

b. BPA and the female reproductive system

Based on a recent review, it was noted that BPA has toxic effects on the ovaries and uterus at doses below NOAEL (5 mg/kg/day) and even below the human safe reference dose (50 μg/kg/day) (Tomza-Marciniak, Stepkowska, Kuba, & Pilarczyk, 2018). In humans, BPA exposure was associated with a higher frequency of premature deliveries and miscarriages (Sugiura-Ogasawara, Ozaki, Sonta, Makino, & Suzumori, 2005; Y. M. Zheng et al., 2012). For instance, a cross-sectional study reported an association between serum BPA levels and recurrent miscarriages whereby mean BPA levels in 45 women with a history of three or more miscarriages were more than three times higher than those in 32 women without fertility problems (Sugiura-Ogasawara et al., 2005).

A study on mice showed that prenatal exposure to BPA increased the frequency of miscarriages not among the mothers but among their offsprings (Rubin, 2011); as a matter of fact, offsprings born to mothers exposed to 0.025 and 25 g BPA/kg/day from gestation day 8 to lactation day 16 had fewer successful pregnancies and delivered a lesser number of pups throughout a 32 week study (N. J. Cabaton et al., 2011). The negative effects of early BPA exposure on the female reproductive capacity are most likely mediated by its actions on the hypothalamic-pituitary-ovarian axis (HPOA), uterus and ovaries (Rubin, 2011; Ziv-Gal & Flaws, 2016). At the level of the HPOA, BPA was associated with changes in histology of the anteroventral periventricular nucleus (AVPV), modifications in gonadotropin release, and alterations in ER expression in the hypothalamus and pituitary (Rubin, 2011). In the uterus, it resulted in morphological and molecular modifications, increase in steroid receptor expression, and increase in response to estrogen (Rubin, 2011).

As for its effect on the ovaries, prenatal BPA exposure in mice diminished the corpora lutea number and increased the unilateral or bilateral filled bursae (Kelley et al., 2015). In mice, BPA also increased the meiotic disturbances and aneuploidy in oocytes which is a known risk factor for miscarriage (Kelley et al., 2015; L. N. Vandenberg, Maffini, Sonnenschein, Rubin, & Soto, 2009; Ziv-Gal & Flaws, 2016).

c. BPA and the male reproductive system

Several cross-sectional studies showed that BPA exposure is associated with less reproductive capacity in males (D. K. Li et al., 2010; D. K. Li et al., 2011; X. Liu et al., 2015; Tomza-Marciniak et al., 2018). For instance, in a group of 427 men, higher urinary BPA levels were associated with erectile problems, decreased libido and lower ejaculation intensity (D. K. Li et al., 2010). Another study showed that higher urinary BPA concentrations are associated with lower sperm count and motility (D. K. Li et al., 2011). In a third study, although urine and semen BPA levels were correlated, semen but not plasma BPA levels were associated with less sperm concentration and count and altered sperm morphology. BPA exposure was also associated with more sex steroid binding globulin levels, less androstenedione levels, and less free androgen index in blood of 592 men that could potentially mediate BPA-induced male infertility (X. Liu et al., 2015).

BPA was also shown in sertoli cell cultures to induce apoptosis. Underlying potential mechanisms were elevation of Pten expression with concomitant inactivation in Akt, activation of caspase3, stimulation of JNKs/p38 MPAK pathway, and translocation of

nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Tomza-Marciniak et al., 2018). Besides, BPA at an environmentally relevant concentration (10nmol/l) decreased basal testosterone secretion of explanted and cultured human fetal testes (Eladak et al., 2015).

d. BPA and the endocrine system

Large cross-sectional population-based studies reported that BPA exposure is associated with higher incidence of obesity, type II diabetes mellitus, and metabolic syndrome. For instance, a study on 4389 adults older than 20 years observed that higher urinary BPA levels were associated with more occurrence of type II diabetes mellitus and higher hemoglobin A1c levels. Besides, another study on 282 healthy premenopausal non-obese women aged between 20 and 55 revealed that urinary BPA is positively associated with body weight, body mass index, fat mass and serum leptin levels. Moreover, BPA was linked to increase in inflammatory markers. As such, higher serum BPA levels were associated with higher incidence of insulin resistance and elevation in markers of chronic inflammation in 60 women aged between 23 and 33 years (Caporossi & Papaleo, 2017).

Animal studies showed that body weights of mice or rats increase upon exposure to BPA during both gestation only or gestation and lactation. Cell culture studies showed that BPA increases adipocyte differentiation and accumulation of lipids in 3T3-L1 preadipocytes. Animal studies also showed that BPA altered glucose homeostasis; as such, exposure to 100 µg BPA/kg/day for 4 days in adult male mice elevated pancreatic insulin content, produced

hyperinsulinemia, and induced insulin resistance (Rubin, 2011). Besides, exposure of pregnant mice to 10 µg or 100 µg BPA/kg during gestation resulted in increased insulin resistance and elevated plasma insulin, triglycerides and leptin levels in mothers, and increased insulin resistance and plasma insulin levels in male offsprings at 6 months of age (Alonso-Magdalena et al., 2010).

e. BPA and the cardiovascular system

Some human studies showed an association between BPA and higher incidence of cardiovascular diseases including hypertension, angina, heart attack, and coronary and peripheral arterial disease (Caporossi & Papaleo, 2017). For example, a cross-sectional study showed that higher urinary BPA levels were associated with elevated blood pressure in 521 adults older than 60 (Bae, Kim, Lim, Park, & Hong, 2012). Importantly, analysis of the association between the urinary BPA levels and cardiovascular risks in 1380 individuals from the National Health and Nutritional Examination Survey (2003-2004) revealed a statistically significant positive association between higher BPA levels and hypertension (Shankar & Teppala, 2012).Consistently in a sample of 501 Lebanese individuals, urinary BPA levels were significantly higher among individuals with diagnosed hypertension or with elevated blood pressure reading upon recruitment (Mouneimne et al., 2017).

Ex-vivo animal studies showed that acute exposure to low-concentration BPA (10^{-9}M) enhanced occurrence of cardiac arrhythmia in female rat hearts, and chronic exposure to low-dose BPA (5 µg/kg body weight/day) resulted in cardiac remodeling and altered blood

pressure in mice. Proposed potential mechanisms involve changes in cardiac calcium handling, ion channel inhibition/activation, and production of reactive oxygen species (X. Gao & Wang, 2014).

f. BPA and the nervous system

Human studies indicate that early life exposure to BPA impacts neurological development which manifests as increased aggressive behaviors and increased anxiety symptoms in children. For instance, higher gestational BPA levels were associated with more anxious and depressive symptoms and worse emotional controls among girls at 3 years of age (Braun et al., 2011), but these changes were not observed among boys. However, there were discrepancies among the studies, whereby another study showed that higher prenatal BPA levels were associated with more aggressive behaviors and poor emotional control among boys of 3-5 years and not girls (Perera et al., 2012). The reason for this discrepancy is unknown, but it may be due to differences in BPA measurement methods, BPA levels, and study population (Inadera, 2015).

Cell culture of human cortical neurons showed that BPA significantly decreased potassium chloride cotransporter 2 (KCC2) mRNA expression and hence would lead to neurodevelopmental toxicities (Yeo et al., 2013). Another study on human fetus-derived neural progenitor cell line (ReNcell) showed that BPA decreased the gene expression of neural markers (β III-tubulin and glial fibrillary acidic protein),eventually, disrupting the differentiation of neurons during fetal brain development (Fujiwara, Miyazaki, Koibuchi, &

Katoh, 2018).

BPA at doses below the NOAEL completely blocked the synaptogenic effects of estradiol and testosterone in the brains of rats and non-human primates. BPA thus acts as an estrogen and androgen antagonist in this context. BPA antagonism of estrogen and androgen receptors was dose dependent. Of note, BPA dose of 40 μg/kg (which is lower than the current human safe reference dose) reduced the density of spine synapses (Hajszan & Leranth, 2010). The reduction in the spine synapse density observed with BPA administration in animals was in line with the impairment in learning behaviors reported in both male and female rodents treated with BPA at doses lower than the acceptable human daily dose during gestation, lactation or puberty (Carr et al., 2003; Della Seta et al., 2006; Dessi-Fulgheri, Porrini, & Farabollini, 2002; Farabollini, Porrini, Della Seta, Bianchi, & Dessi-Fulgheri, 2002; Farabollini, Porrini, & Dessi-Fulgherit, 1999).

8. Stance of the regulatory authorities towards BPA

Based on two multi-generation studies and several other studies, the Food and Drug Administration (FDA) set the no-adverse event level of BPA at 5mg/kg/day (FDA, 2008). In 2014, FDA reviewed the literature on BPA toxicity between year 2010 and 2014 using weight of evidence approach and concluded that there are no safety issues at current human BPA exposure levels. Despite this conclusion, a long term study is currently conducted by the FDA, and the agency is still providing animal and tissues to grantees from the National Institutes of health (NIH) who are studying BPA safety (FDA, 2014).

In contrast to the FDA, the US Environmental Protection Agency (USEPA) set the chronic oral reference dose (defined as the exposure level per day that will not result in deleterious effects on health during lifetime) at 50 µg/kg/day. The same dose was set as the tolerable daily intake by the European Food Safety Agency (EFSA). This dose is 100 times lower than the NOAEL and around 100 times the WHO-estimated 95th percentile of BPA intake in adults of 1.5 µg/kg/day, and 20 times the FDA-estimated BPA intake in infants (Shelnutt, Kind, & Allaben, 2013). In response to the several studies showing adverse health effects induced by low-dose BPA exposure (vom Saal et al., 2007), EFSA decreased the tolerable daily intake to 4 µg/kg/day. This dose is ~3-5 times higher than the current human BPA exposure which is dependent on the age group. The agency also reported potential further lowering of this dose in response to results of an on-going long term study. Importantly, BPA has been recommended with "high priority" for the evaluation of its carcinogenic effects by 2019 by the International Agency for Research on Cancer (IARC) monographs (Monographs, April 2014).

9. BPF and BPS associated health outcome

Toxicological studies on BPF and BPS are scant. However, since their chemical structure, mechanism of action and metabolism are similar to BPA, it is hypothesized that exposure to BPF and BPS results in similar adverse events to BPA. The majority of cell culture studies focused on the estrogenic activity of BPF and BPS, and showed that, similarly to BPA, BPF and BPS increase the proliferation of ER-positive breast cancer cell line MCF-7 but not of the ER-negative breast cancer cell line MDA-MB-231 cells, concluding that these

compounds increase the cellular proliferation by acting through ER (Stroheker, Picard, Lhuguenot, Canivenc-Lavier, & Chagnon, 2004). The remaining few animal and cell culture studies showed adverse effects of BPF and BPS on the reproductive and nervous systems, that were in line with the estrogenic and anti-androgenic actions reported in cell culture studies for these compounds. One human study associated the levels of BPA, BPF and BPS with body mass index (BMI) and waist circumference (WC) in 1,521 adults. In this study, although BPA, BPF and BPS levels were higher in obese than non-obese adults, only BPA was statistically significantly associated with higher BMI and larger WC after adjustment for demographic, socioeconomic and lifestyle factors, as well as urinary creatinine levels (B. Liu et al., 2017).

A cell culture study showed that BPS at low concentrations comparable to those of human exposure impaired pig oocyte maturation (Zalmanova et al., 2017). Low concentration BPS was also reported to impair reproduction in zebra fish whereby exposure of zebra fish to BPS (0.5 µg/l) for 21 days reduced testosterone levels in males, decreased egg production rate in females and resulted in delayed hatching time and less hatching rates. Continuous exposure to BPS in the offsprings resulted in more decrease in hatchability and higher malformation rates (Ji, Hong, Kho, & Choi, 2013). BPF (100 mg/kg/day) and BPS (20mg/kg/day) administered subcutaneously for three consecutive days increased uterine weight in female rats. Moreover, Eladak *et al.* showed that, similarly to BPA, treatment of explanted and cultured human fetal testes with 10nmol/l of BPF or BPS decreased basal testosterone secretion (Eladak et al., 2015). Higashihara *et al.* showed that oral administration of BPF to young adult rats at several doses (20, 100 and 500 mg/kg/day) for

28 days decreased body weight and decreased levels of serum cholesterol, glucose and albumin at all doses in female rats (Higashihara et al., 2007). In contrast to other studies, in their study, there were no abnormal endocrine-related findings i.e. no altered estrous cycle and no changes in sperm morphology/count or uterine weights.

One study evaluated the behavioral effects of BPF and showed that oral administration of BPF (10 mg/kg/day) to female mice from gestation day 11.5 to 18.5 aggravated anxiety and depression states in offsprings at postnatal week 10. In the same study, a similar dose of BPA resulted in weaker effects than BPF (Ohtani et al., 2017).

10. Effects of BPA and its analogues BPF and BPS on breast cancer development and progression

Estrogen and estrogen signaling pathways play critical roles in the development of the mammary gland and breast carcinogenesis. ER1 is first expressed during the embryonic life and drives morphogenetic alterations throughout mammary gland development (H. Gao et al., 2015). For instance, mammotropic hormones including estrogen regulate proliferation, apoptosis and gene expression at the cellular level, as well as ductal elongation, branching and lobule development at the tissue level of the mammary gland (Neville, McFadden, & Forsyth, 2002). During these morphogenetic processes, the mammary tissue is highly responsive to neoplastic stimuli as shown by the observed increased incidence of breast cancer in subjects exposed to DES during fetal life (Reed & Fenton, 2013). Accordingly, it

has been postulated that mammary tissue may also be sensitive to circulating EDCs such as BPA and its analogues BPF and BPS.

a. Epidemiological studies

To date, two epidemiological cross-sectional studies evaluated the potential effect of BPA on breast cancer in women, and none is so far available for BPF and BPS. Yang *et al.* (2009) measured the levels of BPA from 70 breast cancer cases and 82 age-matched controls, and observed no association between BPA levels and breast cancer risk (M. Yang, Ryu, Jeon, Kang, & Yoo, 2009). Similarly, Trabert *et al.* (2014) analyzed the urinary BPA metabolite, BPA-glucuronide G, in 575 post-menopausal breast cancer cases and 575 controls matched by age and study site, and found no statistically significant differences in BPA levels between cases and controls; in addition, there were no differences in BPA levels between ER-positive and ER-negative breast cancer cases (Trabert et al., 2014). However, both studies measured BPA levels at one-time point only and after breast cancer development, and did not associate BPA with different breast cancer subtypes. With no data yet available on BPA exposure levels before development of breast cancer, and with no data on cumulative lifetime exposure to BPA, such studies fail to accurately discern the impact of BPA on the critical windows of mammary gland development. Interestingly however, Sprague *et al.* (2013) observed that high BPA levels were significantly associated with increase in mammographic breast density, a risk factor for breast cancer, among postmenopausal women (N=264) following adjustment for age, BMI, and other confounding factors (Sprague et al., 2013).

b. Animal studies

Despite the lack of valid epidemiologic evidence, multiple animal studies have shown the effect of BPA on the development of mammary gland carcinogenesis using several animal models (**Table 2**), though only one study is currently available for BPS ("DES daughter,") and so far none is for BPF. The majority of the studies with BPA used rat models, and showed that BPA exposure during fetal or early postnatal life is associated with development of pre-neoplastic and neoplastic lesions in the mammary gland (Acevedo, Davis, Schaeberle, Sonnenschein, & Soto, 2013; Seachrist et al., 2016; L.N. Vandenberg et al., 2008; L.N. Vandenberg et al., 2007; Wadia et al., 2013; Wadia et al., 2007). For example, when Wistar Furth rats were exposed *in-utero* to BPA through mini-osmotic pumps, there was an increase in hyperplastic ducts at postnatal day 50 and 95. *ER1* gene (*ESR1*) expression was significantly overexpressed in these hyperplastic lesions when compared to normal ducts in controls, indicating an increase in proliferation and estrogenic activity (Murray, Maffini, Ucci, Sonnenschein, & Soto, 2007). Similarly, Sprague Dawley rats exposed to BPA doses ranging from 0.25-250 µg/kg/day via osmotic pumps during gestation, or both gestation and post-natal period through mother milk, showed macroscopic tumors by postnatal day 90 at an incidence of 3.5% (Acevedo et al., 2013). Interestingly, when BPA was combined with the carcinogens *N*-nitroso-*N*-methylurea (NMU) or dimethylbenzanthracene (DMBA), several studies reported an increase in mammary tumor formation and decrease in tumor latency (Betancourt, Eltoum, Desmond, Russo, & Lamartiniere, 2010; Durando et al., 2007; Jenkins et al., 2009; Lamartiniere, Jenkins, Betancourt, Wang, & Russo, 2011). Besides, a number of studies using murine models have shown that 0.025 and 25 µg/kg/day of BPA delivered at gestation via osmotic

pumps increases the sensitivity of the mammary gland to estradiol and induces morphological changes during puberty, including increased lateral branching, increased lobuloalveolar development, and intraductal hyperplasia (L.N. Vandenberg et al., 2008; L.N. Vandenberg et al., 2007; Wadia et al., 2013; Wadia et al., 2007). Because of these established effects of very low doses of BPA in animal mammary glands, and since human exposure was measured to be higher (vom Saal et al., 2007), it has been concluded with almost certainty that BPA exposure ought to be associated with organizational changes in the mammary gland such as those with laboratory animals (Vogel, 2009). As for BPS, only one recent animal study examined the effect of BPS exposure on mammary gland development whereby pregnant mice were given BPS (0.05, 0.5, 5mg/kg/day) via oral gavage from gestation day 10 to 17. Exposed female offsprings showed adverse mammary gland morphology (undifferentiated duct ends, lobuloalveolar hyperplasia and perivascular inflammation) and adenocarcinomas. These adverse effects were more observed at BPS dose of 0.5 mg/kg/day ("DES daughter,").

Table 2. Animal studies showing effects of BPA exposure on the mammary gland

Reference	Animal model	BPA dose (µg/kg/day)	Route of exposure	Exposure period	Treatment duration	Co-treatment	Mammary gland modifications (dose in µg/kg/day)
Acevedo *et al.* 2013 (Acevedo et al., 2013)	Sprague Dawley CD rats	0.25, 2.5, 25, 250	SC (Alzet osmotic pumps)	pre and postnatal	GD 9-birth and GD9-PND21	NA	Increase in intraductal hyperplasia (BPA25)
							Increase in atypical ductal hyperplasia (BPA2.5; BPA25)
							increase in tumor incidence (BPA0.25; BPA2.5; BPA25; BPA250)
Jenkins *et al.* 2011 (Jenkins, Wang, Eltoum, Desmond, & Lamartiniere, 2011)	MMTV-erB2 mice	0.5, 5, 50, 500	Oral (drinking water)	postnatal	56-252 days	NA	Increase proliferation (BPA0.5; BPA5; BPA50; BPA500)
							Increase in tumor multiplicity and decrease in tumor latency (BPA2.5; BPA25)
							Increase in metastasis (BPA2.5; BPA25)
Jenkins *et al.* 2009 (Jenkins et al., 2009)	Sprague Dawley CD rats	25, 250	Oral (intragastric lavage)	prenatal	PND 2-20	DMBA (30 mg/kg) at PND 50	Increase in proliferation and decrease in apoptosis (BPA250)
							Increase in tumor multiplicity and decrease in tumor latency (BPA250 with DMBA)
Betancourt *et al.* 2010(Betancourt et al., 2010)	Sprague-Dawley CD rats	25, 250	Oral (intragastric lavage)	prenatal	GD 10-21	DMBA (30 mg/kg) at PND 50 or 100	Increase in proliferation (BPA250)
							Increase in tumor incidence and decrease in tumor latency (BPA250 with DMBA)
Jones *et al.* 2010 (Jones et al., 2010)	BRCA1 mutant and wild type	0.25	SC (Alzet osmotic pumps)	postnatal	90-118 days	NA	Increase in proliferation (BPA0.25)
							Increase in intraductal

	C57Bl/6 mice						hyperplasia (BPA0.25)
Vandenberg *et al.* 2008 (L.N. Vandenberg et al., 2008)	CD-1 mice	0.25, 2.5, 25	SC (Alzet osmotic pumps)	pre and postnatal	GD 8-PND 16	NA	Increase in alveolar buds (BPA0.25)
							Increase in intraductal hyperplasia (BPA0.25; BPA2.5)

CD: cluster of differentiation; C57Bl/6: C57 black 6; BW: body weight; SC: subcutaneous; GD: gestation day; PND: post-natal day; NA: not available; DMBA: 7,12‑dimethylbenz(a)anthracene; MMTV: mouse mammary tumor virus

c. Cell culture studies

i. Cancer cell proliferation

While only few cell culture studies were performed for BPF and BPS, several were conducted to evaluate the effect of BPA on breast cancer cell lines proliferation. For instance, an increase in cell proliferation was observed in breast cancer cell lines (MCF-7 or MCF-7 clonal variant (CV)) treated with BPA (Aghajanpour-Mir et al., 2016; H. S. Lee et al., 2014; Miyakoshi, Miyajima, Takekoshi, & Osamura, 2009; Pfeifer, Chung, & Hu, 2015; Pisapia et al., 2012; Ricupito et al., 2009; H. Song et al., 2015; Stroheker et al., 2004; W. Zhang et al., 2012), BPF (Pisapia et al., 2012; Stroheker et al., 2004) or BPS(J. Y. Kim et al., 2017). Interestingly, some data showed that the BPA-induced cell proliferation in MCF-7 cells was abolished in the presence of the ER antagonist ICI (182,780), implicating a role of ER in BPA-induced cell proliferation in these cells (H. S. Lee et al., 2014; Qin et al., 2012). In support of the role of ER in BPA-induced cell proliferation, BPA was shown to decrease apoptosis and increase chemoresistance in various ER-positive breast cancer cells (Lapensee, Tuttle, Fox, & Ben-Jonathan, 2009). Moreover, some studies have shown that BPA and BPF were not associated with any increase in cell proliferation of the breast cancer cell line MDA-MB-231 that is ER-negative (Stroheker et al., 2004; W. Zhang et al., 2012; X.L. Zhang, Liu, Weng, & Wang, 2016).

ii. Cell cycle analysis of cancer cells

Few cell culture studies focused on the effects of BPA, BPF and BPS on cell cycle progression in breast cells. For cell cycle phase analysis, an increase in the cell percentages in the S phase was observed in non-cancerous human high-risk donor breast epithelial cells (HRBEC) and T47D breast cancer cells treated with 100nM BPA for 1 week (Dairkee, Luciani-Torres, Moore, & Goodson, 2013). Only one study performed cell cycle analysis in MCF-7 cells treated with several concentrations of BPA or BPF (10^{-5}, 10^{-6}, 10^{-7} M) for 24 hrs and observed a decrease in the percentages of cells in the G0/G1 phase and increase in the percentages of cells in the S and G2/M phases at BPA and BPF concentrations of 10^{-5}M and 10^{-6} M but not at 10^{-7} M (Pisapia et al., 2012). No study performed cell cycle analysis for BPS-treated breast cell lines. However, one recent study showed an increase in the protein expression of cell-cycle progression genes, *cyclin D1* and *cyclin E1* in MCF-7 CV cell line treated with10^{-5} M of BPA, BPF or BPS for 48 hrs (J. Y. Kim et al., 2017).

iii. Cancer cell migration and invasion

As for studies on cancer cell migration and invasion in breast cancer cell lines, few studies reported that BPA increases the migration of ER-negative breast cancer cell lines MDA-MB-231 through either GPER/ERK2 or ERR-ɣ pathway, and one reported that BPA increases the invasion of these cells via ERR-ɣ pathway, indicating a potential role of ER-independent mechanism in BPA-induced cancer progression (Castillo, Gomez, & Perez, 2016; X. L. Zhang et al., 2016; X. L. Zhang, Wang, Liu, & Ge, 2015). Only two studies were performed to test the effect of BPA on migration of ER-positive breast cancer cell

lines (MCF-7 CV and MCF-7), and one of these also tested the effects of BPF and BPS. As such, Kim *et al.* (2017) showed that 10^{-5} M of each of BPA, BPF and BPS increased the migration of MCF-7 CV cells and altered the expression of epithelial to mesenchymal transition markers (increased the ratio of N-cadherin to E-cadherin). The alterations in N-cadherin and E-cadherin protein expression levels were restored to control levels in the presence of the ER antagonist, ICI 182,780 (J. Y. Kim et al., 2017). Lee *et al.* (2017) reported that 10^{-6} M of BPA increases the migration and invasion of MCF-7 cells in an ER-dependent mode, and when MCF-7 cells were treated with the same concentration of BPA for 72 hrs, BPA decreases the protein expression of E -cadherin but increases the expression of N -cadherin, snail and slug, which was not observed upon co-treatment with the ER antagonist, ICI 182,780. These molecular alterations indicate that BPA potentially induces the epithelial to mesenchymal transition process via ER-dependent pathways (G. A. Lee, Hwang, & Choi, 2017).

iv. Normal-like breast cell proliferation, cell cycle and invasion

As for normal-like breast epithelial cells, studies are available for BPA only whereby it was shown to lead to an increase in MCF-10A cell proliferation (Pfeifer et al., 2015). BPA exposure was also associated with increase in cell proliferation and colony formation of primary human mammary epithelial cells (HMEC), which could be mediated by the observed downregulation of inhibitors of G1-S transition (*CCNE1, CCNA2* and *CDKN2A*) (Qin et al., 2012). Besides, one study evaluated the effects of BPA on invasion of MCF-10F, and showed that BPA increased the invasive potential of these cells, though this

increase did not reach statistical significance (Fernandez & Russo, 2010). Moreover, BPA treatment of high-risk cells derived from the contralateral tissue of patients with breast cancer induced a gene expression profile similar to that observed in breast tumors with high histological grade and large tumor size (Dairkee et al., 2008).

In conclusion, more cell culture studies are warranted to elucidate the role of these three bisphenols in breast cancer development and progression.

C. Telomeres and telomerase

1. Telomere

Human chromosomes include non-coding repeated TTAGGG DNA sequences at their ends (telomeres) that not only maintain genomic integrity through protecting the chromosomal ends from being recognized as double strand breaks that require repair, but also provide a solution for the inability of the replication machinery to replicate the chromosomal ends. The estimated telomeric length ranges from 2 to 20 kilo base pairs, depending on factors such as age and tissue type. Because of the end-replication problem, 50-200 base pairs of the end of telomeric DNA are lost with each cell division until the telomeres reach a critical length that triggers cell senescence (Mu & Wei, 2002). In humans, maintenance or lengthening of the telomeres mainly occurs by mechanisms involving overexpression or reactivation of telomerase (Mu & Wei, 2002; Rubtsova et al., 2012).

2. Telomerase

Telomerase is the enzyme that compensates for telomere loss and prolongs the lifetime of the cell (N. W. Kim et al., 1994). It is a ribonucleoprotein composed of a catalytic telomerase protein which is the *human* telomerase reverse transcriptase (*h*TERT), and the functional template which is the telomerase RNA (TR) of sequence 5' -CUAACCCUAAC-3' complementary to the telomeric repeat (Feng et al., 1995; Lingner et al., 1997). Telomerase activity is maintained in highly proliferating cells such as keratinocytes and activated lymphocytes (Masutomi et al., 2003), germ cells, some stem cells such as hematopoietic progenitor cells or embryonic stem cells (E. Hiyama & Hiyama, 2007) and cancer cells (N. W. Kim et al., 1994). Telomerase adds telomere repeats during S or G2/M phase, and it preferentially acts on the shortest telomere subset (Marcand, Brevet, Mann, & Gilson, 2000; Teixeira, Arneric, Sperisen, & Lingner, 2004). Telomerase was found to increase the telomere subset of 100 nucleotides by 42-46%, while 300-nucleotide length telomere is accompanied by only 6-8% increase in telomere length (Teixeira et al., 2004). Nevertheless, Zhao *et al.* (2009) showed that telomerase in human cancer cells acts on elongating telomere subsets at most of the chromosomes, without having preference for shortest telomeres (Zhao et al., 2009). Telomerase has also been reported to have other functions such as nuclease and transferase activities inside eukaryotic cells. Although telomerase is not active in somatic cells, it was found that *h*TERT protein can be expressed in the nucleus, cytoplasm and mitochondria, and it plays an important role in protecting cells during oxidative stress (Rubtsova et al., 2012).

3. Telomerase and breast cancer

Telomerase is detectable in around 80-95% of many cancer types including breast cancer (Carey et al., 1998; E. Hiyama, Hiyama, Yokoyama, & Shay, 2001). Bieche *et al.* (2000) quantified the *h*TERT mRNA levels in tissue samples from 134 invasive primary breast cancer patients using reverse transcription-polymerase chain reaction (RT-PCR), and showed that 75.4% of breast cancer patients were *h*TERT-positive, and they had shorter relapse-free survival period after surgery when compared to *h*TERT-negative patients (Bieche et al., 2000). Moreover, increased telomerase activity was associated with differences in breast tumor size, lymph node status, and disease stage in breast cancer patients; suggesting that telomerase could be a useful prognostic marker for breast cancer (Hoos et al., 1998; Mokbel, Parris, Ghilchik, Amerasinghe, & Newbold, 2000).

As for cell lines, telomerase activity was assessed in MCF10A (a normal-like immortalized breast cell line), MCF7 (a non-invasive breast cancer cell line) and HTB26 (an invasive breast cancer cell line), and all three were shown to express *h*TERT. When compared to the cancerous cell lines MCF7 and HTB26, MCF10A that is contact inhibited and does not develop into tumors when injected in an organism showed less *h*TERT expression. These data are consistent with the previous results on breast tissues suggesting telomerase activity as an indicator of breast cancer development and metastasis (Collado, 2006).

In conclusion, telomerase was shown to be expressed in breast cancer cells and associated with higher stage and grade of the disease indicating that it can be a marker of poorer prognosis.

4. Relative telomere length (RTL) and cancer

Chromosomes with very short telomeres are recognized by DNA repair enzymes as double strand breaks, which may result in sister chromatid fusions, prolonged breakage/fusion/bridge cycles and chromosomal rearrangements. This genomic instability is commonly associated with increased cancer risk (Bailey & Murnane, 2006). Thus, short telomeres have been linked to a number of cancer types such as colon, prostate and ovarian cancers (Cui et al., 2012; A. K. Meeker, 2006; Mirabello et al., 2010; A. J. Pellatt, Wolff, Lundgreen, Cawthon, & Slattery, 2012). A meta-analysis performed in 2015 showed that short RTL was associated with increased cancer mortality and poor cancer prognosis. RTL was an independent predictor of overall cancer survival in chronic lymphocytic leukemia, esophageal cancer and colorectal cancer (C. Zhang et al., 2015). However, long telomeres have also been linked to some cancer types such as melanoma (Han et al., 2009; Nan et al., 2011). This was explained as being secondary to the higher mutation potential as a result of the higher number of cycles the cell is allowed to undergo before its telomeres reach a critical length at which cell senescence occurs. As such, a prospective study indicated that both very short and long telomeres are associated with increased colorectal cancer risk (Cui et al., 2012).

5. RTL and breast cancer

Clinical studies on the association between RTL in blood and breast cancer showed inconsistent results. This inconsistency may be attributed to the difference in the ethnic and genetic background, sample type (whole blood vs. leukocytes only) and pathology of the

breast cancer (De et al., 2009; Martinez-Delgado et al., 2011; A.J. Pellatt et al., 2013; Qu et al., 2013; Y. L. Zheng et al., 2010). For instance, Pellatt *et al.* (2013) investigated the association between RTL in whole blood and breast cancer risk in US non-Hispanic White (1481 cases, 1586 controls) and US Hispanic and Mexican women (2111 cases, 2597 controls). Longer telomeres in whole blood were linked to breast cancer risk, particularly among women with high indigenous American ancestry (A.J. Pellatt et al., 2013). However, Martinez Delgado *et al.* (2011) showed an association between shorter telomeres in peripheral blood leukocytes and breast cancer risk in families carrying *BRCA1* or *BRCA2* mutations, but not among families carrying none of these mutations (Martinez-Delgado et al., 2011). Some studies observed no association between RTL and breast cancer; such as De vivo *et al.* who measured RTL in peripheral blood leukocytes derived from postmenopausal breast cancer patients (N= 1122) and controls (N= 1147) (De Vivo et al., 2009). Interestingly, a large prospective study performed on 601 cases (who later developed breast cancer) and 695 controls from the Shanghai Women's Health Study (1997-2009) showed a statistically significant association between short leukocyte RTL in peripheral blood (withdrawn before breast cancer development) and higher breast cancer risk, and a potential association between long RTL (RTL in the top quintile) and higher breast cancer risk (Qu et al., 2013).

There are 4 reports on RTL in breast cancer tissues, three of which revealed an association between shorter RTL and breast cancer. For instance, Rashid-Kolvear *et al.* (2007) compared RTL in normal breast tissues *versus* cancerous breast tissues derived from 18 individuals and showed an association between breast cancer and short RTL (Rashid-

Kolvear, Pintilie, & Done, 2007). In addition, Looi *et al.* (2010) analyzed RTL and telomerase activity in 29 infiltrating ductal carcinoma (IDC) tissues and 22 benign non-lesional controls, and revealed higher telomerase activity and shorter RTL in IDC tissues when compared to benign non-lesional controls (Looi et al., 2010). Besides, Meeker *et al.* (2004) analyzed RTL in normal breast epithelial tissues and in cancerous breast tissues derived from 114 individuals with invasive breast carcinoma, 29 with carcinoma in situ and 10 with benign proliferative lesions. The majority of invasive carcinomas had marked telomere shortening, and some showed moderate telomere shortening, yet few demonstrated an increase in RTL. Most ductal carcinoma *in-situ* cases showed marked or moderate telomere shortening (A.K. Meeker et al., 2004). In contrast, a recent study by Thriveni *et al.* (2018) measured RTL in tumor tissues and normal adjacent breast tissues derived from 98 breast cancer patients with invasive ductal carcinoma, and observed that 47 patients had shorter RTL in tumor than normal adjacent tissues, while 51 patients had longer telomeres in tumor than normal adjacent tissues. In their study, RTL was influenced by clinicopathological characteristics of breast cancer, whereby shorter RTL was associated with early stage, low grade, small tumor size (< 5cm), and lymph node-negative breast cancer, while longer RTL was associated with advanced stage, high grade, large tumor size (> 5cm) and lymph node-positive breast cancer (Thriveni, Raju, Kumar, Krishnamurthy, & Chaluvarayaswamy, 2018).

In conclusion, epidemiological studies showed inconsistencies regarding the association between peripheral blood RTL and breast cancer; however, the majority of studies in tissues (three out of four) showed shorter RTL in cancerous tissues compared to normal adjacent

tissues.

6. RTL and telomerase in breast cancer

Telomerase is reactivated in cancer cells by genomic mutations and plays a role in maintenance of telomere length. Studies showed that telomere inhibition leads to progressive loss of telomeric repeats and thereby cancer cell death (Jafri, Ansari, Alqahtani, & Shay, 2016).

In breast cancer patients, few studies investigated the association between telomerase expression/activity and telomere length. Although Looi *et al.* showed higher telomerase activity and shorter telomere length in IDC tissues when compared to benign non-lesional controls (Looi et al., 2010), the recent study by Thriveni *et al.* (2018) observed a correlation between higher telomerase expression and longer RTL. In their study and as noted above, patients with advanced stage, high grade and lymph-node positive breast cancer had higher telomerase expression and longer RTL than patients with early stage, low grade and lymph-node negative breast cancer. However, some patients (13% of the overall breast cancer cases) had long telomere length despite presence of low telomerase expression; in these patients lengthening of telomeres was proposed to be mediated through alternative lengthening mechanism (ALT) that is not dependent on telomerase, but on homologous chromosomal recombinations (Thriveni et al., 2018). It is generally estimated that in 10 to 15% of cancer cases, lengthening of RTL occurs through ALT mechanism (Cesare & Reddel, 2010).

In conclusion in breast cancer, maintenance or lengthening of telomeres occurs mainly through telomerase and sometimes by ALT mechanism. Reactivation of telomerase is more observed in cancerous breast tissues of breast cancer with high stage and grade.

7. Factors influencing RTL

Although telomere is shortened with age, telomere length is also influenced by several dietary, behavioral and environmental factors. For instance, epidemiological-based studies showed that RTL in leukocytes was positively associated with fiber intake but inversely associated with intake of polyunsaturated fatty acids, particularly linoleic acid. Larger WC and higher BMI were associated with shorter RTL (Cassidy et al., 2010; Gielen et al., 2018). Poor sleep quality, short sleep duration (≤ 6hrs), personality traits such as pessimism, stress (i.e. higher urinary catecholamines), and psychological stress such as abusive relationship were all associated with shortened RTL in leukocytes (Starkweather et al., 2014). Of interest, leukocyte RTL was reported to be associated with the living environment whereby people living in disordered or old regions of the city had shorter RTL than those living in more ordered or newly renovated regions with green spaces (Starkweather et al., 2014). Some studies showed an association between moderate levels of physical activity and longer leukocyte RTL. Moreover, increased duration of exposure to several pesticides such as metolachlor and 2,4-dichlorophenoxyacetic acid, was associated with shorter RTL in buccal cells (Andreotti et al., 2015; Hou et al., 2013).

However, to the best of our knowledge, no epidemiological study investigated the association between the three bisphenols (BPA, BPF or BPS) and RTL.

8. Bisphenols and telomerase and RTL

There is very little literature on BPA and telomere/telomerase in breast or other tissue types. One old study on MCF-7 cells treated with BPA and diethylstilbestrol (DES) showed a dose-dependent increase in telomeric associations that have been implicated in increased genomic instabilities (Roy, Colerangle, & Singh, 1998). However, BPA was associated with increase in telomerase expression in MCF-7 cells (Takahashi et al., 2004). Therefore, whether BPA exposure is associated with change in telomere length in breast epithelial cells remain to be elucidated. Interestingly, other estrogen-like chemicals were found to be associated with telomere length reduction. For instance, it was shown that Noble rats exposed to DES exhibited different changes in their mammary glands, whereby cell cycle alterations were reported along with increased cell proliferation and reduction in telomere length (Roy et al., 1998).

It has been shown that *h*TERT promoter contains an estrogen response element that is activated by ligand bound ER, therefore few investigators studied the effect of estrogen and estrogen-like compounds on *h*TERT transcription. One study in mice showed that estrogen deficiency resulted in decrease in cell proliferation, inhibition of *h*TERT expression and shortening of RTL in the adrenal gland, and these were restored after estrogen administration (Bayne et al., 2008). Estrogen and the endocrine disrupting agent 2,3,7,8-tetrachlorodibenzo-p-dioxin increased the telomerase activity in trophoblast-derived

choriocarcinoma cell line (BeWo), suggesting that BPA may also upregulate telomerase activity (Sarkar, Shiizaki, Yonemoto, & Sone, 2006). Treatment with BPA and estrogen was associated with increased expression of *h*TERT in HeLa (human cervical carcinoma), H1299 (human lung carcinoma), and MCF-7 (human breast cancer) (Takahashi et al., 2004). BPA also increased the proliferation of hepatoblastoma HepG2 cells by enhancing telomerase activity through an ER-dependent pathway (B. L. Xu, Zhao, Gao, & Hou, 2015). However, no study examined the effect of BPF or BPS on telomerase expression and RTL in the breast or other tissue type.

In conclusion, although two *cell culture* studies showed that BPA increases telomerase expression in different cell lines including breast cancer cells, no *cell culture* study examined the effect of BPF or BPS on telomerase expression in any tissue type. In addition, to date, no epidemiological study investigated the association between the three bisphenols (BPA, BPF or BPS) and RTL in any tissue type. The three bisphenols could be associated with lengthening of RTL through increased expression of telomerase or with shortening of RTL through other factors associated with telomere attrition, secondary to potential increase in proliferation and oxidative stress following exposure to the three bisphenols (Gassman, 2017; Macczak, Cyrkler, Bukowska, & Michalowicz, 2017; von Zglinicki, 2002).

D. Epigenetics

Epigenetics is the study of inherited changes in gene expression occurring without alterations in the DNA sequence (Sharma, Kelly, & Jones, 2010). It includes DNA methylation, non-coding RNAs and histone modifications, which are intricately connected. These processes are known to be involved in genomic imprinting, gene silencing, X chromosome inactivation, reprogramming, and the progress of carcinogenesis (C. J. Li, 2013; Sharma et al., 2010). Till now, DNA methylation is the most studied epigenetic mechanism.

1. DNA methylation

DNA methylation is an epigenetic mark that occurs in approximately 70 to 80% of cytosine residues present in a CpG context in human somatic cells (C. J. Li, 2013). When present in a gene promoter, DNA methylation can repress gene expression by affecting transcription factor binding and/or chromatin structure. DNA methylation is established through complex DNA methylation/demethylation machinery and is maintained in a stable manner throughout the lifetime of the organism. Alterations in DNA methylation can occur as a consequence of aging or exposure to endogenous hormones or environmental chemicals such as EDCs through the same complex machinery (Bergman & Cedar, 2013).

In humans, DNA methylation is catalyzed by three enzymes collectively termed as DNA methyltransferases (DNMTs). DNMT1 is responsible for the faithful maintenance of DNA methylation patterns after DNA replication and is directed by methyl residues in the

hemimethylated strands, while DNMT3A and DNMT3B catalyze *de novo* DNA methylation. DNA demethylation can be achieved either passively, by failure to maintain DNA methylation pattern in the new DNA strand after replication, or actively by a replication-independent pathway. Active DNA demethylation is mediated by the ten–eleven translocation (TET) family of enzymes (TET1, TET2, TET3). These hydroxylate the methylated cytosines that are then replaced by unmethylated ones through the DNA repair machinery (C. J. Li, 2013).

2. DNA methylation aberrations in cancer

a. Tumor suppressor genes and cancer

The transformation process of normal cells into cancer cells occurs as a result of several genetic and epigenetic modifications. Genetic mutations in tumor suppressor genes are central for cellular transformation since they lead to aberrations in DNA repair and induction of mutations in several genes. It is also recognized that DNA hypermethylation of key genes such as tumor suppressor and DNA repair genes, play a role in the development of cancer. Increased expression of *DNMT1* was reported in several cancer types while its decreased expression was linked to protective effect. Besides, *DNMT3B* was also overexpressed in several tumors, and its suppression led to apoptosis of tumor cells (Pouliot, Labrie, Diorio, & Durocher, 2015). As for the DNA demethylation enzymes, TET1 mediates hypomethylation and leads to overexpression of oncogenic genes such as PI3K, EGFR, and PDGF(Good et al., 2018). However, TET2 may suppress cancer

development as it was found to be mutated and inactive in several cancer types (Pouliot et al., 2015).

b. Global DNA methylation and cancer

Besides hypermethylation in promoters of tumor suppressor genes, overall loss of DNA methylation (global hypomethylation) especially in centromeric regions has been linked to genomic instability. Global DNA methylation is often assessed by measuring the methylation of repetitive sequences in the genome, such as the *long interspersed nuclear element-1 retrotransposons* (*LINE-1*). *LINE-1* constitutes around 17% of the human genome with a significant number of CpG dinucleotides and is thus considered as a surrogate marker of global DNA methylation (Baba et al., 2018).

When hypomethylated and expressed, active *LINE-1* sequences use the "copy and paste" mechanism to integrate into the genome via reverse transcription of their RNA intermediates, and thereby causing mutation in neighboring genes. In brief, *LINE-1* is comprised of a 5' untranslated region (UTR), two open reading frames (ORF1 and ORF2), and a 3' UTR containing two promoter regions (one sense and another antisense). RNA polymerase II binds to the sense promoter and initiates transcription resulting in a full length LINE-1 mRNA. In the cytoplasm, LINE-1 mRNAs are then either degraded or translated into ORF1 and ORF2. Both ORF proteins bind to LINE-1 mRNA which returns to the nucleus to be integrated into the genome by the reverse transcription action of ORF2. This "copy and paste" mechanism reshapes the genome and gives rise to genetic mutations

that could play a role in the transformation of normal cells into cancer cells (Xiao-Jie, Hui-Ying, Qi, Jiang, & Shi-Jie, 2016).

Several studies investigated the association between *LINE-1* methylation and cancer, hypothesizing that global hypomethylation is associated with cancer development, either through direct activation of transposable elements or through indirect association with whole genome DNA hypomethylation at other regions of the genome such as centromeres. A systematic review and meta-analysis conducted in 2014 on 19 articles showed that *LINE-1* methylation was statistically significantly lower in colorectal and gastric cancer tissues when compared to controls, yet the association between *LINE-1* methylation and cancer risk was not significant in other cancer types including breast cancer (Barchitta, Quattrocchi, Maugeri, Vinciguerra, & Agodi, 2014).

3. DNA methylation aberrations in breast cancer

a. Tumor suppressor genes and breast cancer

Similar to other cancer types, regional hypermethylation of tumor suppressor genes were reported in breast cancer (Delgado-Cruzata et al., 2012; Kuchiba et al., 2014; Radpour et al., 2011). For instance, DNA methylation of a panel of tumor suppressor genes was measured in circulating cell-free (cf) DNA isolated from serum of breast cancer patients in a test set (112 breast cancer, 102 normal, 20 benign breast disease and 27 colon cancer) and in an independent validation set (138 breast cancer, 135 normal, 39 benign breast disease and 31 colon cancer). Three tumor suppressor genes: *inter-alpha-trypsin inhibitor heavy*

chain family, member 5 (*ITIH5*), *dickkopf WNT signaling pathway inhibitor 3* (*DKK3*) and *Ras association domain family member 1 (RASSF1A)* were hypermethylated in breast cancer patients when compared to controls (Kloten et al., 2013). Besides, eight tumor suppressor genes (*APC, BIN1, BRCA1, CST6, GSTP1, P16, P21* and *TIMP3*) were hypermethylated in cf DNA isolated from plasma of breast cancer patients (N=36) when compared to controls (N=30), and all except *P21* genes were hypermethylated in tumor tissues (N=20) when compared to matched-normal tissues (N=20) from non-familial breast cancer patients (Radpour et al., 2011). Moreover, hypermethylation of *kinesin family member 1A* (*KIF1A*) was observed in plasma from breast cancer patients (N=89) when compared to controls (N=85) (Guerrero-Preston et al., 2014). Furthermore, a study on identical twin pairs (test set : 15 twin pairs, independent validation set: 21 twin pairs) showed that *docking protein 7* (*DOK7*) promoter was hypermethylated in blood of breast cancer patients when compared to their twin (Heyn et al., 2013).

Overexpression of *DNMT*s was also reported in breast cancer, and DNMT3B was proposed to play a predominant role over DNMT1 and DNMT3A. To illustrate, while *DNMT3B* was overexpressed in about 30% of breast cancers, *DNMT1* and *DNMT3A* were overexpressed in only 5 and 3% of breast cancers, respectively. In addition, *DNMT3B* had a higher increase in expression than *DNMT1* and *DNMT3A* (Subramaniam, Thombre, Dhar, & Anant, 2014). As for the DNA demethylation enzymes, TET1 was reported to promote tumorigenesis through demethylation of genes involved in oncogenic pathways such as PI3K, EGFR, and PDGF. A cell culture study showed that *TET1* deletion silences these genes, enhances the immune response and reduces cell proliferation in triple negative breast

cancer cells (Good et al., 2018). TET2 was found inactive in several cancer types, and its activation in breast cancer suppresses tumorigenesis potentially through expression of caspase-4 (X. Zhu & Li, 2018).

b. Global DNA methylation and breast cancer

A number of case-control studies investigated the association between global DNA methylation and breast cancer risk (Tang, Cheng, Cao, Surowy, & Burwinkel, 2016), and few investigated the association of particularly *LINE-1* methylation with breast cancer but with discrepant results (S. Cao et al., 2017; Choi et al., 2009; X. Xu et al., 2012). According to a systematic review published in 2016, less than one-half of 11 reviewed studies showed that global DNA hypomethylation is significantly associated with breast cancer, while the remaining showed no association with the exception of one study that observed an association between global DNA hypermethylation and breast cancer. These studies differed in several aspects including the choice of target genes as surrogate markers of global DNA methylation (Tang et al., 2016).

In conclusion, in addition to hypermethylation of tumor suppressor genes, global DNA hypomethylation enhances cancer development. Studies on breast cancer patients showed hypermethylation of tumor suppressor genes but there were inconsistencies regarding alterations in global DNA methylation.

4. Epigenetic mechanisms of BPA

Epigenetic effects of BPA have been first demonstrated in the Agouti (ASIP) gene model whereby maternal exposure to BPA shifted the coat color distribution of agouti mouse offspring toward yellow through decreasing the DNA methylation of a retrotransposon located upstream of the Agouti gene (Dolinoy, Huang, & Jirtle, 2007). Currently, DNA methylation aberrations in response to BPA exposure have been studied in multiple tissues and different organisms. However, to the best of our knowledge, no study has investigated the DNA methylation aberrations induced by BPA analogues, BPF and BPS.

Multiple animal studies reported that exposure to BPA *in-utero* and in early postnatal period may impair brain development, sexual differentiation and behavior through disruption of epigenetic programming of certain genes (Anderson et al., 2012; Chao et al., 2012; Doshi, D'Souza, Dighe, & Vanage, 2012; Doshi, Mehta, Dighe, Balasinor, & Vanage, 2011; M. Hiyama et al., 2011; Jang et al., 2012; Wolstenholme et al., 2011; H. Q. Zhang et al., 2012). Besides, being an estrogenic monomer, the potential effect of BPA on the ER has also been investigated in rat and mice models. As such, neonatal BPA exposure was found to be associated with increased *ER1* promoter methylation and decreased global DNA methylation in the testis of rats, which was associated with alterations in *DNMT* expression (Abdel-Maksoud, Leasor, Butzen, Braden, & Akingbemi, 2015; Anderson et al., 2012; Doshi et al., 2011; M. Hiyama et al., 2011). Besides, in mice oocytes, neonatal BPA exposure was associated with hypomethylation of imprinted genes (*Insulin-like growth factor II receptor "IGF2R"* and *paternally expressed 3 "PEG3"*) and increased ER (mRNA and protein) expression (Chao et al., 2012).

There are also few studies performed on human tissues, some of which used high throughput methylation arrays. For instance, Hanna *et al.* (2012) evaluated DNA methylation in blood samples from 43 women scheduled for *in-vitro* fertilization and revealed a lower methylation of a promoter CpG site at the *TSP50* gene in those with higher urinary BPA levels (Hanna et al., 2012). In addition, high urinary BPA levels were associated with global hypomethylation in spermatozoa in male factory workers (Miao et al., 2014). More recently, Kim *et al.* used salivary samples from 60 pre-pubescent girls and found that lower genomic methylation was generally associated with higher urinary BPA levels. Pathway analyses revealed that genes showing lower DNA methylation in association with higher BPA levels were involved in immune responses, metabolism, and transport (J. H. Kim et al., 2013). Moreover, BPA levels in fetal liver tissues derived from voluntary terminations of pregnancy were categorized into tertiles (n=6 for each group) and reported to be significantly associated with linear and non-monotonic aberrations in DNA methylome in liver tissues (Faulk et al., 2015). In addition, higher BPA levels were associated with DNA hypermethylation in CpG islands but with DNA hypomethylation in CpG shores, shelves and repetitive regions.

5. BPA and DNA methylation in breast cells

Some cell culture and animal studies have been performed to test the effect of BPA on DNA methylation in breast cells (L. Camacho et al., 2015; Dhimolea et al., 2014; Fernandez et al., 2012; Mine Senyildiz, 2015; Qin et al., 2012; Weng et al., 2010) (**Table 3**),while only one region of interest (ROI) assay was peformed for BPS (Huang et al., 2019)

and none for BPF. Two recent studies on rodents did not observe changes in global genomic DNA methylation in rats exposed prenatally to BPA, though region-specific DNA methylation changes were reported (78;79). In cell culture studies, a functional analysis confirmed that the expression of lysosomal-associated membrane protein 3 became epigenetically silenced in breast epithelial cells after exposure to BPA and probably through an ER-dependent pathway (Weng et al., 2010). Furthermore, one recent study showed that BPA exposure is associated with decreased global DNA methylation in MCF-7 cells (Mine Senyildiz, 2015).

Only one methylome-wide profiling and few targeted assays were performed to examine BPA DNA methylation aberrations in breast cells (Fernandez et al., 2012; Qin et al., 2012; Wang, Wei, Guo, Yuan, & Zhao, 2018). In the methylome-wide analysis, MCF-7 cells were treated with BPA (10^{-5} M or 10^{-6} M) for 5 weeks, and DNA methylation analysis using the older generation Infinium HumanMethylation450 arrays showed that BPA induces hypermethylation of tumor suppression genes and hypomethylation of genes involved in tumor development and growth (Wang et al., 2018). As for the targeted assays, hypermethylation of proapoptotic genes and hypomethylation of DNA repair genes were observed in MCF10-F after treatment with BPA (10^{-5} M or 10^{-6} M) for 2 weeks (Fernandez et al., 2012), and these DNA aberrations were reported to result in increased proliferation and DNA repair. It was postulated that DNA repair mechanisms are elicited in the cells in response to BPA-induced DNA damage and formation of adducts (Fernandez et al., 2012). Another targeted DNA methylation assay showed that tumor suppressor genes, which were also reported to be hypermethylated in breast cancer, were hypermethylated in HMEC cells

treated with 10^{-8} M of BPA for 7 days (Qin et al., 2012). These findings in breast epithelial cells suggest the role of epigenetic disruption in BPA-induced breast cancer promoting effects (Qin et al., 2012). As for BPS, its DNA methylation aberrations in breast cells were only recently investigated in one study on the breast cancer cell line MCF-7. As such, MCF-7 cells were treated with 10^{-6} M BPS for 24 hrs, and DNA methylation analysis was performed for eight transposons and 22 ROI with reported tumor suppressor role. Results showed hypermethylation of two transposons (MaLR and Mariner 2) and of three tumor suppressor genes (*CDH1, SFN, TNFRSF10C*) in treated *versus* untreated cells (Huang et al., 2019).

Table 3. Cell culture, animal and clinical studies investigating the DNA methylation aberrations associated with bisphenol A (BPA) in multiple tissue types

Study type	Reference	Model	Target tissue type	BPA concentration or dose	Treatment Duration	Study Endpoint	Method	Methylation changes
Cell culture	Wang *et al.* 2018 (Wang et al., 2018)	MCF-7	Breast cancer cells	10^{-5} M	5 weeks	5 weeks	HPLC-MS	No change: Global methylation
								No change: Global hydroxymethylation
							Infinium Human methyation 450K array	Hyper: 182 genes
								Hypo: 134 genes
				10^{-6} M	5 weeks	5 weeks	HPLC-MS	No change: Global methylcytosine
								No change: Global hydroxymethylcytosine
							Infinium Human methyation 450K array	Hyper: 159 genes
								Hypo: 137 genes
	Yin *et al.* 2016 (Yin et al., 2016)	GC-2	Mouse spermatocyte cells	$2 * 10^{-5}$ M	2 days	2 days	5-methylcytosine DNA blot hybridization	No change: Global methylation
							Affymetrix mouse promoter 1.0R array	Hyper: *Myosin-binding protein H*
				$4 * 10^{-5}$ M	2 days	2 days	5-methylcytosine DNA blot hybridization	Hyper: Global methylation
							Affymetrix mouse promoter 1.0R array	Hyper: *Myosin-binding protein H*
				$8 * 10^{-5}$ M	2 days	2 days	5-methylcytosine	Hyper: Global

							DNA blot hybridization	methylation
							Affymetrix mouse promoter 1.0R array	Hyper: > 1000 DMPs (mostly hyper)
								Hyper: Myosin-binding protein H
								Hypo: *Protein kinase C*
	Senyildiz *et al.* 2015 (Mine Senyildiz, 2015)	MCF-7	Breast cancer cells	100 nM	48 hrs or 96 hrs	48 hrs or 96 hrs	5-mC Elisa kit	Hypo: Global methylation (48 and 96 hrs)
				1 uM	48 hrs or 96 hrs	48 hrs or 96 hrs		
	Kitraki *et al.* 2015 (Kitraki, Nalvarte, Alavian-Ghavanini, & Ruegg, 2015)	HT-22	Mouse hippocampal neuronal cells	10nM	2 days	2 days	Bisulfite pyrosequencing	Hyper: *Fkbp5*
				1000 nM	2 days	2 days		Hyper: *Fkbp5*
	Fernandez *et al.* 2012 (Fernandez et al., 2012)	MCF-10F	Immortalized normal-like breast cells	10^{-5} M	2 weeks	2 weeks	Methylated DNA IP-on-chip	Hyper: 1266 genes
								Hypo: 154 genes
				10^{-6} M	2 weeks	2 weeks	Methylated DNA IP-on-chip	Hyper: 633 genes
								Hypo: 155 genes
	Qin *et al.* 2012 (Qin et al., 2012)	HMEC	Primary normal breast cells	10^{-8} M	7 days	2-5 weeks	Methyl-Profiler DNA Methylation array (24 gene promoters)	Hyper: *BRCA1, CCNA1, CDKN2A, THBS1, TNFRSF10C,*

								TNFRSF10D
								Hypo: *H1C1*
Animal studies	Jadhav *et al.* 2017 (Jadhav et al., 2017)	Sprague-Dawley rats	Mammary tissue	250 µg/kg/day	PND2 - PND20	PND100	MBDCap-seq	Hyper: 4028 genes
						PND100		Hypo: 91 genes
	Cheong *et al.* 2016 (Cheong et al., 2016)	Sprague-Dawley rats	Prostate tissue	10 µg/kg	PND1,3,5	PND90	MIRA-assisted Rat ChIP 385K array	Change: 86 genes
	Kitraki *et al.* 2015 (Kitraki et al., 2015)	Wistar rats	Hippocampus	40 µg/kg/day	Pregnancy and lactation	PND46	Bisulfite pyrosequencing	Hyper: *Fkbp5*
	Dhimolea *et al.* 2014 (Dhimolea et al., 2014)	Wistar-Furth rats	Mammary tissue	250 µg/kg/day	GD1 - PND1	PND4	Nimblegen ChIP array	Hyper: 812 regions
						PND4		Hypo: 675 regions
						PND21		Hyper: 1904 regions
						PND21		Hypo: 1787 regions
						PND50		Hyper: 1072 regions
						PND50		Hypo: 1162 regions
	Yaoi *et al.* 2008 (Yaoi et al., 2008)	Mice	Forebrain	20 µg/kg/day	E0 - E 12	E12.5	Methylation sensitive qPCR	Hyper: 12 spots
						E12.5		Hypo: 10 spots
				20 µg/kg/day	E0 - E 14	E14.5	Methylation sensitive qPCR	Hyper: 9 spots
								Hypo: 9 spots
	Dolinoy *et al.* 2007 (Dolinoy et al., 2007)	A^{vy} mice	tail, brain, liver, kidney	50 mg/kg/day	2 weeks before mating and throughout	PND22	Bisulfite sequencing	Hypo: *Agouti*

					pregnancy and lactation			
Clinical studies	Tian *et al.* 2018 (Tian et al., 2018)	Male factory workers	Semen	BPA exposed: GM (SD) 158.41 (17.92) µg/g creatinine vs. BPA unexposed: 0.84 (6.53) µg/g creatinine	Environmental exposure	NA	EpiMark 5-hmC analysis Kit	Hyper: *LINE-1* hydroxymethylation
	Faulk *et al.* 2015 (Faulk et al., 2015)	Normal liver tissues from pregnancy terminations (GD70 - 120)	Liver	< LOD (0.4 ng/ml) - 96.76 ng/g (liver)	Environmental exposure	NA	Methyl-Plex-next generation sequencing	Change: 6286 DMRs (no BPA *versus* low BPA)
								Change: 7340 DMRs (no BPA *versus* high BPA)
	Kim *et al.* 2013 (J. H. Kim et al., 2013)	Prepubescent Egyptian girls	Saliva	< LOD (0.4 ng/ml) - 12 ng/ml (urine)	Environmental exposure	NA	Infinium HumanMethylation27 BeadChip	Change: 1439 DMPs (low *versus* high BPA)

HPLC-MS: high performance liquid chromatography-mass spectroscopy, IP: immunoprecipitation, ChIP: chromatin immunoprecipitation, mC: methylcytosine, MIRA: Methylated CpG island recovery assay, MBDCap-seq: methyl-binding domain capture sequencing, qPCR: quantitative polymerase chain reaction, E: embryonic day, GM: geometric mean, SD: standard deviation, LOD: level of detection, PND: postnatal day, DMP: differentially methylated probes, DMRs: differentially methylated regions, NA: not available

In conclusion, although the DNA methylation effects of BPA were investigated in few cell culture targeted assays and in one methylome-wide profiling assay in breast cell lines, only one recent study was performed for BPS using targeted assay, and no studies were performed for BPF in breast cells. Hence, the methylome-wide aberrations induced by BPA analogues, BPF and BPS in breast cells are not yet addressed.

CHAPTER II

SPECIFIC AIMS

Breast cancer is the most common and fatal cancer type among females globally including Lebanon (IARC, https://gco.iarc.fr/today/home Last accessed: Jan-2019). Knowing that estrogens are key players in the development of cancer in estrogen-responsive organs such as the breast (Horn & Vatten, 2017; Jeronimo et al., 2017; Lakkis et al., 2010), it has been postulated that EDCs with estrogenic activity induce carcinogenic effects in these organs (Macon & Fenton, 2013). As a matter of fact BPA, an EDC with estrogenic activity, has been linked to the development of breast cancer in several cell culture and animal studies (Acevedo et al., 2013; Aghajanpour-Mir et al., 2016; Fernandez & Russo, 2010; H. S. Lee et al., 2014; Miyakoshi et al., 2009; Murray et al., 2007; Pfeifer et al., 2015; Pisapia et al., 2012; Ricupito et al., 2009; Seachrist et al., 2016; H. Song et al., 2015; Stroheker et al., 2004; W. Zhang et al., 2012). Breast cancer has been associated with a number of molecular changes such as short RTL and global DNA hypomethylation (Looi et al., 2010; A.K. Meeker et al., 2004; Rashid-Kolvear et al., 2007; Tang et al., 2016); Although BPA was shown in few cell culture studies to be associated with increased telomerase expression and decreased *LINE-1* methylation (Mine Senyildiz, 2015; Takahashi et al., 2004; B. L. Xu et al., 2015), no such data are available from epidemiological studies. A recent study conducted on a community cohort of Lebanese individuals of whom ~65% were females

revealed that urinary BPA levels are comparable to those of other populations (Mouneimne et al., 2017). However, whether BPA is associated with alterations in RTL and global DNA methylation and whether these molecular alterations are associated with breast cancer have not been previously investigated in the Lebanese. In addition, there is limited knowledge regarding BPA analogues (BPF and BPS) exposure risk and the associated molecular changes in breast cancer.

The research questions are hence whether BPA/F/S exposure promotes breast cancer and results in changes in RTL and DNA methylation aberrations that are associated with breast cancer. Due to the unavailability of a longitudinal cohort and biorepository of women with data on BPA/F/S exposure and incidence of breast cancer, we decided to address the question indirectly by conducting a dual epidemiological-cell culture approach. In the epidemiological part, we aimed to validate the RTL and *LINE-1* DNA methylation aberration results shown in previous studies in biological samples of Lebanese women with breast cancer compared to Lebanese women without breast cancer. We also aimed to measure RTL and *LINE-1* methylation in peripheral blood of a cohort of Lebanese women for whom urinary BPA levels were available for association analysis. The cell culture part was conducted with the aim to evaluate the tumor promoting potential, including ER-dependence and associated telomerase-linked and global (using *LINE-1* as a proxy) and genome-wide methylation mechanisms, of BPF and BPS in comparison to BPA in breast cells.

PART I. EPIDEMIOLOGICAL PART

We aimed to investigate whether BPA exposure is associated with alterations in RTL and *LINE-1* methylation and whether these alterations can act as biomarkers of breast cancer risk.

We measured RTL and *LINE-1* methylation in peripheral blood of Lebanese women without breast cancer, with the aim to test the hypothesis that high BPA urinary levels are associated with altered RTL (**H1**) and *LINE-1* hypomethylation (**H2**) in peripheral blood of these subjects.

We also measured RTL and *LINE-1* methylation in peripheral blood of Lebanese women with breast cancer and compared them to the levels measured above in the Lebanese women without breast cancer with the aim to test the hypotheses that breast cancer is associated with altered RTL (**H3**) and *LINE-1* hypomethylation (**H4**), and that these alterations are in the same direction as those associated with higher BPA exposure.

We also measured RTL and *LINE-1* methylation in breast tissues with the aim to test the hypotheses that RTL (**H5**) and *LINE-1* DNA methylation differences (**H6**) are in the same direction in cancerous tissues versus normal adjacent tissues when compared to results from peripheral blood of women with breast cancer versus women without breast cancer.

PART II. CELL CULTURE PART

Despite the lack of valid epidemiologic evidence, multiple animal studies have shown the effects of BPA on the development of mammary gland carcinogenesis using several animal models (**Table 2**), though only one study is currently available for BPS ("DES daughter,")

and so far none is for BPF. Several cell culture studies showed that BPA increases the proliferation of ER-positive breast cancer cell lines proliferation (Aghajanpour-Mir et al., 2016; H. S. Lee et al., 2014; Miyakoshi et al., 2009; Pfeifer et al., 2015; Pisapia et al., 2012; Ricupito et al., 2009; H. Song et al., 2015; Stroheker et al., 2004; W. Zhang et al., 2012) and few showed that this increase is ER-dependent (J. Y. Kim et al., 2017; G. A. Lee et al., 2017). However, few cell culture studies were performed for BPF and BPS, and all showed similar results to BPA (J. Y. Kim et al., 2017; Pisapia et al., 2012; Stroheker et al., 2004). Only two studies tested the effect of BPA on the migration of ER-positive breast cancer cell lines (MCF-7 and MCF-7 CV), and one of these also tested the effects of BPF and BPS. Both studies reported an increased migration of cells and altered expression of epithelial to mesenchymal transition markers in response to treatment with BPA (J. Y. Kim et al., 2017; G. A. Lee et al., 2017), and similar results were reported with BPA analogues (BPF and BPS) (J. Y. Kim et al., 2017). Thus, more cell culture studies are warranted to compare the effects of the three bisphenols on breast cancer progression.

To date, only one cell culture study performed epigenome-wide DNA methylation profiling (Wang et al., 2018), and one cell culture study showed an increase in telomerase expression in breast cells treated with BPA (Takahashi et al., 2004). As for BPA analogues, BPF and BPS, their effects on telomerase and genome-wide DNA methylation have not been addressed.

Hence, in breast cells, little is known about the telomerase- and whole genome DNA methylation-linked mechanisms associated with the cancer promoting potential of BPA, and these mechanisms were not addressed for BPA analogues. In order to unravel these

mechanisms and to assess the contribution of ER, we aimed to test the below hypotheses on two breast cancer cell lines (ER-positive: MCF-7 and ER-negative: MDA-MB-231). We initially also aimed to run the same types of experiments on normal-like breast epithelial cell lines (MCF-10A and MCF-10F). These aims were however unfortunately aborted due to the embargo on cholera toxin by the Lebanese authorities, a toxin that is crucial for the growth of these cell lines.

H1. BPA and its analogues (BPF and BPS) induce an ER-dependent increase in the metabolic activity, viability, cell cycle progression, and migration of breast cancer cells.

We aimed to first measure the metabolic activity and viability of breast cancer cell lines treated with BPA and its analogues BPF and BPS with and without ERI (ICI 182,780) using MTT assay and trypan blue cell exclusion assay. We sought to replicate the previous several studies performed in the literature on cell proliferation and the implicated role of ER pathway regarding BPA (Aghajanpour-Mir et al., 2016; H. S. Lee et al., 2014; Miyakoshi et al., 2009; Pfeifer et al., 2015; Pisapia et al., 2012; Ricupito et al., 2009; H. Song et al., 2015; Stroheker et al., 2004; W. Zhang et al., 2012) and the little studies regarding BPF and BPS (J. Y. Kim et al., 2017; Pisapia et al., 2012; Stroheker et al., 2004), and to select the optimal treatment concentration and duration for other assays. First, concentrations ranging from human exposure concentration to very high concentrations were assessed for effects on cell metabolic activity and viability using MTT and trypan blue cell exclusion assays. The minimum functional concentration, human exposure concentration and optimal treatment time points associated with marked increase in cell metabolic activity and

viability were selected for other assays including cell cycle analysis and cell migration (cell scratch) assays.

H2. BPA and its analogues (BPF and BPS) induce an ER-dependent increase in telomerase expression and activity and alteration in RTL in breast cancer cells.

We aimed to determine the effects of BPA and its analogues (BPF and BPS) with and without ERI on telomerase gene expression and RTL and the potential contribution of ER in these effects. In breast cells, one cell culture study showed that BPA increases telomerase expression in MCF-7 cells (Takahashi et al., 2004), so we wanted to replicate their finding and evaluate the effects of BPF and BPS on telomerase expression. We also measured the RNA expression and protein activity of *h*TERT and RTL in these breast cancer cells.

H3. BPA and its analogues (BPF and BPS) alter the RNA expression and enzymatic activity of DNA methylation and demethylation in breast cancer cells, potentially through an ER dependent pathway.

We aimed to examine the effects of BPA, BPF and BPS with and without ERI on RNA expression and enzymatic activity of DNA methylation and demethylation enzymes in breast cancer cells, and this aim was not addressed before for the three bisphenols in breast cells. We measured RNA expression and enzymatic activity of genes involved in DNA methylation (*DNMT1, DNMT3A, DNMT3B)* and demethylation (*TET* enzymes (1, 2 and 3)) in MCF-7 breast cancer cells.

H4. BPA and its analogues (BPF and BPS) decrease the global DNA methylation in breast cancer cells, potentially through an ER dependent pathway.

We aimed to assess the effects of BPA, BPF and BPS with and without ERI on global DNA methylation in breast cancer cells. In the literature, one cell culture study showed that BPA decreases the global DNA methylation in MCF-7 cells (Mine Senyildiz, 2015), but this was never addressed for BPF and BPS. We measured *LINE-1* methylation in these breast cancer cells treated with BPA, BPF and BPS with or without ERI for two time points. The time point associated with the highest change in *LINE-1* methylation in response to BPA, BPF and BPS was selected for the epigenome-wide DNA methylation profiling.

H5. BPA and its analogues (BPF and BPS) disrupt the DNA methylation of sites and regions in genes involved in cancer promoting pathways, potentially through an ER dependent pathway.

We aimed to assess the epigenome-wide DNA methylation effects of BPA, BPF and BPS with and without ERI in MCF-7 breast cancer cells. We detected differentially methylated sites and regions in response to BPA, BPF and BPS with or without ERI using DNA methylome-wide analysis and performed pathway analysis for these genes using online pathway analysis tools.

H6. BPA and its analogues (BPF and BPS) disrupt the DNA methylation of genes that are similarly differentially methylated in cancerous tissues when compared to normal adjacent tissues in breast cancer patients

We aimed to detect the clinical relevance of the differentially methylated sites and genes associated with BPA and its analogues BPF and BPS in breast cancer development. For this aim, we used publicly available methylation data of 21986 CpG sites in 595 ER-positive breast cancer and 124 adjacent-normal breast tissues of breast cancer patients based on The Cancer Genome Atlas (TCGA) database (The Cancer Genome Atlas et al., 2012).

CHAPTER III

MATERIAL AND METHODS

PART I. EPIDEMIOLOGICAL PART

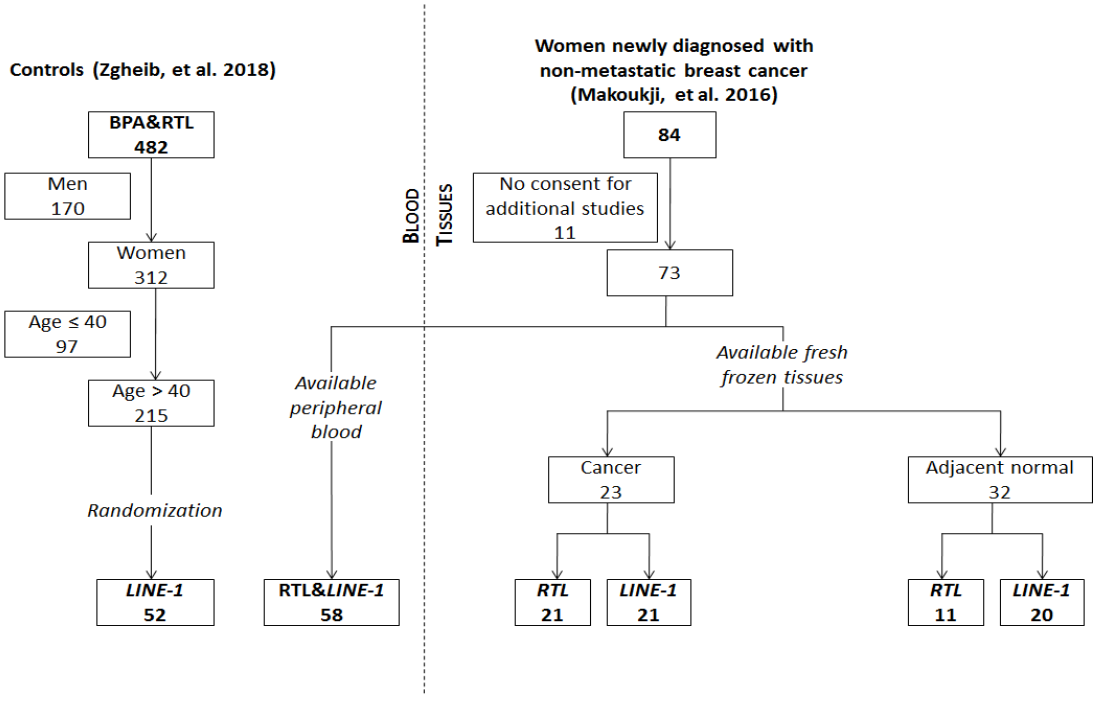
A. Study Participants

This study was approved by the Institutional Review Board (IRB) and builds on two previously recruited and described cohorts (**Figure 2**).

The first cohort involved clinical data and stored peripheral blood DNA from a community of 482 Lebanese individuals who did not have a cancer diagnosis (controls) and who were recruited between February and June 2014. All subjects signed an informed consent. Noteworthy, individuals working in plastic manufacturing were not eligible for the study. Non-occupational BPA measurements were available for all study participants (Mouneimne et al., 2017), and we measured RTL in blood of all participants. However, for *LINE-1* we were limited on budget. Since almost all of the potential participants with breast cancer were diagnosed at age 40 years or above, and since it is known that both DNA methylation and RTL are affected by aging (Hannum et al., 2013; Zgheib et al., 2018), we deliberately randomly extracted the final sample (N=52) from the subsample of female participants who were 40 years of age or above (**Figure 2**).

The second cohort entailed clinical data, stored peripheral blood and fresh frozen tissues from 84 Lebanese women newly diagnosed with non-metastatic breast cancer (cases) who were recruited between September 2012 and May 2014 (Makoukji et al., 2016). All subjects signed an informed consent for the initial study but, and as seen in **Figure 2**, not all agreed to be included in further studies. In addition, no left over peripheral blood and tissues were available for a number of subjects. We were, hence, left with a final sample size of 58 for peripheral blood, and 23 cancerous and 32 normal adjacent tissues. The sample sizes in some downstream analyses decreased further due to suboptimal experimental results and quantity/quality of extracted DNA.

Figure 2. Diagram of study participants and biological samples



BPA: Bisphenol A, RTL: relative telomere length, LINE-1: long interspersed nuclear element-1

B. DNA isolation, quantification and bisulfite conversion

DNA from blood samples was isolated using Flexigene DNA isolation kit from Qiagen (cat # 51206, Hilden, Germany) and DNA from tissue samples was isolated using trizol reagent (Sigma-Aldrich, Taufkirchen, Germany) as per manufacturer's protocol. DNA was then measured using a Denovix DS-11 spectrophotometer (Denovix Inc., Wilmington, DE, USA), and both 260/230 and 260/280 ratios were detected for assessment of the purity of samples. Bisulfite conversion of a portion of the isolated DNA was performed using EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) as per manufacturer's protocol. Both bisulfite converted and non-converted DNA samples were stored at -20°C until further assays.

C. RTL measurement

RTL was measured on the isolated DNA using quantitative PCR (qPCR) as previously described by Cawthon et al. (2002) (Cawthon, 2002). *Telomere* and single copy gene (SCG) PCR were performed in separate 384 well plates using the *telomere* and *human ß-globin (SCG)* primer pairs shown in **Table 4**. In both PCR experiments, telomere and SCG of a 3 µl DNA sample (10ng/µl) were amplified using the corresponding primer pairs and SYBR green master mix (cat# 1708882, Bio-Rad, Hercules, CA, USA). The thermal cycling protocol for telomere was: 50°C for 2 min, 95°C for 2 min, then two cycles of 95°C for 15 sec, 49°C for 15 sec, then 35 cycles of 95°C for 15 sec, 62°C for 10 sec, and 74°C for 15

sec. For SCG: 50°C for 2 min, 95°C for 2 min, then 35 cycles of 95°C for 15 sec and 58°C for 1 min. A melt curve was generated to detect any primer dimer formation.

In addition to the samples, standards of concentrations ranging from 0.312 ng/µl to 40 ng/µl were prepared from a pool of DNA samples and run in every plate along with a NTC. Standard curves for each of telomere and SCG ct with log (standard concentration) were ideal with an $r^2 > 0.9$. Samples and standards were run in triplicates, and samples with intra-standard deviation of > 1 were excluded.

D. Bisulfite pyrosequencing of LINE-1

LINE-1 region of interest was amplified on the bisulfite converted DNA and sequenced as previously described (Daskalos et al., 2009; Ghantous et al., 2014) using Hot-Start Taq Master Mix from Qiagen (Hilden, Germany) and the primers listed in **Table 4**. This was carried out at IARC on the PyroMark Q96 ID (Qiagen, Hilden, Germany), and the methylation % of 5 CpG sites in the *LINE-1* gene were measured.

Table 4. Primers used in the different assays (Part I and II)

	Primers	Reference
LINE-1 pyrosequencing		Daskalos *et al.* (2009)
Forward primer	5’ btn-TAG GGAGTGTTAGATAGTGG 3’	
Reverse primer	5’ AACTCCCTAACCCCTTAC 3’	
Sequencing	5’ CAAATAAAACAATACCTC 3’	
Relative telomere length		Cawthon *et al.* (2002)
Telomere		
Forward primer	5’ACACTAAGGTTTGGGTTTGGGTTTGGGT TTGGGTTAGTGT 3’	
Reverse primer	5’TGTAGGTATCCTATCCCTATCCCTATCC TATCCCTAACA 3’	
human ß-globin		
Forward primer	5’ GCTTCTGACACAACTGTGTTCACTAG 3’	
Reverse primer	5’ CACCAACTTCATCCACGTTCACC 3’	
Gene expression		
DNA methyltransferase 1 (DNMT1)		Sheng *et al.* (2013)
Forward primer	5’ AAACCCCTTTCCAAACCTCG 3’	
Reverse primer	5’ CTGGTGCTTTTCCTTGTAATCC 3’	
DNA methyltransferase 3A (DNMT3A)		Sheng *et al.* (2013)
Forward primer	5' CCAAGTTCAGCAAAGTGAGGAC3'	
Reverse primer	5' TGGACTGGGAAACCAAATACC3'	
DNA methyltransferase 3B (DNMT3B)		Sheng *et al.* (2013)
Forward primer	5' TCCCAGCTCTTACCTTACCATC 3'	
Reverse primer	5' ATCTCCACTGTCTGCCTCCA 3'	
Ten-eleven translocation 1 (TET1)		Guidotti *et al.* (2012)
Forward primer	5’ CCCGGGCTCCAAAGTTGTG 3’	
Reverse primer	5’ GCAGGAAACAGAGTCATTGGTCCT 3’	
Ten-eleven translocation 2 (TET2)		Guidotti *et al.* (2012)
Forward primer	5’ GAAAGGAGACCCGACTGCAACTG 3’	
Reverse primer	5’ GCAGCTCAGTCCCTTACTGCTC 3’	
Ten-eleven translocation (TET3)		Guidotti *et al.* (2012)
Forward primer	5’ CAGTGGCTTCTTGGAGTCACCTC 3’	
Reverse primer	5’ GGATGGCTTTCCCCTTCTCTCC 3’	
Telomerase		Zhu *et al.* (2006)
Forward primer	5’ CGTCGAGCTGCTCAGGTCTT 3’	
Reverse primer	5’ AGTGCTGTCTGATTCCAATGCTT 3’	
ß2-microglobulin		Sheng *et al.* (2013)
Forward primer	5' TGCTGTCTCCATGTTTGATGTATCT3'	
Reverse primer	5' TCTCTGCTCCCCACCTCTAAGT 3'	
Telomerase activity		Yaku *et al.* (2017)
Forward TS	5’ AATCCGTCGAGCAGAGTT 3'	
Reverse ACX	5’ GCGCGG(CTT ACC)$_3$CTAACC 3'	

E. Statistical Analysis

Data were analyzed using Statistical Package for Social Sciences version 24.0 for Windows (SPSS, Chicago, IL.SPSS) and displayed graphically using GraphPad Prism version 6.0 (La Jolla, CA, USA). A P value less than 0.05 was considered statistically significant.

Categorical variables are presented as number and percent, whereas continuous ones are presented as mean ± SD. BPA urinary levels were adjusted for urinary creatinine and calculated as μg/g creatinine. BPA and RTL were categorized into tertiles. Multinomial logistic regression was then carried out for the association between BPA and RTL. Multivariate analysis was performed to adjust for potentially confounding variables: those that showed statistical significance (P value < 0.05) at the univariate analysis and those that were considered clinically important though not statistically significant; these included age, BMI and WC. Dose-response relationship was assessed by carrying out trend analyses where the P value was used to indicate statistical significance. Analyses were performed on the total sample and stratified by gender. As for the association between urinary BPA levels adjusted for urinary creatinine and peripheral blood *LINE-1* methylation, results were not categorized because of the small sample size. Only CpG sites whose DNA methylation was detected with good quality were included in the analysis, and the association between BPA and *LINE-1* methylation (at every CpG site and average of all CpGs) was tested using linear regression.

Breast cancer patients and controls were compared for age, BMI, alcohol consumption, smoking history and premenopausal status using student's t-test or chi-square test as appropriate. *LINE-1* methylation and RTL were compared between breast cancer patients

and controls and between cancerous and normal adjacent breast tissues using student's t-test. Logistic regression analysis was also performed to detect their association with breast cancer before and after adjustment for potential co-variates (BMI, smoking, and alcohol consumption). *LINE-1* methylation and RTL in cancerous breast tissues were tested for correlation with respective levels in corresponding blood samples using bivariate Pearson correlation. No paired analysis was performed for the tissue samples as very few cancer and normal adjacent tissues originated from the same patient. Crude and adjusted linear regression models were also used to identify whether clinicopathological characteristics of breast cancer patients are predictors of *LINE-1* methylation and RTL in breast tissues.

PART II. CELL CULTURE PART

MCF-7 and MDA-MB-231 breast cancer cell lines were treated with BPA, BPF and BPS ranging from non-occupational environmental exposure concentrations (10^{-8}M for BPA and BPF; 10^{-9}M for BPS) to high concentration (10^{-4}M) in the presence and absence of ERI for 24, 48 and 72hrs, and assessed for cell metabolic activity and viability using MTT and trypan blue assay, respectively. Non-occupational human exposure concentrations (10^{-8}M for BPA and BPF; 10^{-9}M for BPS) and minimum functional concentrations (10^{-6}M for BPA and BPF; 10^{-5}M for BPS) which noteworthy approached occupational concentrations for BPA (Ribeiro et al., 2017) were used in cell cycle, cell scratch and molecular assays. These included RT-PCR measurement of the gene expression of telomerase and DNA (de)methylation enzymes, qPCR measurement of RTL, telomeric repeat amplification protocol (TRAP) for telomerase activity, measurement of the activity of DNA methylation and demethylation enzymes, *LINE-1* pyrosequencing and methylome-wide profiling using

Infinium MethylationEPIC microarrays. Bisphenol-induced differentially methylated genes were compared with those differentially methylated in ER-positive breast cancer patients relative to normal adjacent tissues from The Cancer Genome Atlas (TCGA) database.

A. Bisphenol reagents and related chemicals

BPA (cat#239658), BPF (cat# 51453) and BPS (cat# 43034) were purchased from Sigma-Aldrich (Taufkirchen, Germany), and ER inhibitor (ERI) fulvestrant, ICI 182,780 (cat# sc-203435) was purchased from SantaCruz Biotechnology, Inc. (Dallas, Texas, USA). BPA, BPF and BPS were dissolved in either absolute DMSO (cat# 41640, Sigma-Aldrich, Taufkirchen, Germany) or ethanol (cat# ET0006, Scharlab S.L., Barcelona, Spain) at stock concentrations of 1 M, and ERI was dissolved in absolute DMSO at stock concentration of 100 µM. Stock solutions were stored in aliquots at -20 °C.

B. Choice of concentrations

Epidemiological studies detected BPA and its analogues BPF and BPS in a large number of plasma and/or urine samples from human individuals (Mouneimne et al., 2017; Padmanabhan et al., 2008; Sprague et al., 2013; Thayer et al., 2016; Ye et al., 2015; Q. Zhou et al., 2013). Non-occupational plasma and urine levels of BPA ranged roughly from < LOD to 9.6×10^{-8} M (Padmanabhan et al., 2008; Sprague et al., 2013; Q. Zhou et al., 2013), but those of BPS were 10 folds lower than BPA (Thayer et al., 2016). To date, no report is available concerning the plasma level of BPF; however, its urine levels were

comparable to those of BPA in epidemiological studies (Ye et al., 2015; T. Zhang et al., 2016). Hence, we considered plasma and/or urine levels of 10^{-8}M BPA, 10^{-8} M BPF and 10^{-9} M BPS as human exposure concentrations and tested them in our study. For selection of the concentration that may induce phenotypic and, hence, molecular changes in breast cancer cell lines, concentrations ranging from 10^{-4} M (very high) to human exposure concentration (10^{-8} M for BPA and BPF, 10^{-9} M for BPS) were tested in MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and trypan blue assays. The human exposure concentration, together with the minimum functional concentration that was associated with marked increase in cell metabolic activity and viability were then tested in the cell scratch assay.

C. Cell culture and media

MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) cell lines originating from human breast epithelial adenocarcinomas were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured in Dubecco's Modified Eagle's Medium (DMEM) (cat# BE-12-741F, Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS) (cat# F2442, Sigma-Aldrich, Taufkirchen, Germany), 1% penicillin/streptomycin (cat# 17-602E, Lonza, Basel, Switzerland) and 1% sodium pyruvate (cat# S8636, Sigma-Aldrich, Taufkirchen, Germany)at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. Prior to each assay, cells were cultured for 2-3 days in phenol-red free DMEM (cat# BE12-917F, Lonza, Basel, Switzerland) supplemented with

10% charcoal-stripped FBS (cat# F6765, Sigma-Aldrich, Taufkirchen, Germany), 2% L-glutamine (cat# G7513, Sigma-Aldrich, Taufkirchen, Germany) and 1% penicillin/streptomycin, in order to avoid the effects of the estrogenic components of DMEM and FBS. Cells were detached using 0.25% Trypsin (cat# BE17-160E, Lonza, Basel, Switzerland) and 0.53 mM Ethylenediaminetetraacetic acid (EDTA) (cat# AM9260G, Ambion, Waltham, MA, USA) solution.

D. Cell metabolic activity using MTT assay

MTT assay was performed at 24, 48 and 72 hrs for each treatment concentration with and without ERI. In brief, MCF-7 and MDA-MB-231 cells were seeded in a 96-well plate at a seeding density of 6,000 and 4,000 cells, respectively. After overnight incubation, cells were treated in triplicates with different concentrations of BPA (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M), BPF (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M) or BPS (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M) with or without ERI (100nM) for 24, 48 and 72 hrs. Control cells were treated with 0.2% DMSO (BPA control) or both 0.1% ethanol and 0.1% DMSO (BPF and BPS control). After treatment, cells were incubated with 10 µl of MTT reagent for 4 hrs, which was followed by overnight incubation with 100 µl of solubilizing agent (cat# 11465007001, Sigma-Aldrich, Taufkirchen, Germany). Absorbance was detected using an ELISA plate reader at wavelength of 595 nm. After subtraction of absorbance obtained from wells containing no cells (negative control), results were calculated as % of metabolic activity relative to control. Results are presented as mean optical density (OD) or % of OD relative to control

(% metabolic activity) +standard error of the mean (SEM) of at least three independent trials.

E. Cell viability using trypan blue assay

MCF-7 and MDA-MB-231 cells were seeded in a 6-well plate at a seeding density of 3×10^5 and 1×10^5 respectively. After overnight incubation, cells were treated with different concentrations of BPA (10^{-4}, 10^{-6} and 10^{-8} M), BPF (10^{-4}, 10^{-6} and 10^{-8} M) or BPS (10^{-4}, 10^{-5} and 10^{-9} M) with or without ERI for 24, 48, and 72 hrs. Control cells were treated with 0.1% DMSO (BPA control) or both 0.1% DMSO and 0.001% ethanol (BPF and BPS control). After treatment, cells were counted using a hematocytometer using trypan blue dye (cat# T8154, Sigma-Aldrich, Taufkirchen, Germany). Results are presented as mean viable cell count or % of viable cell count relative to control + SEM of at least three independent trials.

F. Cell migration using cell scratch assay

MCF-7 and MDA-MB-231 cells were seeded into 6-well plates at a seeding density of 1.5×10^{-6} and 1×10^{-6} cells, respectively, so that a confluent monolayer is obtained after overnight incubation. Cells were treated with ERI (100nM) added at least half an hour before treatment. Cell layer was scratched with a 10 µl filter tip, and media were

replenished with media containing BPA (10^{-6} and 10^{-8} M), BPF ($10^{-6}$and 10^{-8} M) or BPS (10^{-5} and 10^{-9} M) with or without ERI (100 nM). A particular zone (defined by the intersection between a horizontal line drawn at the other side of the plate and the vertical line of the scratch) was captured by a light microscope at various time points: 0, 2, 4, 6, 8, 10, 12 and 24 hrs. Cell migration was measured as the distance travelled by the cells, which is the distance between injured cells at time "0" minus the distance between these cells at time "t". Results are presented as mean distance travelled (arbitrary unit) ± SEM of at least three independent trials.

G. Cell cycle analysis using flow cytometry

MCF-7 cells were seeded into 6-well plates at a seeding density of 3×10^5 cells. After overnight incubation, cells were treated with BPA (10^{-6} and 10^{-8} M), BPF (10^{-6} and 10^{-8} M) or BPS (10^{-5} and 10^{-9} M) with or without ERI (100 nM) for 24 and 48 hrs. Control cells were treated with 0.1% DMSO. Cells were washed with ice cold 1 × phosphate-buffered saline (PBS) (cat# BE 17-517Q, Lonza, Basel, Switzerland), fixed with ice cold absolute ethanol, stained with propidium iodide (cat# P4170, Sigma-Aldrich, Taufkirchen, Germany) and run onto Guava easyCyte Flow Cytometer (Merck KGaA, Darmstadt, Germany). Data of 10,000 cells were collected, and percentage of cells in each cell cycle phase was analyzed using easyCyte software. Results are presented as mean % of cells within each cell cycle phase + SEM of at least three independent trials.

H. Molecular assays

Since BPA and its analogues BPF and BPS resulted in significant changes in proliferation and migration of MCF-7 cells and not of MDA-MB-231, we assessed the molecular effects of these endocrine disruptors in MCF-7 cells only.

MCF-7 cells were seeded in 6-well plates at a seeding density of 3×10^5 cells. After overnight incubation, cells were treated with BPA (10^{-6} and 10^{-8} M), BPF (10^{-6} and 10^{-8} M) or BPS (10^{-5} and 10^{-9} M) with or without ERI (100 nM) for 24 or 48 hrs. Control cells were treated with 0.1% DMSO or both 0.1% DMSO and 0.001% ethanol.

1. RNA isolation and reverse transcription

RNA was isolated using Trizol-based protocol (Sigma-Aldrich, Taufkirchen, Germany). Isolated RNA samples were treated with DNase using the Dnase treatment and removal kit (cat# AM1906, Invitrogen, Waltham, MA, USA) as per manufacturer's protocol. RNA was then measured using Denovix DS-11 spectrophotometer (Denovix Inc., Wilmington, DE, USA), and both 260/230 and 260/280 ratios were detected for assessment of the purity of samples. RNA samples were then run on an agarose gel to view RNA bands and immediately reverse transcribed to cDNA using the high capacity reverse transcription kit (cat# 4368814, Applied Biosystems, Waltham, MA, USA) as per manufacturer's protocol. cDNA were stored at -20°C until further assays.

2. RNA expression using RT-PCR

RNA expression of *hTERT, DNMT1, DNMT3A, DNMT3B, TET1, TET2* and *TET3* was measured using RT-PCR. In brief, PCR was performed in a 384 well plate using cDNA template equivalent to 50ng RNA, 1 × SYBR Green master mix (cat# 1708882, Bio-Rad, Hercules, CA, USA) and primers shown in **Table 4** (each at a final concentration of 100nM). No template control (NTC) and no amplification control (NAC) were run with the samples. PCR thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each sample was measured in triplicate, and RNA expression of target genes was calculated relative to ß2-microglobulin using the $2^{-\Delta\Delta ct}$ method.

A standard curve was generated using serial dilutions (0.05, 0.5, 5, 50 ng) of an RNA sample, and delta threshold cycle (ct) (ct target gene – ct endogenous control) was calculated and plotted versus log (RNA input amount). The standard curve was ideal with an r^2 approaching 1 for all tested genes.

3. Protein isolation and quantification

Proteins were isolated using 3[(cholamidopropyl)-dimethyl-ammonium]-1-propanesulfonate (CHAPS) lysis buffer (0.5% CHAPS (cat# ab141396, Abcam, Cambridge, MA, USA), 10 mM Tris-HCl (pH 7.5) (cat# 161-0719, Bio-Rad, Hercules, CA, USA), 1 mM MgCl2 (cat# M-2670, Sigma-Aldrich, Taufkirchen, Germany), 1 mM EGTA

(cat# E-4378, Sigma-Aldrich, Taufkirchen, Germany), 5 mM β-mercaptoethanol (cat# 161-0710, Bio-Rad, Hercules, CA, USA), 0.1 mM [4(2-aminoethyl)-benzenesulfonyl fluoride] hydrochloride (cat# P-7626, Sigma-Aldrich, Taufkirchen, Germany) and 10% glycerol (cat# G5516, Sigma-Aldrich, Taufkirchen, Germany). For telomerase activity assay only, an RNase inhibitor (cat# 129916, Qiagen, Hilden, Germany) was freshly added to the buffer prior to the assay. Proteins were incubated with the lysis buffer for 30 min on ice and vigorous vortexing was performed every 10 min interval. The lysate was then centrifuged at 14000 rpm for 30 min at 4°C, and the supernatant was collected.

Protein quantification was performed using Lowry quantification method. In brief, a serial dilution of bovine serum albumin (BSA) (cat# E588, Amresco, Dublin 15, Ireland) was prepared with concentrations ranging from 0.3 mg/ml and 1.5 mg/ml. Then, samples and standards were treated with Lowry reagents (cat# 500-015, cat# 500-0113, cat# 500-0114, Bio-Rad, Hercules, CA, USA), shaked for 1 min and incubated for 15 min. The absorbance was read at 750 nm with an ELISA plate reader. A standard curve was drawn using the absorbance and concentrations of BSA, and sample protein concentrations were calculated from the standard curve.

4. Measurement of telomerase activity

Telomerase activity was measured using TRAP assay (Mender & Shay, 2015). Fresh protein samples (0.25 µg) were added to a reaction mixture containing 1× SYBR Green buffer and 1 µM of forward TS and reverse ACX primers (**Table 4**).The reaction mixture

was first incubated at 37 °C for 30 min during which telomerase in the protein samples was allowed to elongate the TS primer by inserting TTAGGG repeat sequences. Then, the PCR was initiated at 95 °C for 10 min, which inactivates the telomerase enzyme. This was followed by 40 cycles at 95°C for 10 s and 60°C for 60 sec. Telomerase activity was calculated based on the ct at which SYBR green, by binding to the double stranded telomerase product, emits the threshold fluorescence. All samples were run in duplicate. Negative controls were prepared from protein samples incubated at 95°C for 10 min prior to activity measurement and included in the assay.

Serial dilutions of protein extracts (1/625, 1/125, 1/25, 1/5, 1 µg) extracted from fresh HELA cells (ATCC, Manassas, VA, USA) that are known to have high telomerase activity were run with each plate to generate a standard curve. Telomerase activity of samples was calculated from this standard curve and reported as ratio of activity relative to HELA.

5. Measurement of DNMT and TET activity

DNMT and TET activities were measured using EpiQuik DNMT activity/inhibition assay ultra kit (cat# P-3009, Epigentek, Farmingdale, NY, USA) and TET hydroxylase activity quantification kit (cat# ab156912, Abcam, Cambridge, MA, USA), respectively following manufacturer's protocol. In brief, fresh protein samples (20-50 µg) were incubated with the substrate and assay buffer for 3hrs. Wells were then washed and incubated with the capture antibody for one hour. After that, wells were washed and incubated with the detection antibody and enhancer solution. Finally, a color developing solution was added. Positive

and negative controls were run in each assay. Color absorbance was measured at 450 nm wavelength using ELISA plate reader, and background absorbance was measured at 630 nm wavelength. Enzyme activity was calculated as (OD450 – OD630)/[incubation time (hr) × protein amount (mg)].

6. DNA isolation, quantification and bisulfite conversion

DNA was isolated using Flexigene DNA isolation kit from Qiagen (cat # 51206, Hilden, Germany) as per manufacturer's protocol. DNA was then measured using a DenovixDS-11 spectrophotometer (Denovix Inc., Wilmington, DE, USA), and both 260/230 and 260/280 ratios were detected for assessment of the purity of samples. Bisulfite conversion of portion of the isolated DNA was performed using EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) as per manufacturer's protocol. Both bisulfite converted and non-converted DNA samples were stored at -20°C until further assays.

7. RTL measurement

Previously mentioned in Part I.

8. Bisulfite pyrosequencing of LINE-1

Previously mentioned in Part I.

9. Whole genome DNA methylation profiling

Whole genome DNA methylation analysis was performed at IARC using the Infinium MethylationEPIC microarray that covers over 850,000 CpGs (dinucleotides that are the main target for methylation), following manufacturer's protocol (Illumina Inc., San Diego, CA, USA). Each chip encompasses 8 samples, so we used stratified randomization to mitigate the batch effects, ensuring that matched experimental conditions were present on the same chip and that duplicates were distributed between different chips, when possible. For each sample, 250 ng of bisulfite-converted DNA was used for hybridization on Infinium MethylationEPIC bead arrays, following the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). Chips were scanned using Illumina iScan to produce two-color raw data files (.idat format).

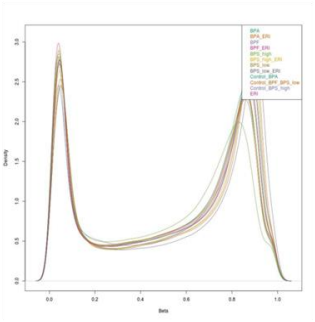
Raw intensity data files (.idat) were handled in R using the minfi package to calculate the methylation level at each CpG as the beta-value (β=intensity of the methylated allele (M)/(intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100)), and the data were exported for quality control and processing. Methylation features were filtered from cross-reactive probes and low-quality probes (probes having bead counts < 3 in at least 5% of samples). Data quality was further assessed using box plots for the

distribution of methylated and unmethylated signals, and multidimensional scaling plots and unsupervised clustering were used to check for sample outliers, which were removed from the analysis along with samples having >1% of CpG sites with a detection p-value >0.05. The remaining dataset was normalized using the funnorm normalization of the minfi package in order to correct for the technical variability between Probe I and Probe II.

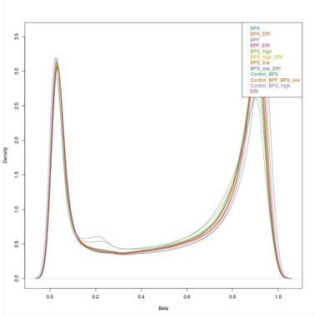
After filtration, density and density bean plots of ß values showed more homogenous distribution, and density bean plots showed roughly similar median ß values among the different treatment conditions (**Figure 3**). Filtered ßs were then corrected for different covariates (sample plate, sentrix position, trial number) using surrogate variable analysis (SVA) method, and log-transformed to M-values. Principal component analysis (PCA) for treatment condition and other covariates was performed before and after SVA correction, and no principal component was significantly associated with the outcome indicating that there is no need to adjust for the different components later in the analysis (**Figure 4**). Hence, crude robust linear model (RLM) was performed on M-values to detect statistically significant differentially methylated CpG probes (DMPs) pertaining to different comparisons. Each treatment condition was compared to corresponding control, and adjusted P value < 0.05 was considered statistically significant. This model was also performed to detect differentially methylated regions (DMRs) using DMRcate bioinformatics package (Peters et al., 2015), whereby the genomic DNA is reduced by a dimension reduction technique into clusters of genomically close (within 1kb distance) and highly correlated CpG sites.

Figure 3. Density plots (A1, A2) and density bean plots (B1, B2) of beta values pertaining to different treatment conditions before and after FunNorm filtration, respectively

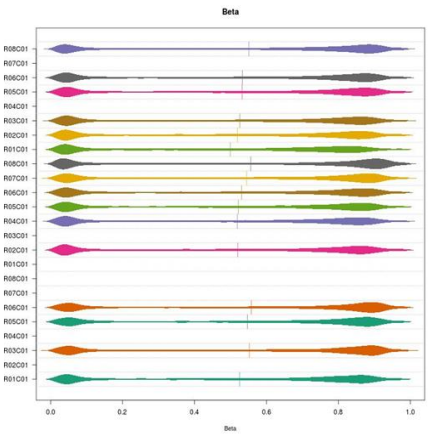
A1.



A2.



B1.



B2.

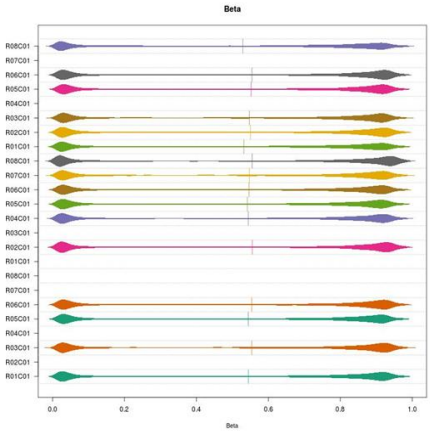
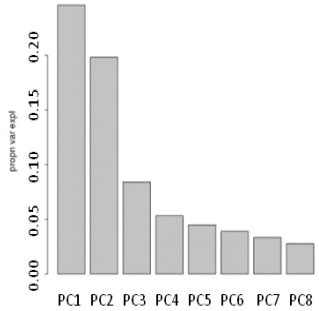


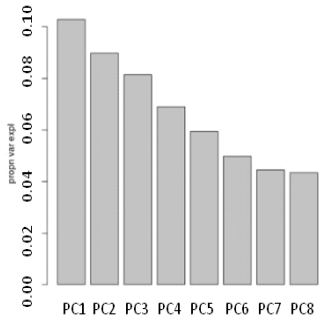
Figure 4. Principal component analysis (PCA) of the contribution of different variables before (A) and after (B) surrogate variable analysis (SVA) correction
1: treatment condition; 2: trial number; 3: sentrix ID; 4: sentrix position

A.



	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
1	0.27	0.15	0.15	0.68	0.28	0.32	0.25	0.38	0.37	0.72
2	0.67	0.75	0.42	0.25	0.09	0.48	0.23	0.20	0.27	0.05
3	0.19	0.45	0.46	0.12	0.03	0.08	0.09	0.03	0.28	0.10
4	0.09	0.04	0.23	0.04	0.52	0.68	0.16	0.98	0.47	0.94

B.



	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
1	0.41	0.17	0.29	0.34	0.31	0.28	0.86	0.44	0.94	0.52
2	0.15	0.14	0.67	0.27	0.31	0.03	0.17	0.49	0.02	0.83
3	0.01	0.09	0.30	0.09	0.03	0.03	0.15	0.50	0.73	0.54
4	0.17	0.32	0.61	0.22	0.93	0.61	0.94	0.49	0.76	0.05

After detecting DMPs and DMRs, these were filtered using the following filtration criteria: for DMPs: mean filtered delta betas (Δßs) ≥ 3%, SDs of filtered ßs in treated group < 10%, SDs of ß values in corresponding control < 10%, and SDs of filtered ßs in all controls < 10%; while for DMRs: mean Δßfc of significant CpGs within the DMR ≥ 3%, mean filtered Δß of significant CpGs within the DMR ≥ 3%, mean SD of filtered ßs in the treated group < 10%, mean SD of filtered ßs in the corresponding control < 10%, and mean SD of filtered ßs in all controls < 10%. Venn diagrams showing the number of DMPs and DMRs in different comparisons (BPA/BPF/BPS compared to their control) were drawn using Venny 2.1.0 online tool ("Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html,").

Pathway analysis of the genes with DMPs in the three comparisons (BPA/BPF/BPS compared to their control) was performed using Enrichr (Ma'ayan Laboratory) (E. Y. Chen et al., 2013; Kuleshov et al., 2016). Pathways with FDR < 0.05 were considered statistically significant, and a graph was drawn using PRISM software (GraphPad6, La Jolla, CA, USA). Similarly, Venn diagrams showing the number of DMPs in different comparisons (BPA/BPA+ERI/ERI compared to control) were drawn using Venny 2.1.0 online tool ("Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html,"), and similar figures were done for BPF and BPS. Concerning the DMPs and DMRs selected for the representative figures, these were statistically significant in either BPA or BPF or BPS treatment conditions when compared to control, with high filtered Δßs, and not statistically significant in the ERI group when compared to control. Completely dependent CpGs were statistically significant in neither the BPA + ERI nor BPF + ERI nor BPS + ERI treatment conditions when compared to control; in contrast, completely independent CpGs were statistically significant in these treatment conditions.

I. Comparison with TCGA dataset

With the aim to test the relevance of discovered DMPs associated with BPA and its analogues BPF and BPS to breast cancer, we accessed publicly available methylation data of 21986 CpG sites in 816 breast cancer and 124 normal adjacent breast tissues of breast cancer patients based on The Cancer Genome Atlas (TCGA) database (The Cancer Genome

Atlas et al., 2012). Since endocrine disruptors act through ER, analysis was performed on the subset of 595 ER-positive tumor tissues in comparison to the normal adjacent tissues using two-tailed t-test followed by false discovery rate (FDR) correction using Benjamini-Hochberg method (IBM SPSS statistics software, version 24.0, Armonk, NY, USA). An FDR P value $< 3.15 \times 10^{-8}$ was considered statistically significant, and only CpGs with an absolute methylation change of $\geq 3\%$ were reported.

J. Statistical analysis

Data were analyzed using IBM SPSS software version 24.0 (Armonk, NY, USA) and visualized using GraphPad Prism software version 6 (GraphPad6, La Jolla, CA, USA). Results of each assay are presented as mean ± SEM of at least three independent trials. For continuous data, comparisons were performed between each treatment condition and control using one-way ANOVA followed by Dunnett *post-hoc* test and within the same treatment condition with ERI *versus* without ERI using ANOVA followed by Tukey HSD *post-hoc* test. For cell scratch assay, two-way ANOVA was performed to test for the association of time points and treatment conditions with cell migration. A P value of less than 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

PART I. EPIDEMIOLOGICAL STUDIES

We investigated the association between urinary BPA levels and peripheral blood RTL in the whole cohort of non-breast cancer individuals (N=482). These results, along with the association between urinary BPA levels and *ESR1* methylation % that is not included in this thesis, have already been published (Awada et al., 2019). Then, RTL in the peripheral blood of a semi randomly chosen subset of this cohort (N=52 females > 40 years of age) was compared to that in peripheral blood of breast cancer patients (N= 58). As for *LINE-1* methylation, it was measured in the peripheral blood of the same subset of the cohort (N=52 females) and associated with urinary BPA levels and breast cancer status. Of note, we measured *LINE-1* methylation in only a subset of the females rather than all 312 females simply because of budget limitation.

A. Characteristics of non-breast cancer participants

As shown in **Table 5**, mean age ± SD of participants was 44.9 years (± 14.8), and 312 (64.7%) were females with significantly higher urinary BPA levels when compared to males. More than half of the participants were current smokers (64.5%) while 19.1% were current alcohol drinkers, with these behaviors being significantly higher in males.

Table 5. Characteristics of non-breast cancer participants (N=482)

Characteristics		**Total (N=482)**
Age (years)	Mean ± SD	44.9 ± 14.8
Gender		
Male	N (%)	170 (35.3)
Female	N (%)	312 (64.7)
Waist circumference (cm)	Mean ± SD	95.6 ± 15.6
Body mass index (Kg/m^2)	Mean ± SD	29.0 ± 5.8
Smoking		
No	N (%)	124 (25.7)
Ex	N (%)	47 (9.8)
Current	N (%)	311 (64.5)
Alcohol		
No	N (%)	390 (80.9)
Yes	N (%)	92 (19.1)

NA: not available

B. Association of BPA with RTL (H1)

The limit of BPA detection was 0.1µg/l (ppb) based on three times the signal to noise ratio. The internal quality control relative error was 4.68%. Mean ± SD of urinary BPA concentration was 3.71 ± 4.83 (µg/L) with a median of 3.11 µg/L (min-max: <limit of detection (LOD) – 59.71) and mean ± SD of urinary BPA concentration adjusted for urinary creatinine was 2.90 ± 4.81 (µg/g creatinine) with a median of 1.86 µg/g creatinine (min-max: <LOD -69.85).

For RTL, mean ± SD was 1.43 ± 0.84 with a median of 1.28 (min-max: 0.16-10.28). The intra-assay geometric mean of the coefficients of variation for the telomere and single copy gene Ct values were less than 1% for both with a mean ± SD of 7 different assays of 0.92 ±

0.17% and 0.58 ± 0.11% for telomere and the single copy gene, respectively. As for inter-run reproducibility, there was a statistically significant high correlation between RTL of 18 samples that were run on two different occasions (R = 0.88; $P< 0.0001$); in addition, the inter-assay geometric mean of the coefficient of variation was 6.49%.

As seen in **Table 6**, there were no statistically significant associations between BPA and RTL except within the female sub-cohort whereby higher urinary BPA concentrations that were adjusted for urinary creatinine were statistically significantly associated with shorter RTL. This association remained statistically significant in the multivariate multinomial regression: OR (95% CI) with the first RTL tertile: 2.85 (1.34-6.10) and 2.97 (1.45-6.09) for the second and third urinary adjusted BPA tertiles respectively. Moreover, results of trend analysis were consistent with those of logistic regression**.** A similar, though not statistically significant trend was found with the second RTL tertile (**Table 6**). Results of trend analysis are illustrated in **Figure 5** that shows a decrease in mean RTL ± SEM over the three BPA tertiles in females.

Table 6. Associations of creatinine-adjusted urinary BPA (µg/g creatinine) with peripheral blood relative telomere length (RTL) (N=482) presented as OR (95% CI)

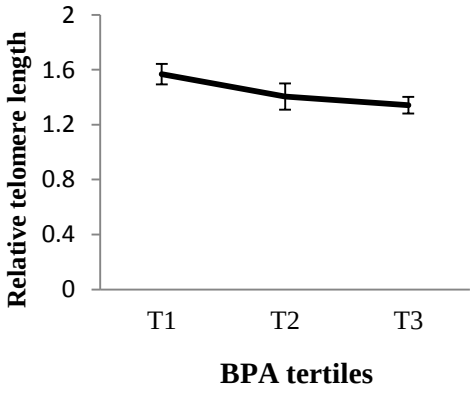
	All		**Males**		**Females**	
	RTL (T1) N=159	**RTL (T2) N=161**	**RTL (T1) N=58**	**RTL (T2) N=57**	**RTL (T1) N=101**	**RTL (T2) N=104**
BPA adjusted for creatinine						
Unadjusted univariate						
T1	Reference	Reference	Reference	Reference	Reference	Reference
T2	1.62 (0.94 – 2.79)	1.11 (0.65 – 1.90)	0.80 (0.35 – 1.85)	0.80 (0.34 – 1.85)	**2.91 (1.38 – 6.16)**	1.37 (0.68 – 2.73)
T3	1.56 (0.90 – 2.67)	1.09 (0.64 – 1.85)	0.44 (0.16 – 1.21)	0.51 (0.19 – 1.37)	**3.19 (1.57 – 6.49)**	1.54 (0.81 – 2.92)
P-value for trend	0.12	0.74	0.12	0.19	**0.002**	0.18
Adjusted multivariate						
T1	Reference	Reference	Reference	Reference	Reference	Reference
T2	1.62 (0.93 – 2.81)	1.12 (0.65 – 1.90)	0.83 (0.35 – 1.94)	0.80 (0.34 – 1.87)	**2.85 (1.34 – 6.10)**	1.36 (0.68 – 2.71)
T3	1.51 (0.87 – 2.62)	1.08 (0.64 – 1.84)	0.40 (0.14 – 1.13)	0.50 (0.18 – 1.36)	**2.97 (1.45 – 6.09)**	1.49 (0.78 – 2.84)
P-value for trend	0.15	0.76	0.10	0.18	**0.005**	0.22

RTL tertiles: T1**:**<1.06; T2: 1.06 – 1.43; T3: >1.43 (reference)

BPA creatinine adjusted tertiles : T1: <1.26; T2: 1.26-2.44; T3:>2.44

Multivariate model is adjusted for: Age, Gender (for the "among all" model only), and WC

Figure 5. Plots of relative telomere length (RTL) (Mean ± SEM) among the three BPA tertiles in females.



C. Association of BPA with *LINE-1* methylation (H2)

In the subcohort of 52 females, mean ± SD of *LINE-1* methylation was 70.87 ± 0.71 with a median of 70.79 (min-max: 68.84-72.76). As shown in **Table 7**, there was no association between *LINE-1* methylation at any of the 4 analyzed CpG sites and urinary creatinine-adjusted BPA concentrations.

Table 7. Association of creatinine-adjusted urinary BPA (µg/g creatinine) with peripheral blood *LINE-1* % methylation (N=52)

	Creatinine adjusted BPA	
	β-value (95% CI)	**P value**
***LINE-1* % methylation**		
First CpG	-0.043 (-0.148 – 0.061)	0.410
Second CpG	-0.027 (-0.079 – 0.024)	0.285
Third CpG	-0.012 (-0.095 – 0.070)	0.765
Fourth CpG	-0.006 (-0.153 – 0.140)	0.931
Average (4 CpGs)	-0.022 (-0.082 – 0.037)	0.452

Linear regression test using crude model

D. Characteristics of breast cancer participants

Comparison of the characteristics of cases with those of controls showed statistically significant lower BMI (mean BMI (kg/m^2) ± SD 28.29 ± 6.69) in breast cancer patients *versus* controls (31.79 ± 6.02),lower percentage of cigarette smokers (36.2 % in breast cancer patients *versus* 59.6 % in controls),and higher percentage of alcohol consumers (24.1% in breast cancer patients *versus* 3.8% in controls). As expected, there was no difference in age between the breast cancer patients and controls (**Table 8**). As for the breast tissue samples, there was no difference in the characteristics of patients for whom cancerous and normal adjacent breast tissues were available for analysis (**Table 9A, Table 9B**).

Table 8. Descriptive statistics of cases and controls for whom peripheral blood samples were evaluated

Variables		Controls	Breast Cancer	P value
Age at recruitment	Mean ± SD	52.65 ± 8.20	53.55 ± 12.82	0.529[a]
BMI	Mean ± SD	31.79 ± 6.02	28.29 ± 6.69	**0.011[a]**
Smoking				
Cigarette	Yes N (%)	31 (59.6)	21 (36.2)	**0.021[b]**
Nargileh	Yes N (%)	4 (7.7)	12 (20.7)	0.059[b]
Alcohol consumption	Yes N (%)	2 (3.8)	14 (24.1)	**0.002[b]**
Pre-Menopausal	Yes N (%)	20 (38.5)	27 (46.6)	0.337[b]
Relative Telomere Length	Mean ± SD	1.19 ± 0.41	0.42 ± 0.10	**0.000[a]**
***LINE-1* methylation**	Mean ± SD			
CpG1		73.52 ± 1.26	74.34 ± 1.29	**0.001[a]**
CpG2		84.43 ± 0.62	85.58 ± 0.59	**0.000[a]**
CpG3		72.59 ± 0.99	72.89 ± 1.01	0.118[a]
CpG4		52.96 ± 1.75	55.23 ± 1.04	**0.000[a]**
Average		70.88 ± 0.71	72.01 ± 0.64	**0.000[a]**

[a]student's t-test, [b]chi-square test

Table 9A. Descriptive statistics of participants for whom breast cancer tissues and normal adjacent breast tissues were evaluated for relative telomere length

		Normal Adjacent Tissues (N=11)	Breast Cancer Tissues (N=21)	P value
Age at recruitment	Mean (SD)	48.46 (14.19)	53.67 (14.83)	0.346[a]
BMI	Mean (SD)	25.53 (6.99)	27.54 (5.15)	0.360[a]
Smoking				
Cigarette	Yes N (%)	5 (45.5)	7 (33.3)	0.703[b]
Narjileh	Yes N (%)	3 (27.3)	4 (19.0)	0.667[b]
Alcohol consumption	Yes N (%)	3 (27.3)	4 (19.0)	0.667[b]
Post-Menopausal	Yes N (%)	5 (45.5)	12 (57.1)	0.712[b]
Pathology	N (%)			0.734[b]
Invasive ductal carcinoma		9 (81.8)	14 (66.7)	
Invasive lobular carcinoma		1 (9.1)	4 (19.0)	
Other		1 (9.1)	3 (14.3)	
Grade	N (%)			0.794[b]
1		3 (27.3)	3 (16.7)	
2		4 (36.4)	9 (50.0)	
3		4 (36.4)	6 (33.3)	
Stage	N (%)			0.584[b]
I		5 (45.5)	6 (31.6)	
IIA		5 (45.5)	7 (36.8)	
IIB		0 (0.0)	3 (15.8)	
IIIA		1 (9.1)	3 (15.8)	
Estrogen receptor	Positive N (%)	8 (72.7)	15 (71.4)	1.000[b]
Progesterone receptor	Positive N (%)	7 (63.6)	13 (61.9)	1.000[b]
HER2	N (%)			0.829[b]
Negative		9 (81.8)	15 (71.4)	
Equivocal		0 (0.0)	2 (9.5)	
Positive		2 (18.2)	4 (19.0)	
Relative Telomere Length	Mean (SD)	2.87 (2.00)	1.75 (1.04)	**0.043**[a]

[a]*student's t-test,* [b]*chi-square test*

Table 9B. Descriptive statistics of participants for whom breast cancer tissues and normal adjacent breast tissues were evaluated for *LINE-1* methylation

		Normal Adjacent Tissues (n=20)	Breast Cancer Tissues (n=21)	P value
Age at recruitment	Mean (SD)	55.5 (12.06)	54.00 (14.70)	0.740[a]
BMI	Mean (SD)	28.46 (7.65)	27.62 (5.10)	0.633[a]
Smoking				
Cigarette	Yes N (%)	6 (33.3)	7 (35.0)	1.000[b]
Narjileh	Yes N (%)	3 (15.0)	4 (19.0)	1.000[b]
Alcohol consumption	Yes N (%)	2 (10.0)	4 (19.0)	0.663[b]
Post-Menopausal	Yes N (%)	13 (65.0)	12 (57.1)	0.751[b]
Pathology	N (%)			0.765[b]
Invasive ductal carcinoma		15 (75.0)	15 (71.4)	
Invasive lobular carcinoma		4 (20.0)	3 (14.3)	
Other		1 (5.0)	3 (14.3)	
Grade	N (%)			0.467[b]
1		5 (27.8)	2 (10.5)	
2		8 (44.4)	11 (57.9)	
3		5 (27.8)	6 (31.6)	
Stage	N (%)			0.959[b]
I		7 (38.9)	6 (31.6)	
IIA		8 (44.4)	8 (42.1)	
IIB		1 (5.6)	2 (10.5)	
IIIA		2 (11.1)	3 (15.8)	
Estrogen receptor	Positive N (%)	17 (85.0)	14 (66.7)	0.277[b]
Progesterone receptor	Positive N (%)	14 (70.0)	11 (52.4)	0.341[b]
HER2	N (%)			0.639[b]
Negative		17 (85.0)	15 (71.4)	
Equivocal		1 (5.0)	2 (9.5)	
Positive		2 (10.0)	4 (19.0)	
***LINE-1* methylation**	Mean (SD)			
CpG1		64.11 (11.32)	65.62 (10.10)	0.898[a]
CpG2		76.07 (13.25)	78.52 (7.53)	0.840[a]
CpG3		67.05 (9.26)	67.50 (8.74)	0.832[a]
CpG4		46.10 (8.23)	49.25 (6.31)	0.256[a]
Average		63.17 (8.69)	64.62 (7.55)	0.830[a]

[a]*student's t-test,* [b]*chi-square test*

E. RTL and breast cancer (H3&H5)

RTL was shorter in peripheral blood of breast cancer patients than in controls (*P*-value < 0.001) with mean ± SD being 0.42 ± 0.10 in breast cancer patients and 1.19 ± 0.41 in controls (**Figure 6A, Table 8**). Similar statistically significant association was obtained

in univariate logistic regression and multivariate logistic regression after adjustment for BMI, smoking, and alcohol consumption (**Table 10**).

Consistent with the findings in peripheral blood, cancerous breast tissue samples had shorter RTL than normal adjacent breast tissues (mean ± SD: 1.75 ± 1.04 in cancerous breast tissues, 2.87 ± 2.00 in normal adjacent breast tissues, *P*-value < 0.05) (**Figure 6B, Table 9B**). However, breast cancerous tissues were only associated with a trend towards shorter RTL in the univariate model (*P*-value < 0.1), and the association became less significant in the multivariate model (**Table 11**). Of note, the correlation between RTL in peripheral blood and cancerous tumor tissues of breast cancer patients approached statistical significance (Pearson correlation coefficient: 0.496, P value: 0.051).

Figure 6. Relative telomere length (mean + SD) in (A) blood samples from breast cancer women (N= 58) and controls (N= 52) and in (B) cancerous breast tissues (N= 21) and normal adjacent breast tissues (N= 11).
**p-value < 0.001 and *p-value < 0.05 (student's t-test)

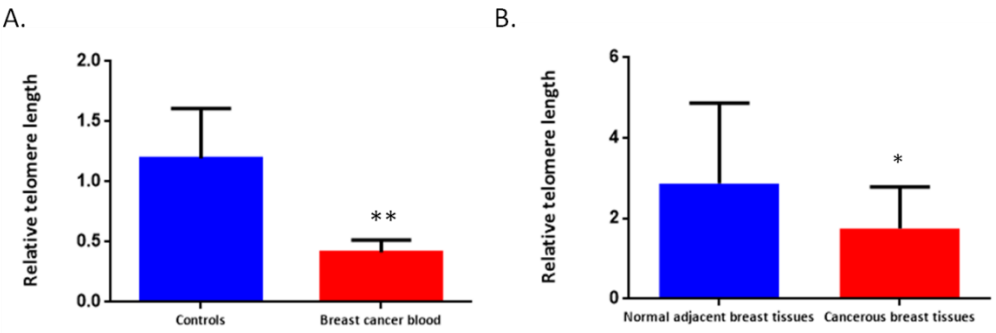


Table 10. Association of peripheral blood relative telomere length (RTL) and *LINE-1* methylation with breast cancer before and after adjustment for potential co-variates

	Univariate[a]		Multivariate[b]		Multivariate[c]	
	OR (95%CI)	**P**	**OR (95%CI)**	**P**	**OR (95%CI)**	**P**
RTL	0.000 (0.000 – 0.000)	**0.000**	0.000 (0.000 – 0.000)	**0.000**	0.000 (0.000 – 0.000)	**0.000**
LINE-1 **methylation**						
CpG1	1.664 (1.200 – 2.307)	**0.002**	1.578 (1.123 – 2.218)	**0.009**	1.651 (1.123 – 2.427)	**0.011**
CpG2	30.802 (8.612 – 110.163)	**0.000**	28.812 (7.924 – 104.759)	**0.000**	39.607 (8.842 – 177.407)	**0.000**
CpG3	1.362 (0.922 – 2.011)	0.120	1.321 (0.884 – 1.974)	0.175	1.401 (0.885 – 2.217)	0.151
CpG4	9.875 (4.224 – 23.087)	**0.000**	9.756 (4.120 – 23.105)	**0.000**	10.120 (3.919 – 26.135)	**0.000**
Average	11.341 (4.731 – 27.184)	**0.000**	10.577 (4.355 – 25.686)	**0.000**	10.373 (4.053 – 26.547)	**0.000**

[a]*Univariate logistic regression model*

[b]*Multivariate logistic regression model adjusted for BMI*

[c]*Multivariate logistic regression model adjusted for BMI, smoking, and alcohol consumption*

Table 11. Association of breast tissues relative telomere length (RTL) and *LINE-1* methylation with breast cancer before and after adjustment of potential co-variates

	Univariate[a]		Multivariate[b]		Multivariate[c]	
	OR (95%CI)	**P**	**OR (95%CI)**	**P**	**OR (95%CI)**	**P**
RTL	0.550 (0.280 – 1.082)	0.083	0.566 (0.275 – 1.164)	0.122	0.554 (0.257 – 1.193)	0.131
LINE-1 methylation						
CpG1	1.014 (0.956 – 1.075)	0.645	1.014 (0.956 – 1.076)	0.642	1.010 (0.948 – 1.076)	0.755
CpG2	1.023 (0.963 – 1.087)	0.463	1.021 (0.960 – 1.085)	0.509	1.017 (0.954 – 1.085)	0.600
CpG3	1.006 (0.938 – 1.079)	0.868	1.004 (0.936 – 1.077)	0.914	1.004 (0.931 – 1.082)	0.917
CpG4	1.064 (0.970 – 1.167)	0.188	1.063 (0.970 – 1.166)	0.191	1.060 (0.964 – 1.164)	0.230
Average	1.009 (0.933 – 1.091)	0.825	1.007 (0.931 – 1.090)	0.858	1.017 (0.937 – 1.104)	0.688

[a]*Univariate logistic regression model*
[b]*Mulitvariate logistic regression model adjusted for BMI*
[c]*Multivariate logistic regression model adjusted for BMI, smoking, and alcohol consumption*

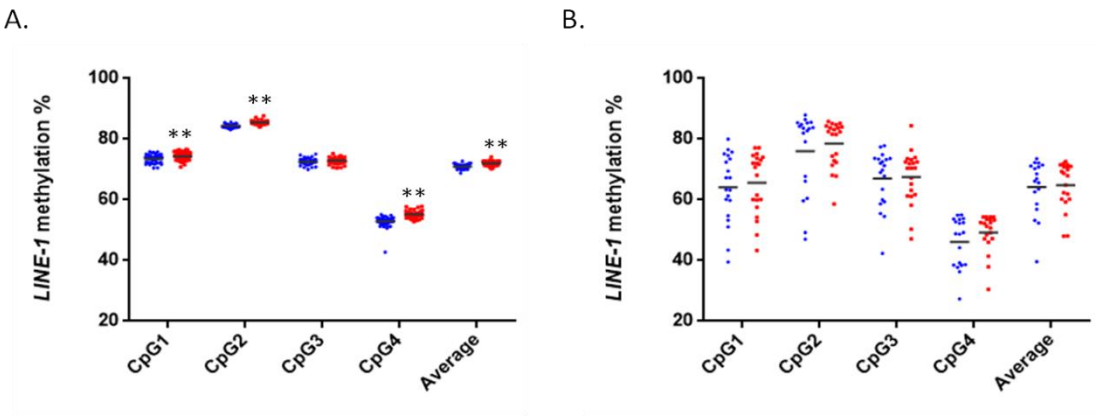
F. *LINE-1* methylation and breast cancer (H4&H6)

For *LINE-1* methylation, all blood samples and the majority of the tissue samples showed good quality at the first 4 CpG sites of the *LINE-1* amplicon. Therefore, only the first 4 CpGs were included in the analysis.

LINE-1 methylation at each of the four CpG sites was lower in peripheral blood samples of breast cancer patients than in controls, although only CpGs 1, 2 and 4 were statistically significant, showing an effect size around 1-2% (P-value < 0.001) (**Figure 7A, Table 8**). Average *LINE-1* methylation was higher in peripheral blood of breast cancer patients than in controls, with mean ± SD being 72.01 ± 0.64 in peripheral blood samples of breast cancer patients and 70.88 ± 0.71 in controls (P-value < 0.001) (**Figure 7A**). Similar results were obtained in univariate logistic regression and multivariate logistic regression after adjustment for BMI, smoking, and alcohol consumption (**Table 10**).

As for tissues, mean values of *LINE-1* methylation at individual and average CpG sites were higher in cancerous breast tissues than in normal adjacent breast tissues, yet results were widely variable and not statistically significant even after adjustment (**Figure 7B, Table 9B&11**).

Figure 7. Global DNA methylation represented by *LINE-1* methylation at CpGs 1-4 in (A) blood samples from controls (**blue**) and breast cancer cases (**red**) and in (B) normal adjacent breast tissues (**blue**) and cancerous breast tissues (**red**)
Mean methylation % at each CpG position is represented by a black dash.
**p-value < 0.001 (student's t-test)



G. Associations of RTL and *LINE-1* methylation with clinicopathologic characteristics of breast cancer

RTL was not associated with any of the clinicopathologic characteristics of breast cancer (**Table 12**). As for *LINE-1* methylation, it was significantly higher in progesterone receptor (PR) positive cancerous breast tissues when compared to PR negative cancerous breast tissues (P value = 0.043) (**Table 12**).

Table 12. Association of clinicopathological characteristics of cancerous breast tissues with relative telomere length (RTL) and *LINE-1* methylation levels in these tissues

	Relative telomere length		**Average *LINE-1* methylation**	
	β-value (95%CI)	**P value[#]**	**β-value (95%CI)**	**P value[#]**
Pathology	-0.169 (-0.830 – 0.492)	0.599	1.082 (-3.915 – 6.079)	0.655
Grade	-0.165 (-0.999 – 0.669)	0.680	1.429 (-4.239 – 7.096)	0.600
Stage	-0.144 (-0.646 – 0.358)	0.552	-0.260 (-3.641 – 3.120)	0.872
Estrogen receptor	-0.674 (-1.703 – 0.354)	0.186	4.570 (-3.222 – 12.363)	0.234
Progesterone receptor	-0.426 (-1.408 – 0.556)	0.376	8.172 (1.888 – 14.457)	**0.014***
HER2	0.039 (-0.574 – 0.652)	0.895	-0.478 (-5.084 – 4.128)	0.830

[#]*Linear regression test using crude model*

PART II. CELL CULTURE STUDIES

A. Bisphenols induced an ER-dependent increase in cell proliferation, migration and S-G2/M cell cycle proportions

We observed a time- and concentration-dependent increase in cell metabolic activity of MCF-7 cells after treatment with BPA, BPF or BPS for 24, 48 and 72 hrs that was completely abolished in the presence of ERI at 48 and 72hrs (**Figure 8**). The lowest concentrations associated with a statistically significant increase in metabolic activity after 48-72 hrs of treatment were 10^{-6} M for BPA and BPF and 10^{-5} M for BPS, and they induced an increase in metabolic activity by 50% to 100% relative to control at 72hrs (**Figure 8**). These concentrations were considered equipotent and used, together with the human exposure concentrations, in the other assays. Consistently, the minimum functional (equipotent) concentrations of the three bisphenols resulted in concentration - and ER- dependent increases in both S and G2/M proportions of the cell cycle at 24hrs of treatment in MCF-7 cells (**Figure 9, Figure 10**), followed by a significant increase in % cell viability at 48 and 72 hrs of treatment (**Figure 11**). This, and the fact that trypan blue and cell cycle results did not show marked changes in cell death, supports the expectation that the increased S-G2/M proportion of treated cells represents increased cell proliferation rather than cell cycle arrest. Both the functional and exposure concentrations of BPA, BPF and BPS increased cell migration in a time- and concentration-dependent manner, with the distance travelled being statistically significant in all conditions except with the exposure concentration of BPF (**Figure 12, Figure 13**). Addition of ERI abolished this increase in the exposure concentrations and

partially prevented it in the functional concentrations of BPA, BPF and BPS (**Figure 12**).

Figure 8. Metabolic activity (MTT assay) of MCF-7 cells following treatment with different concentrations of BPA, BPF and BPS ± ERI for 24hrs (A1, B1, C1), 48 hrs (A2, B2, C2) and 72 hrs (A3, B3, C3), respectively

Metabolic activity was calculated as % relative to control, and data are presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using ANOVA followed by Dunnett *post-hoc* test (* for $p < 0.05$ and ** for $p < 0.001$), and between the same treatment condition ± ERI using ANOVA followed by Tukey HSD *post-hoc* test (# for $p < 0.05$ and ## for $p < 0.001$).

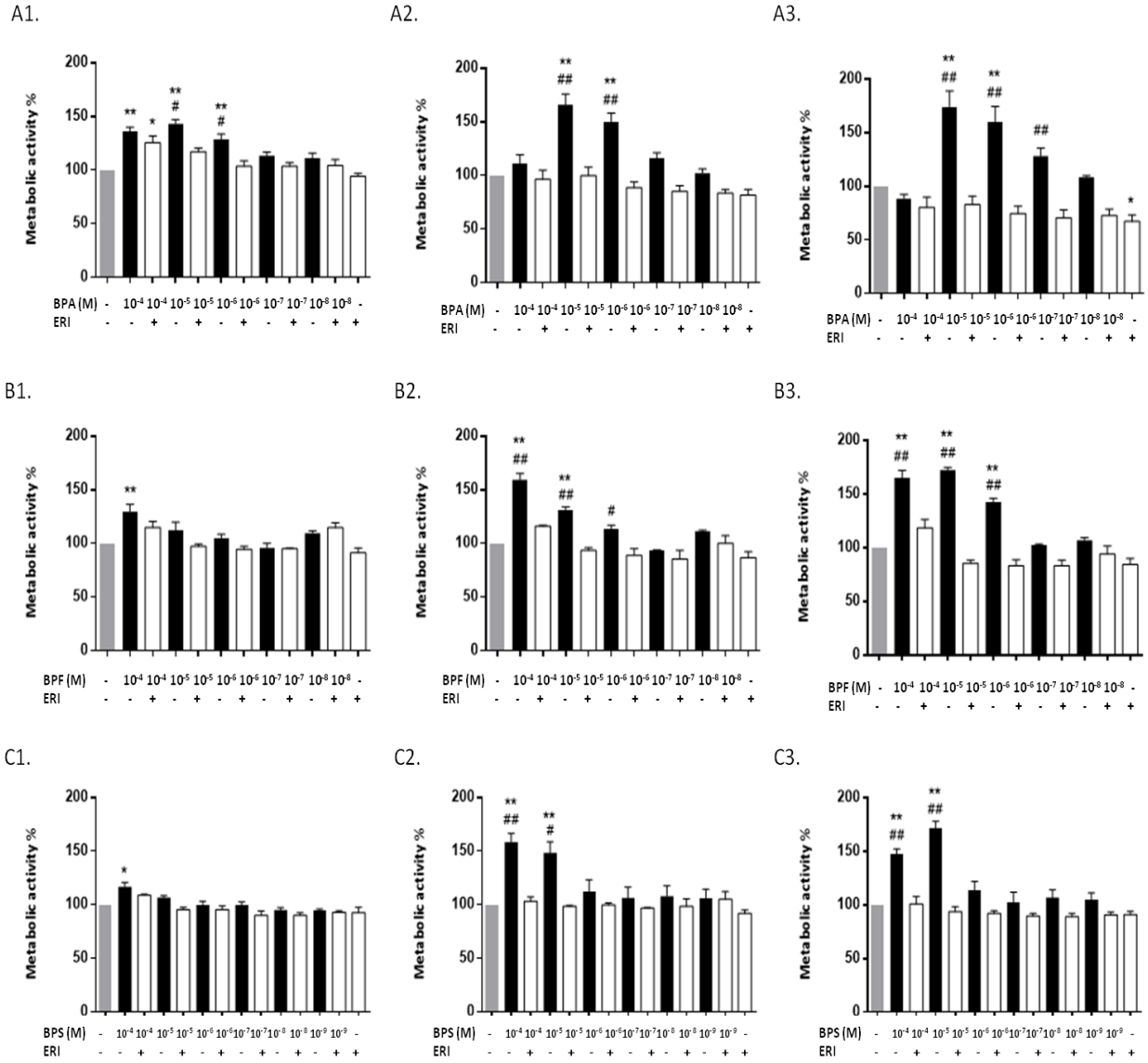


Figure 9. Flow cytometry data showing mean percentage (+ SEM) of MCF-7 cells in different cell cycle phases following treatment with exposure and functional concentrations of BPA, BPF and BPS ± ERI for 24 hrs (A1, B1, C1) and 48hrs (A2, B2, C2), respectively

Figure legend: dark grey for G0/G1, light grey for S and grey for G2/M phases.
Numbers in bars represent mean cell percentage within the corresponding cell cycle phase.

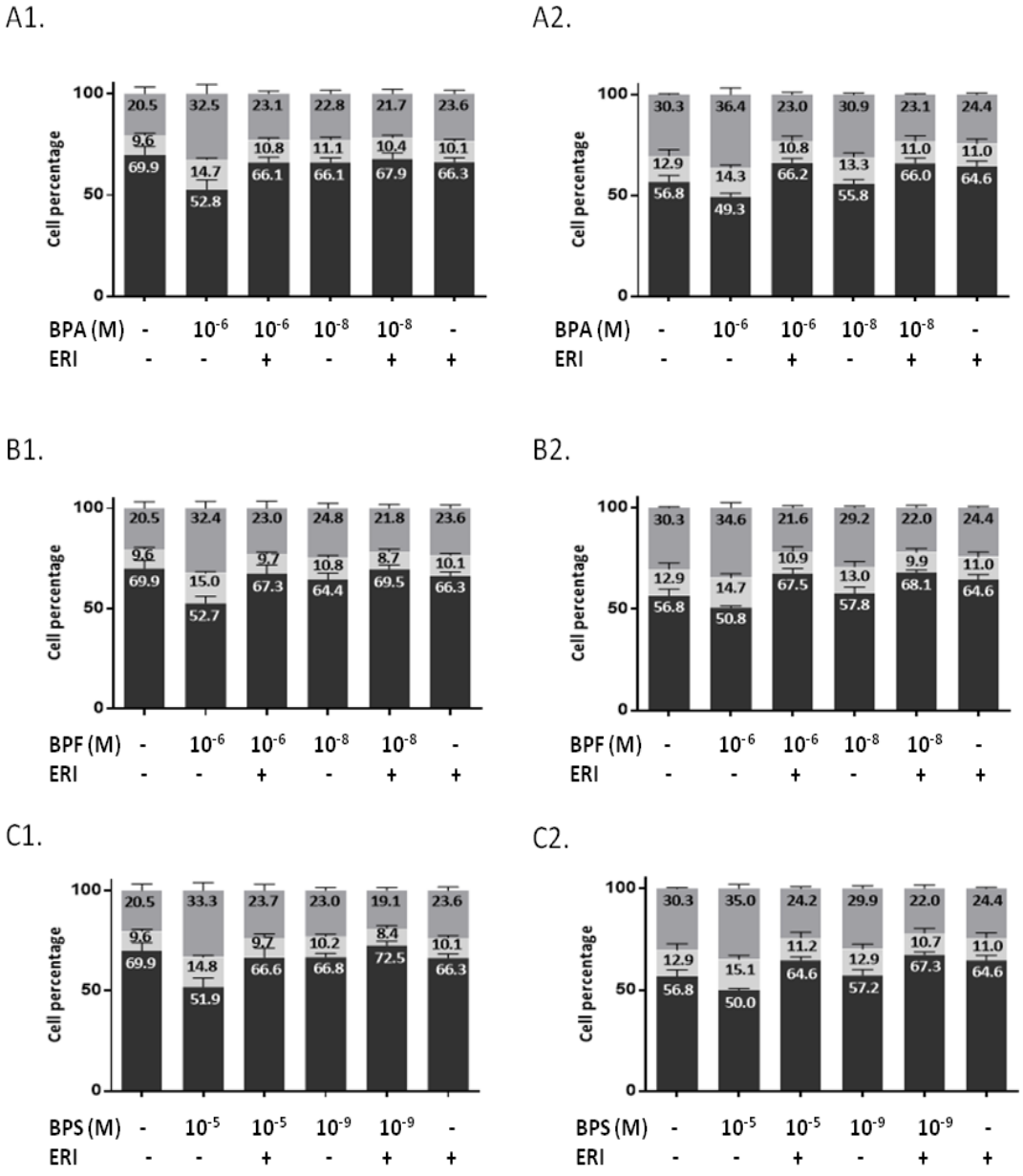


Figure 10. Flow cytometry figures of a representative trial depicting cell cycle phases of MCF-7 cells following treatment with exposure and functional concentrations of BPA, BPF and BPS ± ERI for 24hrs

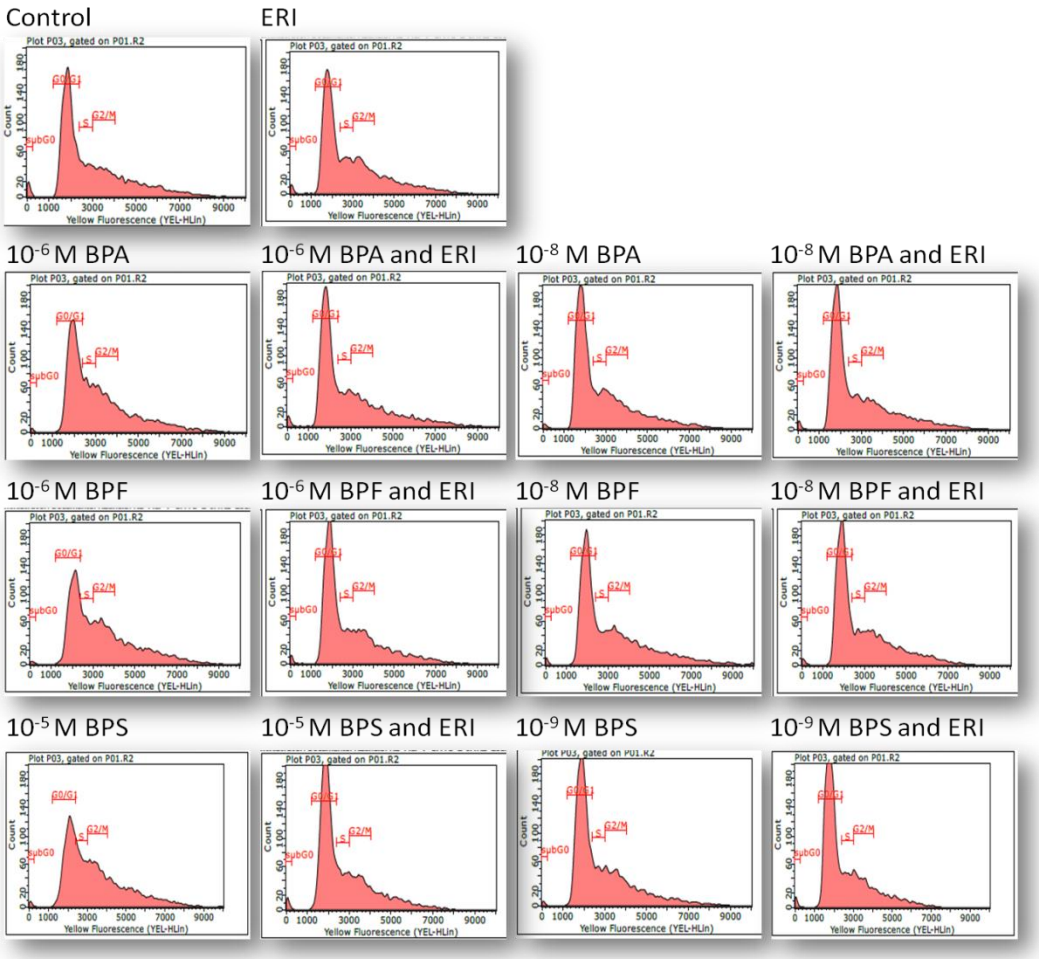


Figure 11. Cell viability (Trypan blue assay) of MCF-7 cells following treatment with different concentrations of BPA, BPF and BPS ± ERI for 24 hrs (A1, B1, C1), 48 hrs (A2, B2, C2), and 72 hrs (A3, B3, C3), respectively

Cell viability was calculated as % relative to control, and data are presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using ANOVA followed by Dunnett *post-hoc* test (* for $p < 0.05$ and ** for $p < 0.001$), and between the same treatment condition ± ERI using ANOVA followed by Tukey HSD *post-hoc* test (# for $p < 0.05$ and ## for $p < 0.001$).

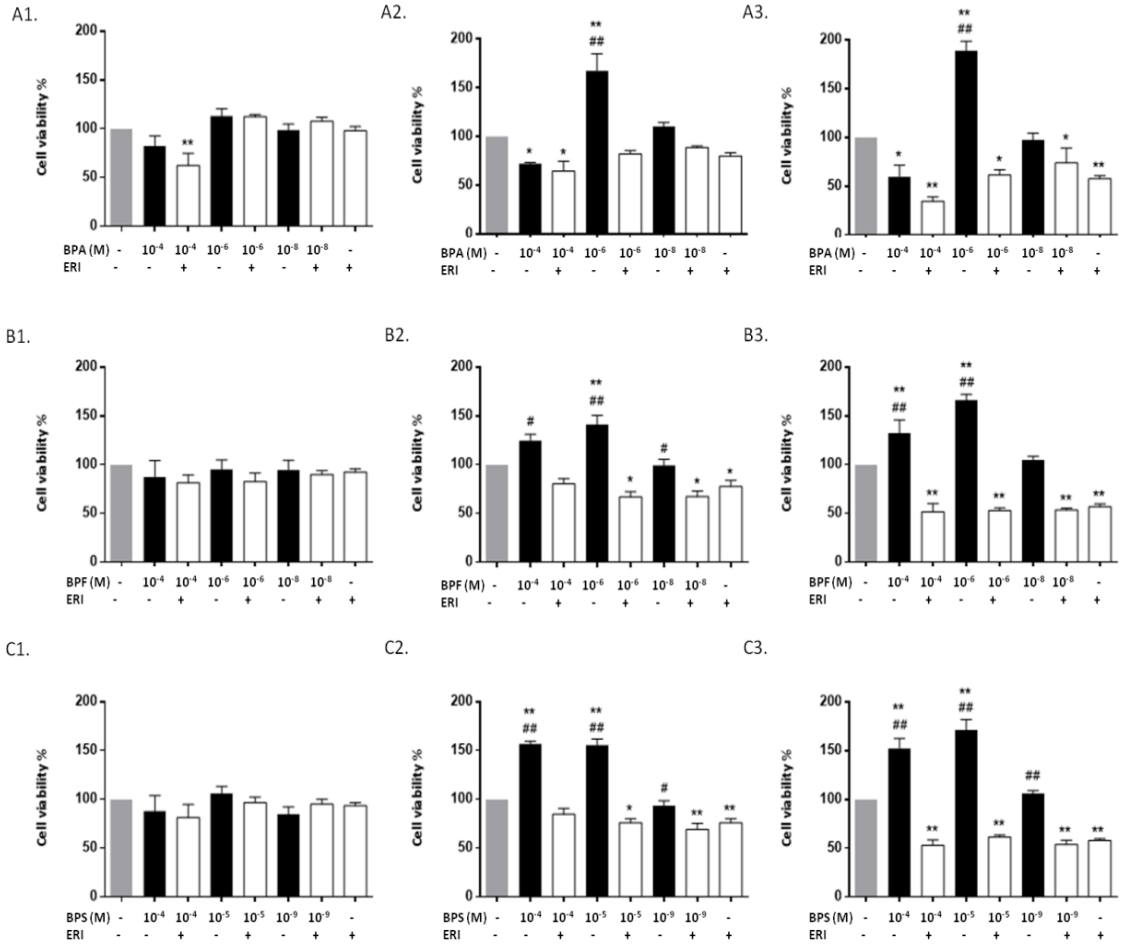


Figure 12. Migration (scratch assay) of MCF-7 cells depicted as mean distance travelled ± SE over time course of treatment with exposure and functional concentrations of BPA (A), BPF (B) and BPS (C)

Data are presented as mean distance travelled (arbitrary unit) ± SEM at time points 0, 2, 4, 6, 8, 10, 12 and 24hrs in at least three independent trials. Comparisons were performed at every time point between each treatment condition and control using two-way ANOVA followed by Dunnett *post-hoc* test (* for $p < 0.05$ and ** for $p < 0.001$), and between the same treatment condition in the presence and absence of ERI using two-way ANOVA followed by Tukey HSD *post-hoc* test (# for $p < 0.05$).

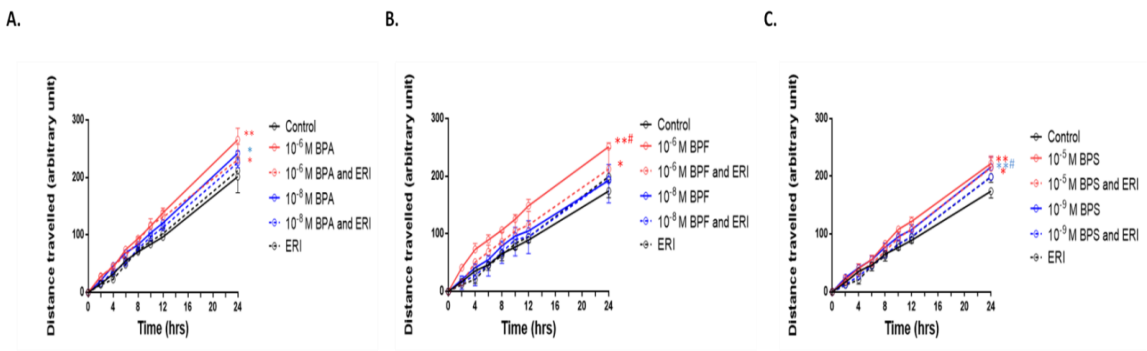
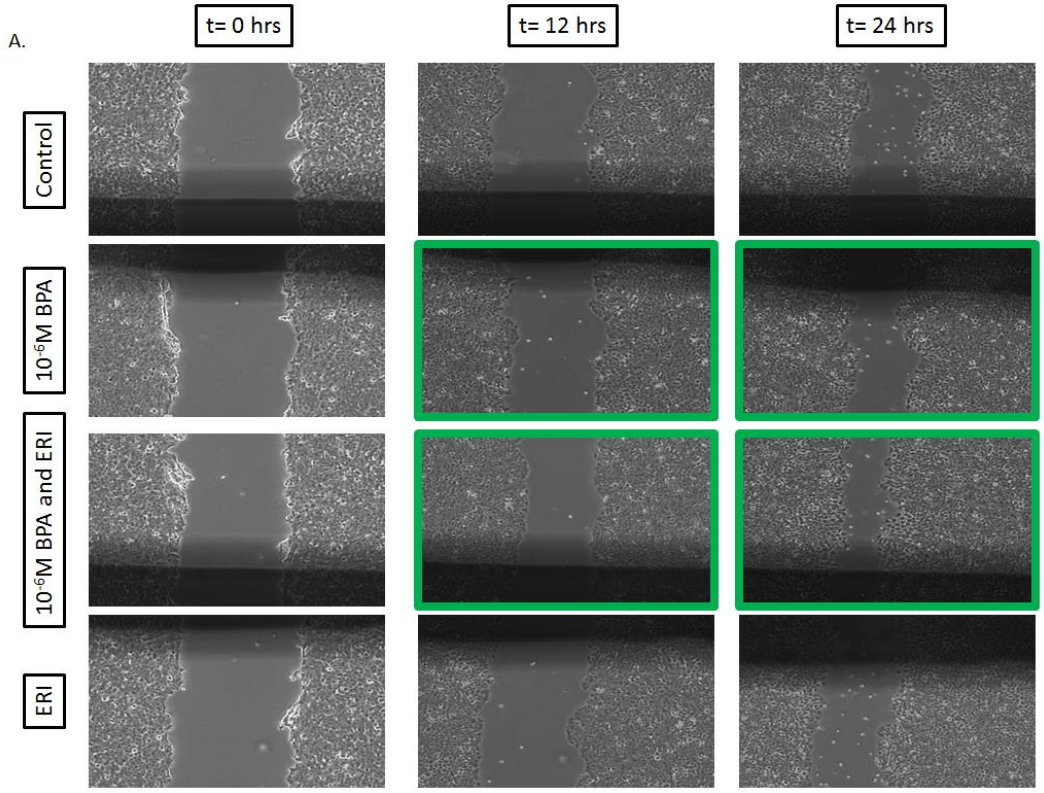
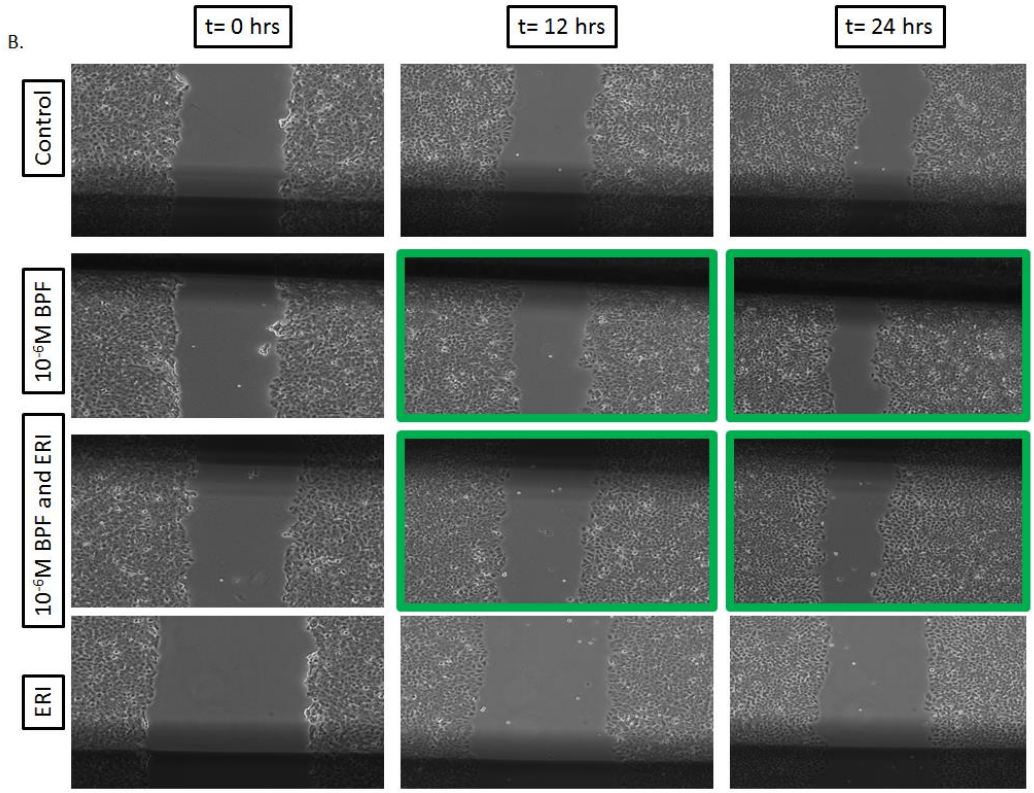


Figure 13. Representative figures of MCF-7 cell migration (scratch assay) showing MCF-7 cells treated with functional concentration s of BPA (A), BPF (B) and BPS (C) at time points 0, 12, 24 hrs

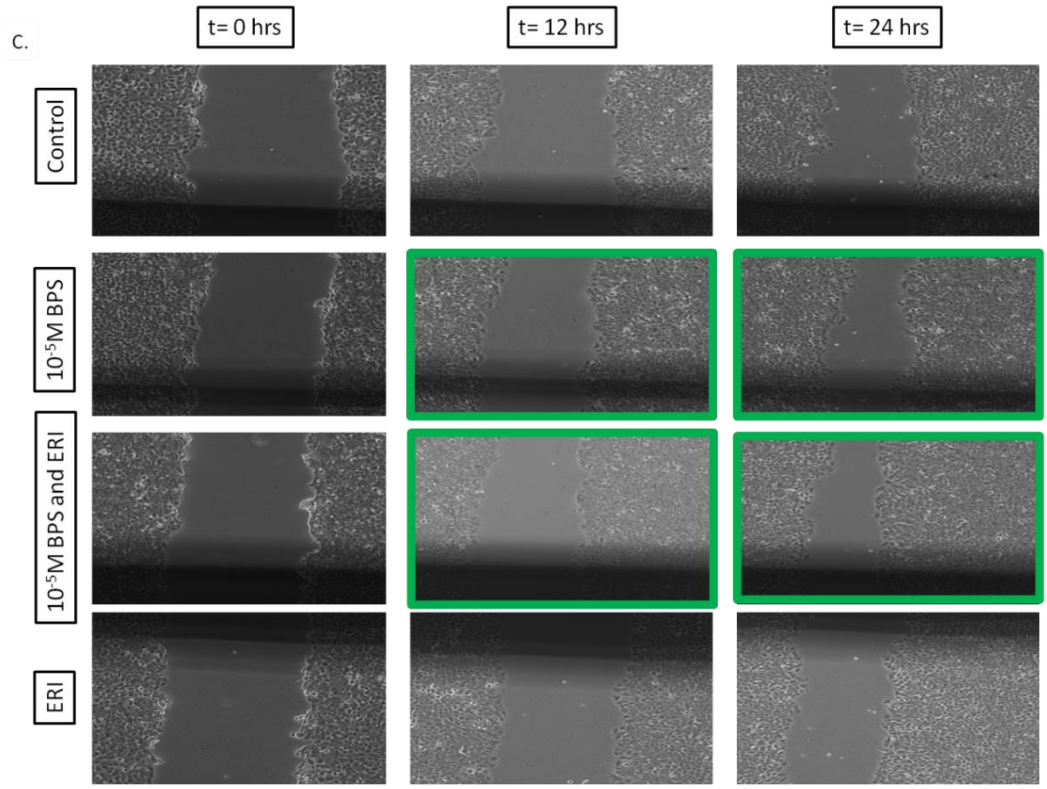
Figures were captured using light microscope (5× magnification), and results of treatment conditions that were significantly different from control are highlighted.



B.


t= 0 hrs
t= 12 hrs
t= 24 hrs
Control
10-6M BPF
10-6M BPF and ERI
ERI

C.


t= 0 hrs
t= 12 hrs
t= 24 hrs
Control
10-5M BPS
10-5M BPS and ERI
ERI

There was no increase in the metabolic activity, viability and migration of MDA-MB-231 cells after treatment with the three bisphenols, hence, confirming the role of ER in mediating the effects of exposure to endocrine disruptors on cell proliferation and migration. Of note, a modest (< 50%) decrease (rather than increase) in cell viability was observed only at late time points and very high concentrations (10^{-4} M) (**Figure 14, Figure 15, Figure 16**).

Figure 14. Metabolic activity (MTT assay) of MDA-MB-231 cells following treatment with different concentrations of BPA, BPF and BPS ± ERI for 24hrs (A1, B1, C1), 48 hrs (A2, B2, C2) and 72 hrs (A3, B3, C3), respectively

Metabolic activity was calculated as % relative to control, and data are presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using ANOVA followed by Dunnett *post-hoc* test (* for $p < 0.05$ and ** for $p < 0.001$), and between the same treatment condition ± ERI using ANOVA followed by Tukey HSD *post-hoc* test.

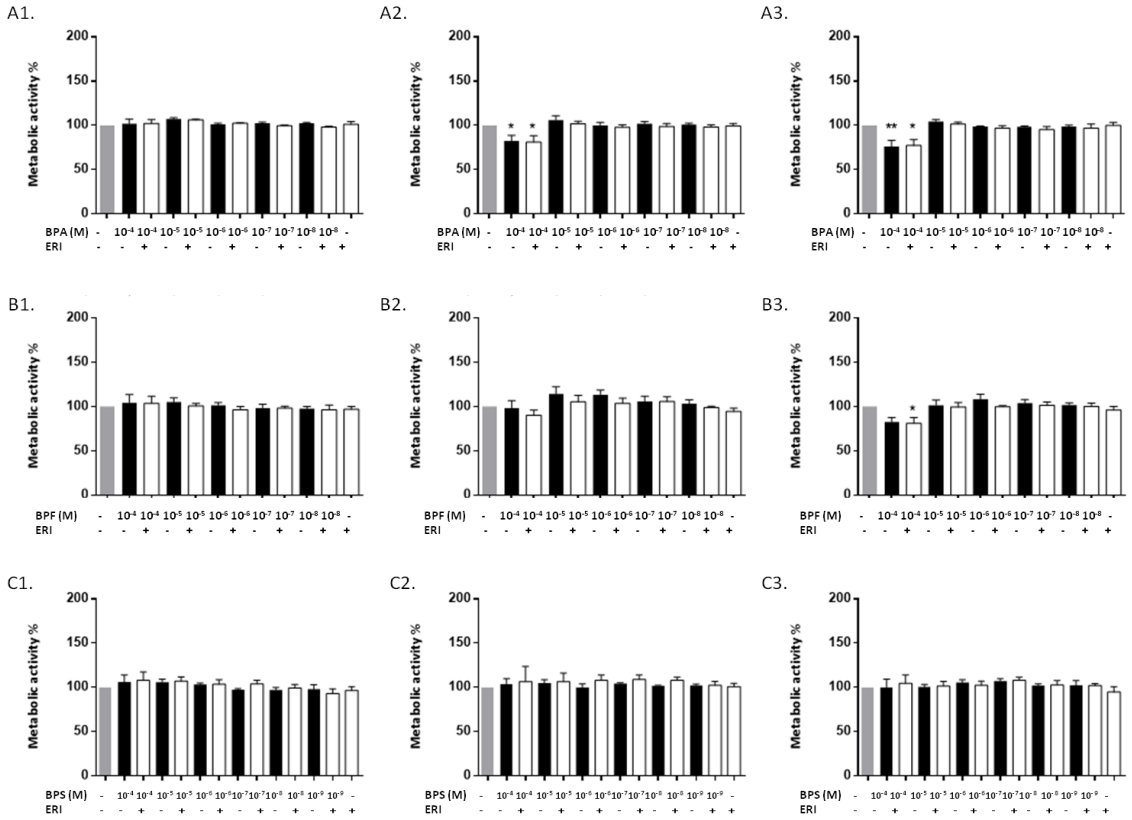


Figure 15. Cell viability (Trypan blue assay) of MDA-MB-231 cells following treatment with different concentrations of BPA, BPF and BPS ± ERI for 24 hrs (A1, B1, C1), 48 hrs (A2, B2, C2), and 72 hrs (A3, B3, C3), respectively

Cell viability was calculated as % relative to control, and data are presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using ANOVA followed by Dunnett *post-hoc* test (* for $p < 0.05$ and ** for $p < 0.001$), and between the same treatment condition ± ERI using ANOVA followed by Tukey HSD *post-hoc* test.

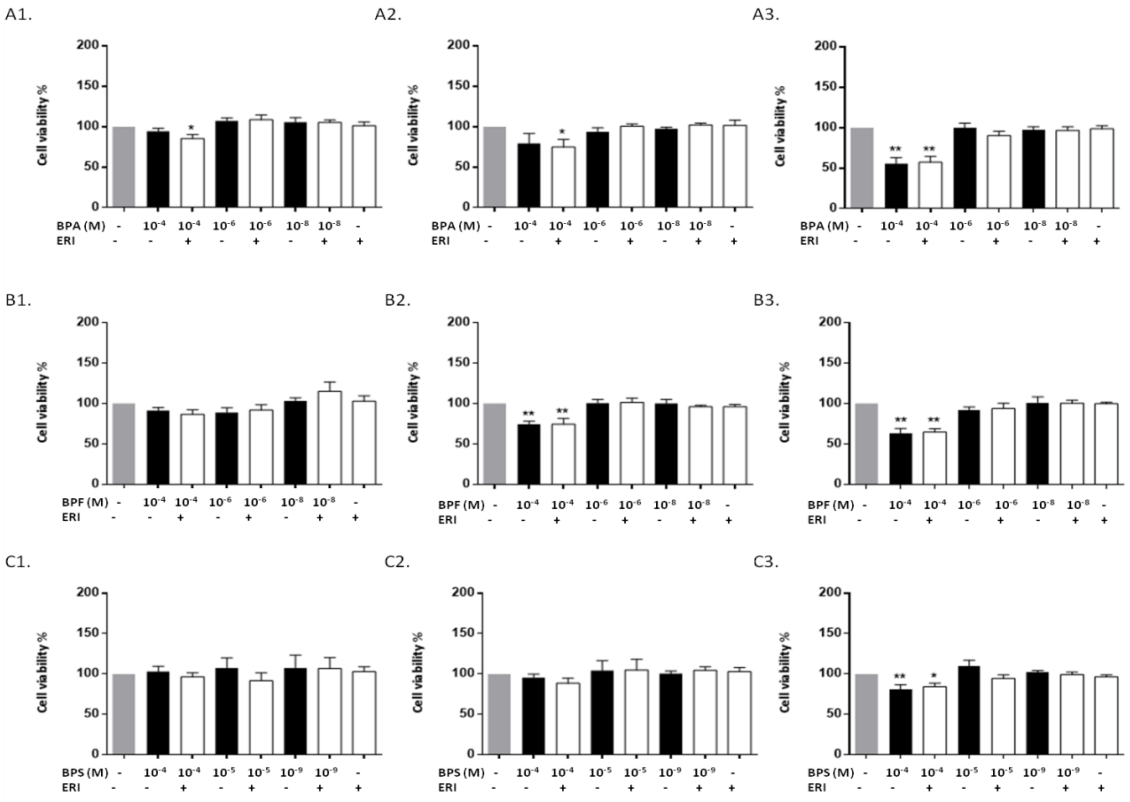
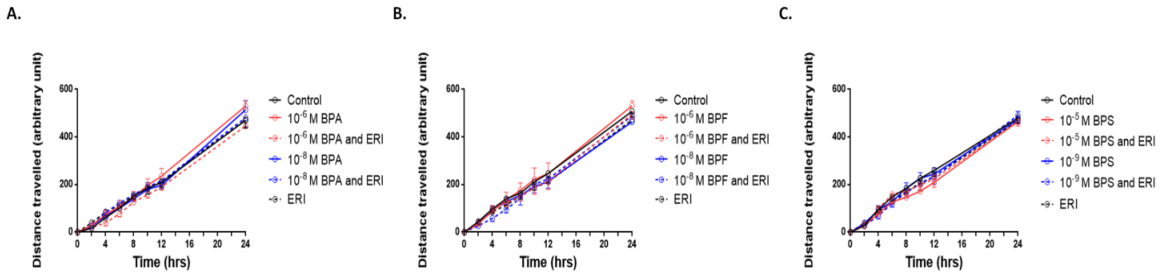


Figure 16. Migration (scratch assay) of MDA-MB-231 cells depicted as mean distance travelled ± SEM over time course of treatment with exposure and functional concentrations of BPA (A), BPF (B) and BPS (C)

Data are presented as mean distance travelled (arbitrary unit) ± SEM at time points 0, 2, 4, 6, 8, 10, 12 and 24 hrs in at least three independent trials. Comparisons were performed at every time point between each treatment condition and control using two-way ANOVA followed by Dunnett *post-hoc* test, and between the same treatment condition in the presence and absence of ERI using two-way ANOVA followed by Tukey HSD *post-hoc* test.



Since the three bisphenols resulted in significant changes in proliferation and migration of MCF-7 cells and not of MDA-MB-231 at 48 and 72 hrs, we assessed the effects of these EDCs on molecular effects in MCF-7 cells only. We also focused on early time points (24 and 48 hrs), coinciding with molecular readouts that likely precede (hence, are causal to rather than resultant from) the observed phenotypic manifestation of cancer progression.

B. Bisphenols induced an ER-dependent increase in telomerase expression and activity but did not change RTL

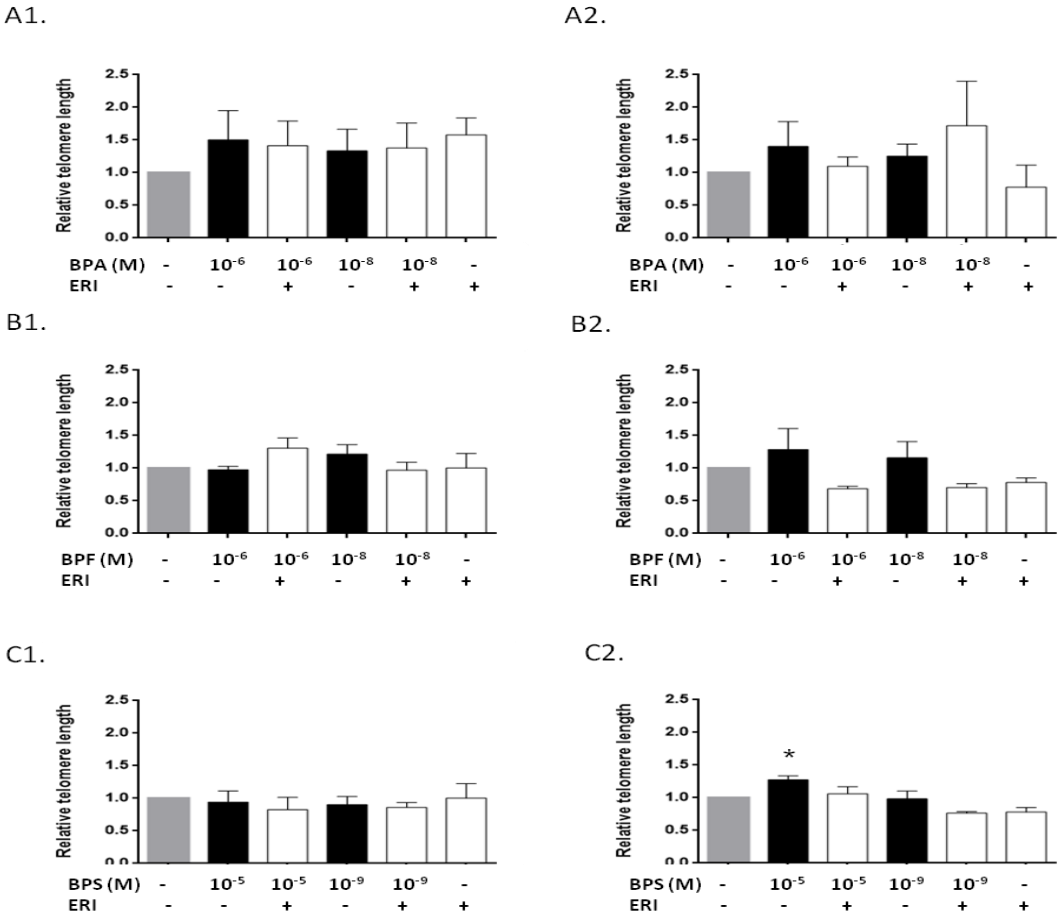
There was no marked change in RTL in MCF-7 cells treated with the functional and exposure concentrations of BPA, BPF and BPS for 24 and 48 hrs (**Figure 17A**). A 2-3 folds increase in telomerase RNA expression and enzymatic activity was observed in

MCF-7 cells after 24 hrs of treatment with the functional concentration of BPA, BPF and BPS; this increase was abolished in the presence of ERI (**Figure 17B**). Similar trends (though not significant) were observed at the exposure concentration of all three bisphenols.

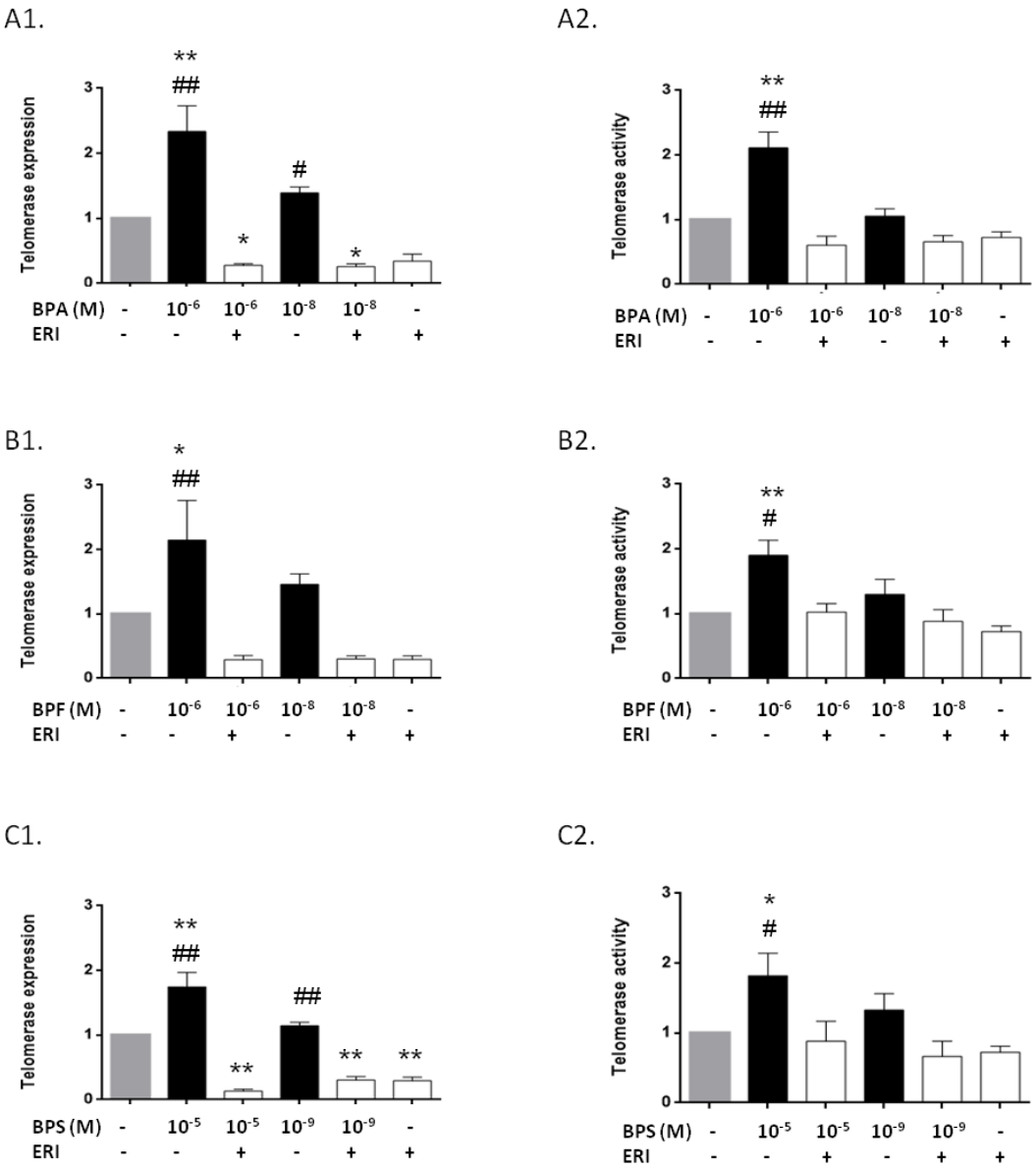
Figure 17. Relative telomere length (**Panel A**) and relative telomerase RNA expression and enzyme activity (**Panel B**) in MCF-7 cells after treatment with exposure and functional concentrations of BPA, BPF and BPS with or without ERI

Results were calculated as relative to control, and presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using ANOVA followed by Dunnett post-hoc test (* for $p < 0.05$ and ** for $p < 0.001$), and between the same treatment condition ± ERI using ANOVA followed by Tukey HSD post-hoc test ($^{\#}$ for $p < 0.05$ and $^{\#\#}$ for $p < 0.001$).

Panel A. Relative telomere length in MCF-7 cells after 24 hrs and 48 hrs of treatment with BPA (A1, A2), BPF (B1, B2) and BPS (C1, C2), respectively



Panel B. Relative telomerase RNA expression and enzyme activity in MCF-7 cells after 24hrs of treatment with BPA (A1, A2), BPF (B1, B2) and BPS (C1, C2), respectively



C. Bisphenols induced an ER-dependent increase in *DNMT1* RNA expression and differential increase in *TETs 2&3* RNA expression but did not change their overall enzymatic activities

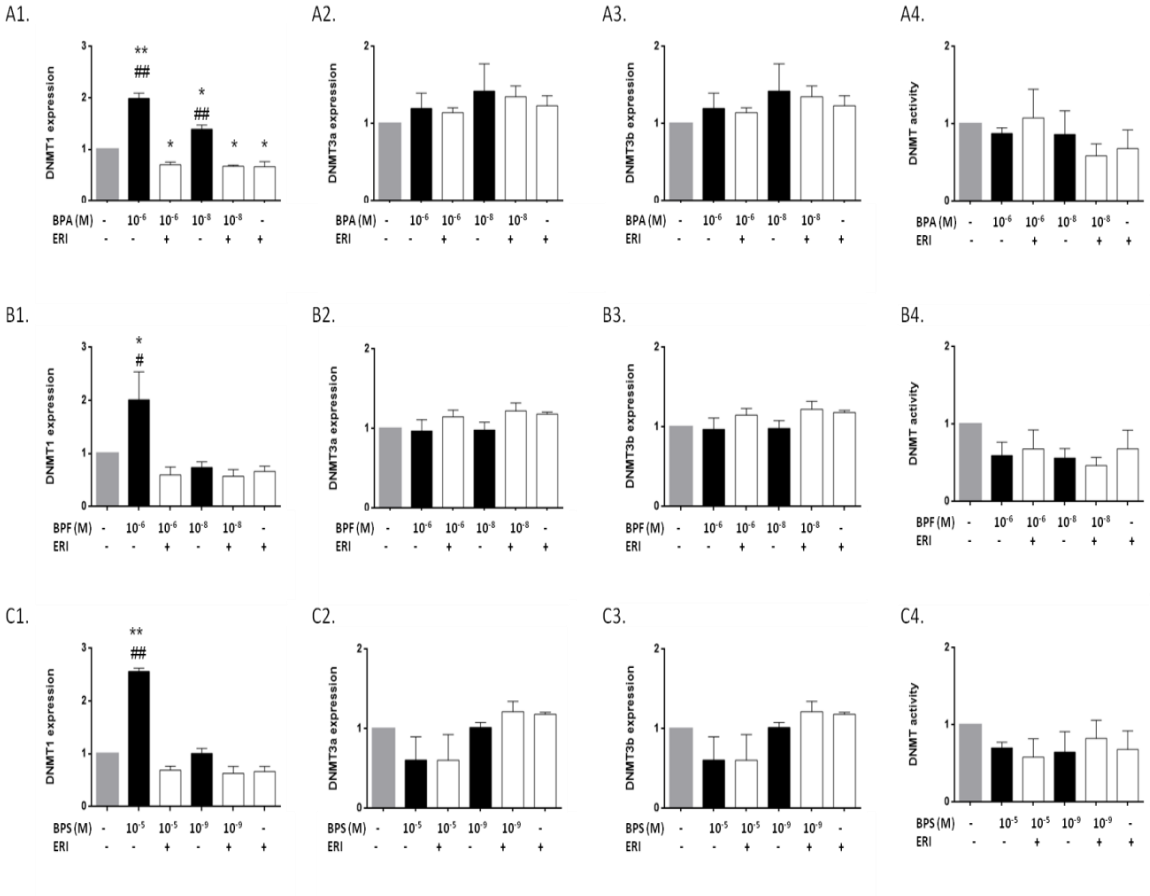
There were modest increases (<2-folds) in *DNMT1* RNA expression in MCF-7 cells after 24hrs of treatment with the functional concentrations of BPA, BPF and BPS and with the human exposure concentration of BPA (except for a 2.5 fold increase with the BPS functional concentration), and these effects were inhibited by ERI. RNA expression levels of *DNMT3A* and *DNMT3B* and overall DNMT enzymatic activity were not altered with the three bisphenols (**Figure 18A**).

Similarly, there were ER-dependent modest increases (<2-folds) in *TET2* RNA expression after 24hrs of treatment with the functional concentration of BPA and BPS and similar increases in *TET3* RNA expression after 24hrs of treatment with the functional and exposure concentrations of BPA. RNA expression levels of *TET1* and overall TET enzymatic activity were not altered with the three bisphenols (**Figure 18B**).

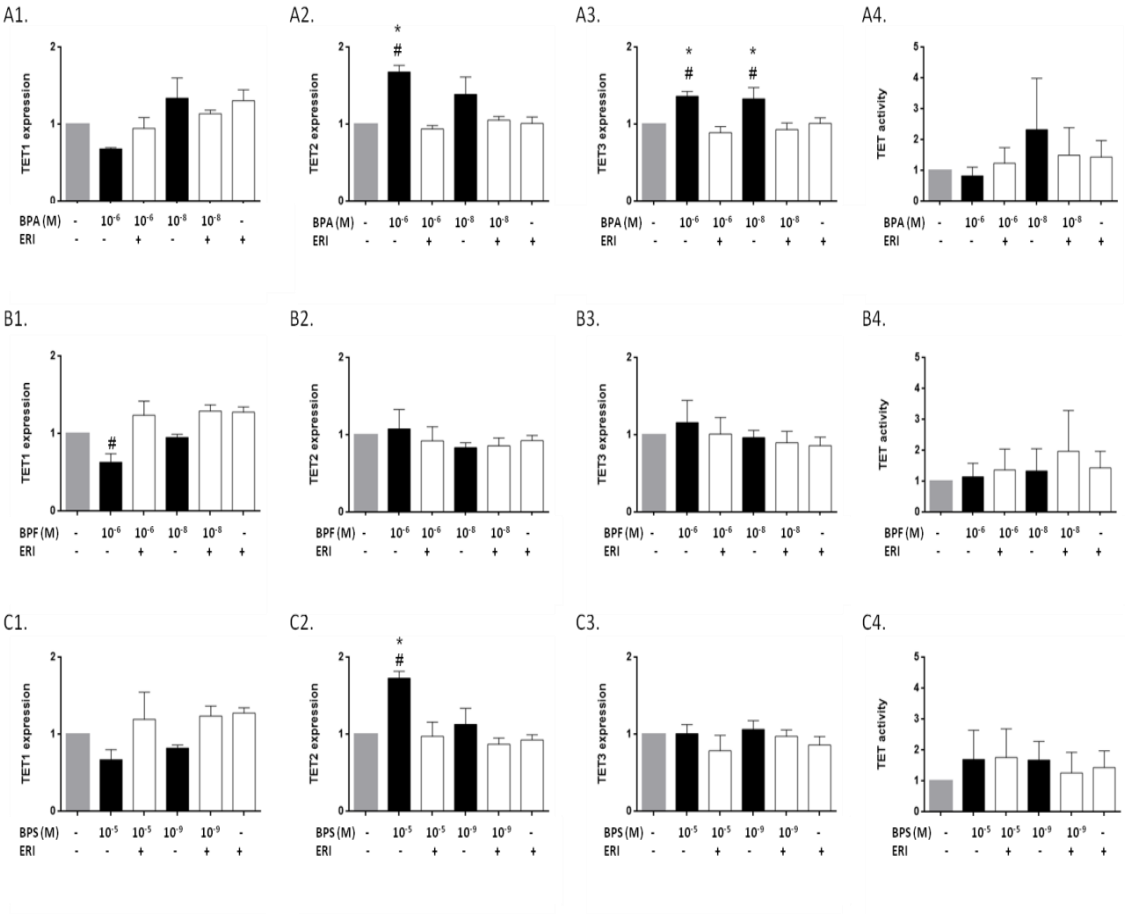
Figure 18. RNA expression and enzymatic activity of DNA methylation (**Panel A**) and demethylation enzymes (**Panel B**) in MCF-7 cells after 24 hrs of treatment with exposure and functional concentrations of BPA, BPF and BPS.

RNA expression and enzymatic activity were calculated as relative to control, and are presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using ANOVA followed by Dunnett *post-hoc* test (* for $p < 0.05$ and ** for $p < 0.001$), and between the same treatment condition ± ERI using ANOVA followed by Tukey HSD *post-hoc* test (# for $p < 0.05$ and ## for $p < 0.001$).

Panel A. RNA expression of DNA methylation enzymes (DNMT1, DNMT3a, DNMT3b)and enzymatic activity of DNMTs in MCF-7 cells after 24 hrs of treatment with exposure and functional concentrations of BPA (A1, A2, A3, A4), BPF (B1, B2, B3, B4) and BPS (C1, C2, C3, C4), respectively.



Panel B. RNA expression of DNA demethylation enzymes (TET1, TET2, TET3) and enzymatic activity of TETs in MCF-7 cells after 24 hrs of treatment with exposure and functional concentrations of BPA (A1, A2, A3, A4), BPF (B1, B2, B3, B4) and BPS (C1, C2, C3, C4), respectively



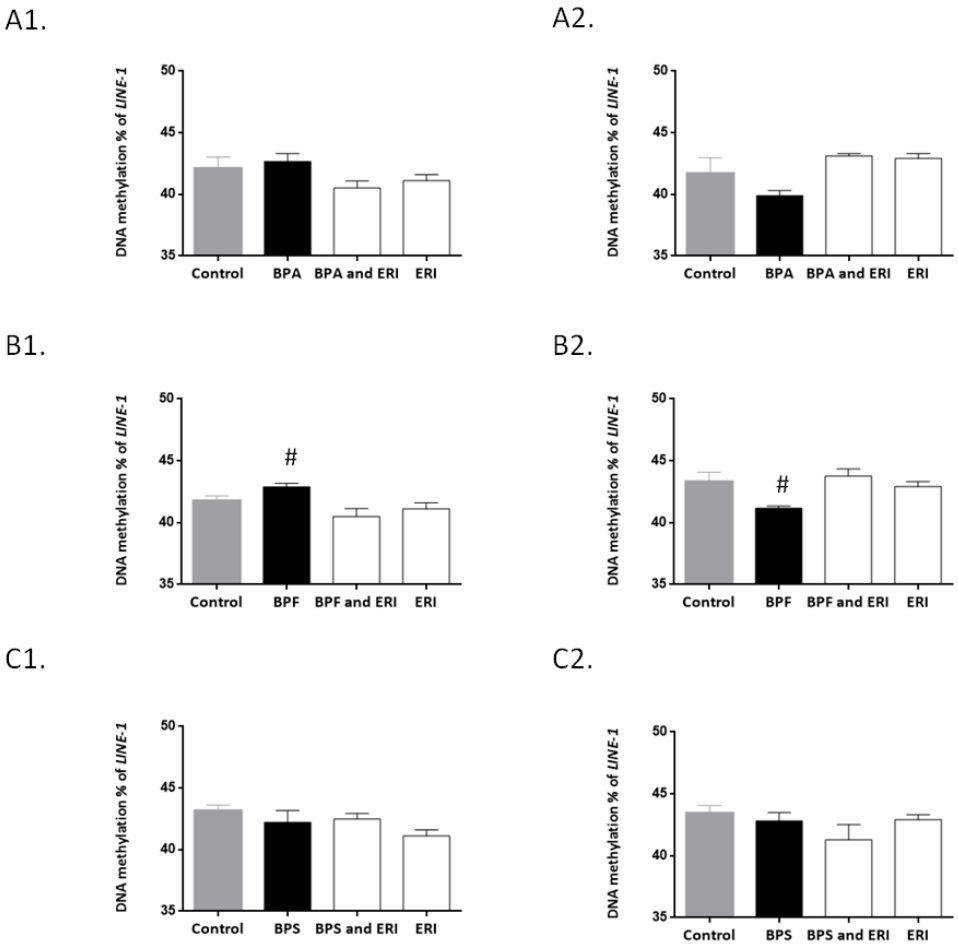
D. Bisphenols showed a trend of *LINE-1* hypomethylation that was ER-dependent with BPA and BPF

We tested the effects of functional concentrations of BPA and its analogues on *LINE-1* methylation, which additionally served as a screening tool to determine the earliest time points and concentrations at which overall methylation alterations occur, so that these conditions can be subsequently analyzed in-depth with genome-wide methylation profiling. BPA and BPF (and to a lesser extent BPS) were associated with a trend

towards decreased *LINE-1* methylation in MCF-7 cells at 48hrs, which was not observed at 24hrs. At 48hrs, mean decrease in *LINE-1* methylation was 1.63% and 2.14% with BPA and BPF, respectively. Of note, this hypomethylation of *LINE-1* with BPA and BPF at 48hrs was completely abolished in the presence of ERI (**Figure 19).**

Figure 19. Global DNA methylation represented as DNA methylation % at 1st CpG site of *LINE-1* gene in MCF-7 cells after treatment with functional concentrations of BPA (A1, A2), BPF(B1, B2) or BPS (C1, C2) with or without ERI for 24 and 48 hrs, respectively

Data are presented as mean + SEM of three independent trials. Comparisons were performed between each treatment condition and corresponding control using one-way ANOVA followed by Dunnett *post-hoc* test, and between the same treatment condition ±ERI using one-way ANOVA followed by Tukey HSD *post-hoc* test ($^{\#}$p-value < 0.05).



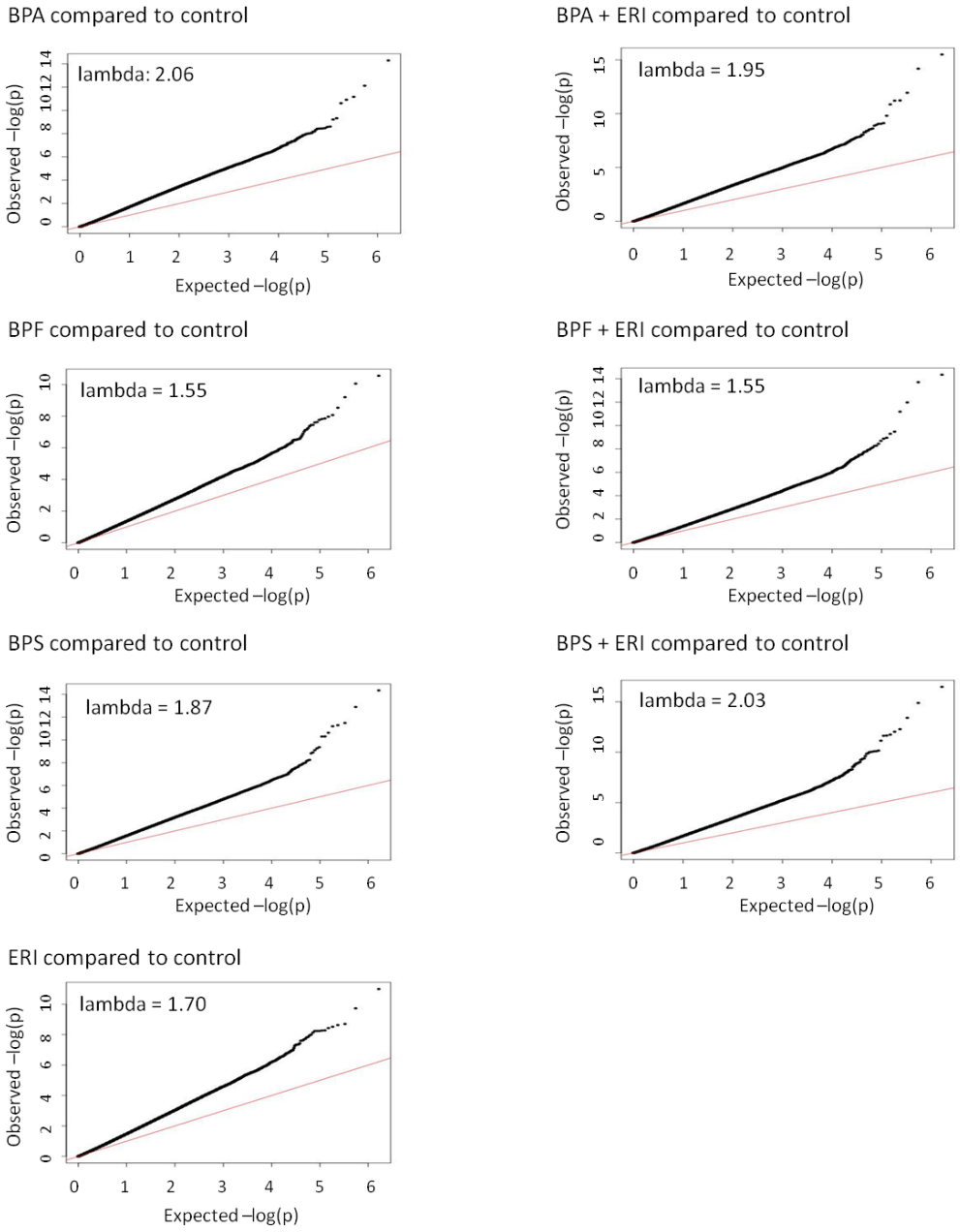
Since there were more changes in *LINE-1* methylation after treatment of MCF-7 cells with functional concentrations for 48hrs compared to 24hrs, the subsequent methylome-wide association experiment was performed at 48hrs.

E. Bisphenols induce differential DNA methylation alterations in several CpG sites and regions located mostly in gene promoters and exons

We used two approaches for the epigenome-wide analysis leading to the identification of differentially methylated CpG probes (DMPs) and differentially methylated regions of CpG clusters (DMRs). Both approaches showed that functional concentrations of each of BPA, BPF and BPS have a profound impact on the DNA methylome, leading to a large number of statistically significant DMPs or DMRs compared to their

corresponding controls, even when using stringent correction (SVA) for batch and confounder effects. Lambda values and q-q plots were not indicative of major inflation (**Figure 20**).

Figure 20. qq plots and lambda values for analysis of differentially methylated probes (DMPs) in MCF-7 cells treated for 48 hrs with functional concentrations of BPA, BPF, BPS with or without ERI or ERI alone when compared to control



Although BPA, BPF and BPS share similar chemical structures (**Figure 1)**, they exhibited differential effects on the methylome at functional concentrations. In particular, both DMP and DMR analyses showed that the effect was consistently strongest for BPA and weakest for BPF (**Table 13)**, and that the majority of identified DMPs, DMRs or genes encompassing the DMPs/DMRs did not overlap between any of the three compounds (**Figure 21, Figure 22)**. A larger proportion of the DMPs, DMRs or differentially methylated genes was hypomethylated in BPA and BPS treatments, while similar proportions of hypo- and hyper-methylation were observed with BPF. Even though BPA, BPF and BPS showed differential epigenetic effects at the gene level, there were similarities observed among the compounds at a more global genomic level. Specifically, the genomic distribution of the DMPs and DMRs were strikingly similar among the three bisphenols, showing enrichment in promoter regions and exons and diminishment in intergenic regions, compared to random probes (**Figure 23**).

Table 13. Number of differentially methylated probes (DMPs) and differentially methylated regions (DMRs) before and after filtration and their corresponding genes in MCF-7 cells treated for 48hrs with functional concentrations of BPA, BPF and BPS with or without ERI when compared to control

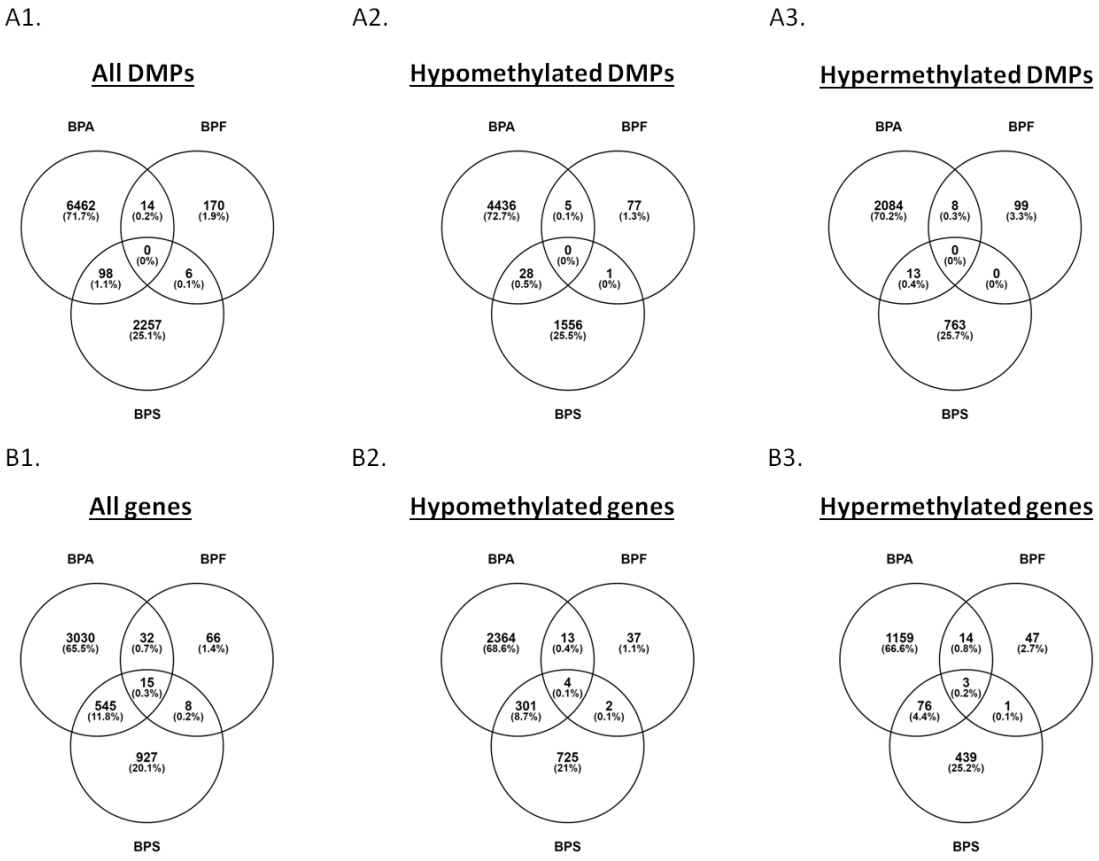
Treatment[†]	DMPs				DMRs			
	Before filtration	After filtration (hypo/hyper)	Genes (hypo/hyper)	Max IΔβI (%)	Before filtration	After filtration (hypo/hyper)	Genes (hypo/hyper)	Max IΔβI (%)
BPA	13366	6574 (4469/2105)	3622 (2682/1252)	22.77	38193	2521 (1972/549)	2203 (1765/523)	13.43
BPA + ERI	9534	4225 (1765/2460)	2478 (1091/1559)	25.06	34076	1419 (379/1040)	1296 (360/983)	18.32
BPF	549	190 (83/107)	121 (56/65)	25.47	15151	721 (414/307)	691 (405/301)	10.92
BPF + ERI	1243	598 (266/332)	409 (172/243)	25.58	17214	1264 (213/1051)	1176 (200/991)	15.77
BPS	5309	2361 (1585/776)	1495 (1032/519)	33.35	30559	1975 (1755/220)	1737 (1562/209)	19.5
BPS + ERI	1097	555 (281/274)	401 (206/198)	18.77	37478	1523 (990/533)	1392 (929/513)	12.05
ERI	2829	1185 (332/853)	764 (208/567)	34.83	23954	1388 (197/1191)	1301 (191/1124)	16.17

[†] Treatment conditions were compared to control using robust linear model (RLM).
Hypo: hypomethylated; hyper: hypermethylated
IΔβI: absolute value of the difference of filtered methylation % between treatment and control

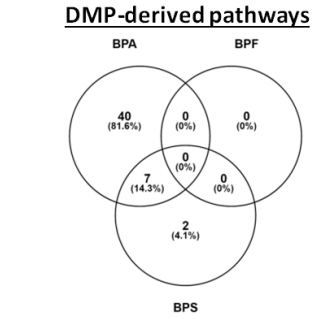
Figure 21. Differentially methylated probes (DMPs) and genes encompassing DMPs in MCF-7 cells treated for 48hrs with exposure and functional concentrations of BPA, BPF and BPS treatment conditions when compared to control

Venn diagrams of DMPs and genes encompassing DMPs of BPA, BPF or BPS are shown in A1-A3 and B1-B3, respectively.

Pathways of genes encompassing DMPs of BPA, BPF and BPS were detected based on KEGG pathway database using Enrichr (http://amp.pharm.mssm.edu/Enrichr/) and shown in C1 and C2.BPF was not included in C2, because its DMP-derived genes were not significantly involved in any pathway (as shown in C1).



C1.


DMP-derived pathways
BPA
BPF
40
(81.6%)
0
(0%)
0
(0%)
0
(0%)
7
(14.3%)
0
(0%)
2
(4.1%)
BPS

C2.

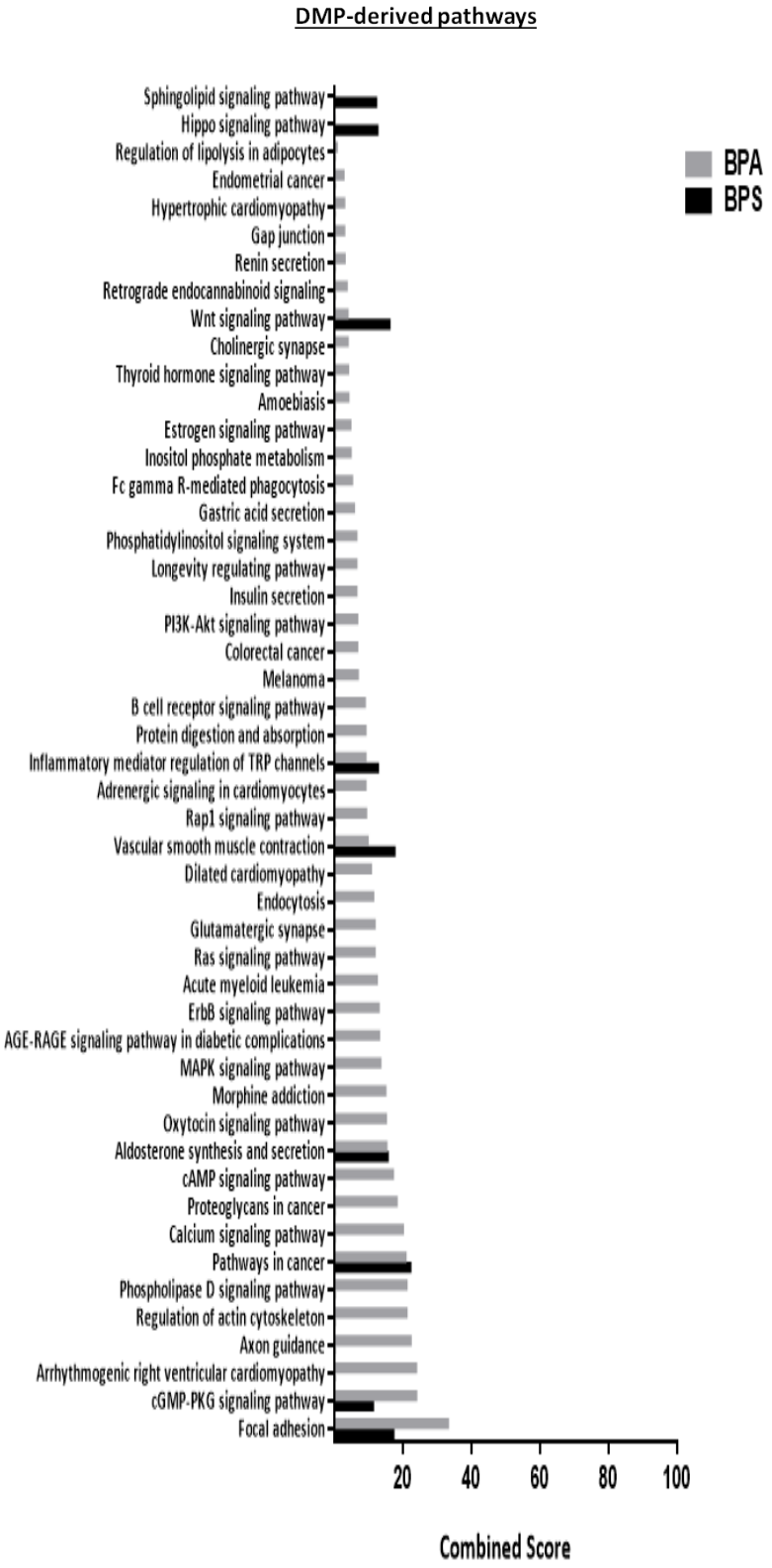
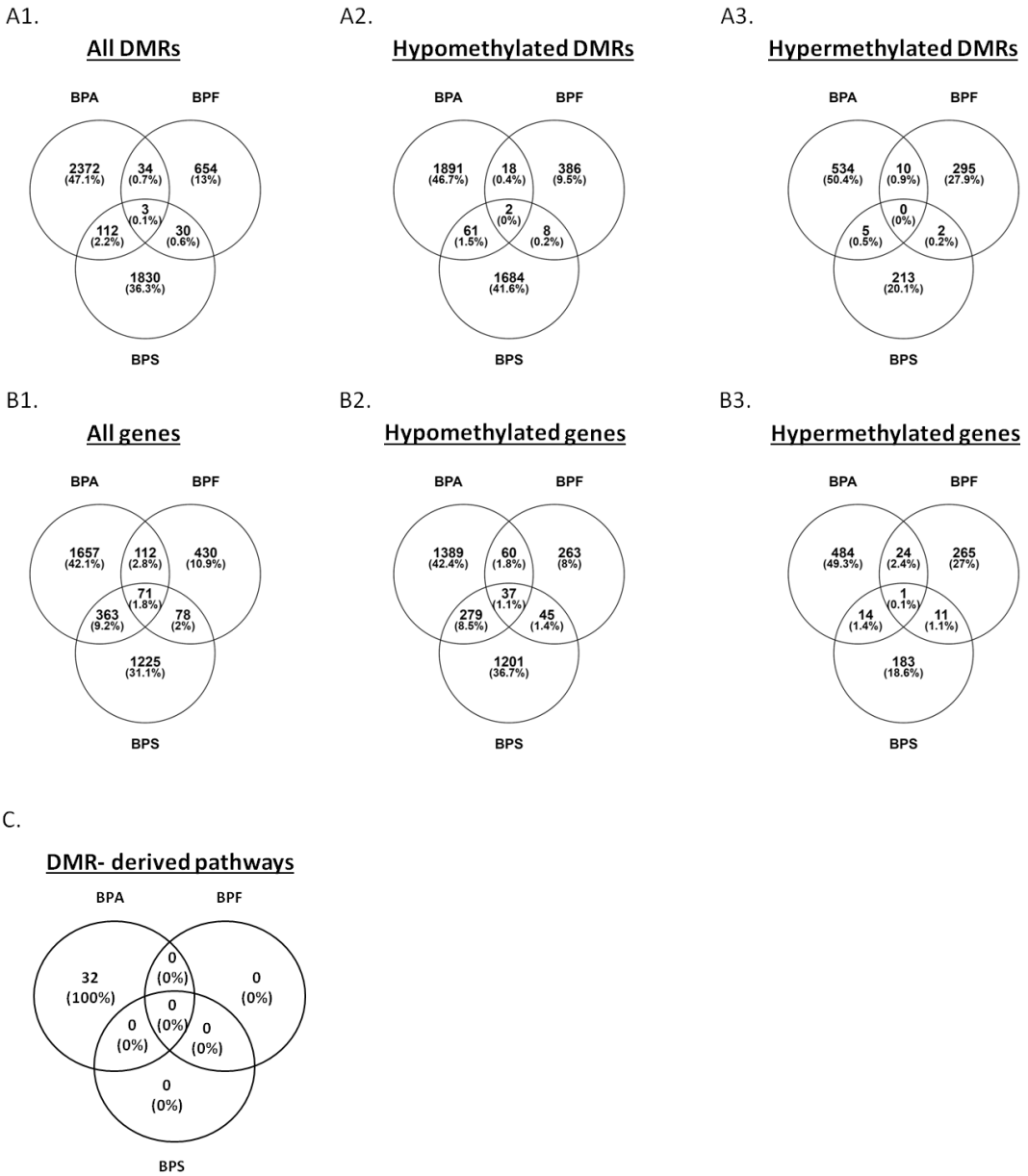

DMP-derived pathways
BPA
BPS
Sphingolipid signaling pathway
Hippo signaling pathway
Regulation of lipolysis in adipocytes
Endometrial cancer
Hypertrophic cardiomyopathy
Gap junction
Renin secretion
Retrograde endocannabinoid signaling
Wnt signaling pathway
Cholinergic synapse
Thyroid hormone signaling pathway
Amoebiasis
Estrogen signaling pathway
Inositol phosphate metabolism
Fc gamma R-mediated phagocytosis
Gastric acid secretion
Phosphatidylinositol signaling system
Longevity regulating pathway
Insulin secretion
PI3K-Akt signaling pathway
Colorectal cancer
Melanoma
B cell receptor signaling pathway
Protein digestion and absorption
Inflammatory mediator regulation of TRP channels
Adrenergic signaling in cardiomyocytes
Rap1 signaling pathway
Vascular smooth muscle contraction
Dilated cardiomyopathy
Endocytosis
Glutamatergic synapse
Ras signaling pathway
Acute myeloid leukemia
ErbB signaling pathway
AGE-RAGE signaling pathway in diabetic complications
MAPK signaling pathway
Morphine addiction
Oxytocin signaling pathway
Aldosterone synthesis and secretion
cAMP signaling pathway
Proteoglycans in cancer
Calcium signaling pathway
Pathways in cancer
Phospholipase D signaling pathway
Regulation of actin cytoskeleton
Axon guidance
Arrhythmogenic right ventricular cardiomyopathy
cGMP-PKG signaling pathway
Focal adhesion
20
40
60
80
100
Combined Score

Figure 22. Differentially methylated regions (DMRs) and genes encompassing DMRs in MCF-7 cells treated for 48 hrs with the functional concentrations of BPA, BPF and BPS when compared to control

Venn diagrams of DMRs and genes encompassing them of BPA, BPF and BPS are shown in A1-A3 and B1-B3, respectively

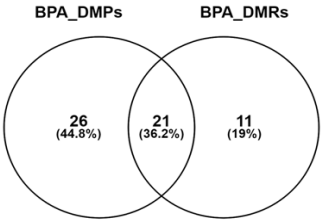
Pathways of genes encompassing DMRs of BPA, BPF and BPS were detected based on KEGG pathway database using Enrichr (http://amp.pharm.mssm.edu/Enrichr/) and shown in C.

Pathways of genes with DMRs of BPA were compared to those with differentially methylated probes (DMPs) of BPA and shown in D1, and common pathways between BPA-related DMPs and DMRs are listed in D2.No figure was drawn for BPF and BPS, because their DMR-derived genes were not significantly involved in any pathway (as shown in C).



D1.

DMP- and DMR- derived pathways



D2.

DMP- and DMR- derived pathways

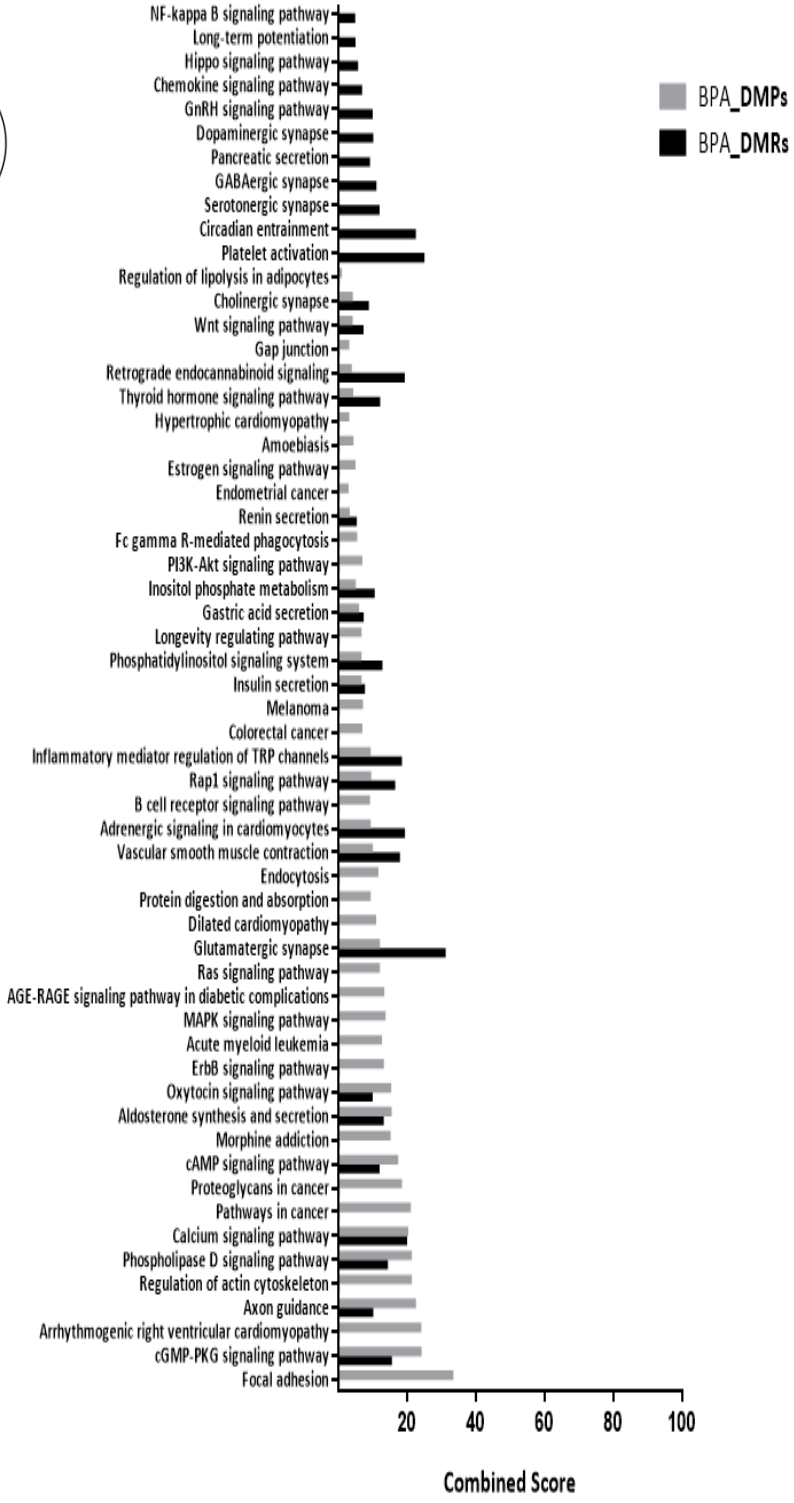
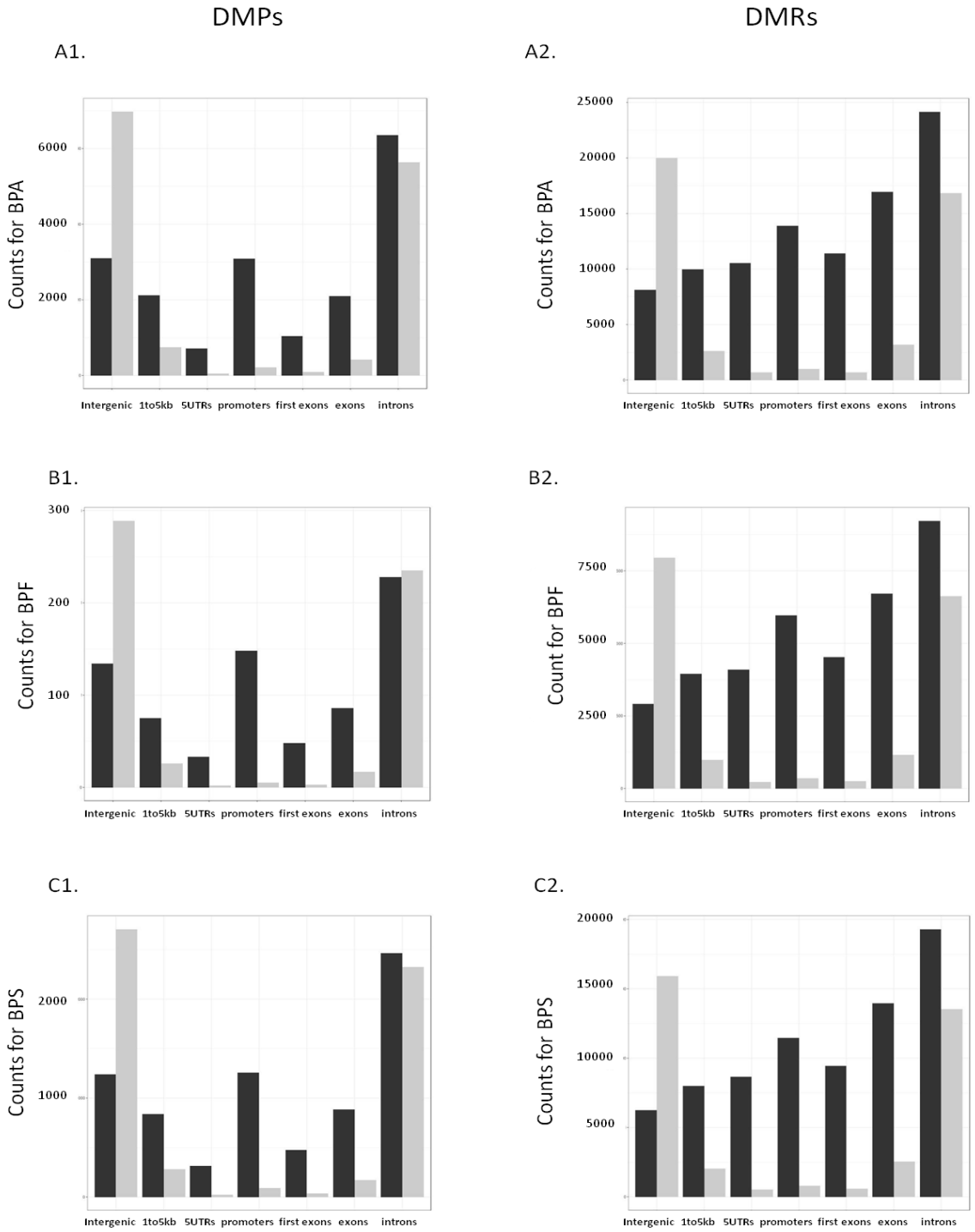


Figure 23. Comparison of the genomic distribution of the differentially methylated probes (DMPs) and differentially methylated regions (DMRs) in MCF-7 cells treated for 48 hrs with the functional concentrations of BPA (A1 and A2), BPF (B1 and B2) and BPS (C1 and C2) (in **black**) with the genomic distribution of randomly selected probes and regions tested by the Infinium MethylationEPIC microarray (in **grey**)



F. Differentially methylated genes of BPA and BPS are involved in cancer-related pathways

Pathway analysis with KEGG database based on the DMP-derived genes showed 47 pathways for BPA, nine for BPS and none for BPF. Seven out of nine pathways of BPS were common with those of BPA, with focal adhesion, cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) signaling and cancer pathways having the highest combined score (**Figure 21C**). Additionally several subsets of cancer pathways were observed with BPA-induced DMPs (Proteoglycans in cancer, Acute myeloid leukemia, Melanoma, Colorectal cancer, and Endometrial cancer). Moreover, the Estrogen signaling pathway was detected with BPA-induced DMPs. As for the DMR-derived genes, using the KEGG database revealed 32 significant pathways for BPA but none for BPF and BPS, so comparison of pathways among the three compounds through this approach was not possible. However, comparing the pathways of genes obtained with DMPs to those obtained with DMRs for BPA revealed 21 out of 32 common pathways, with glutamatergic synaptic, calcium signaling, cGMP-PKG signaling and phospholipase D pathways having the highest combined score. Interestingly, Wnt signaling pathway was common between the pathways derived from genes encompassing DMPs and DMRs induced by BPA (**Figure 22D**).

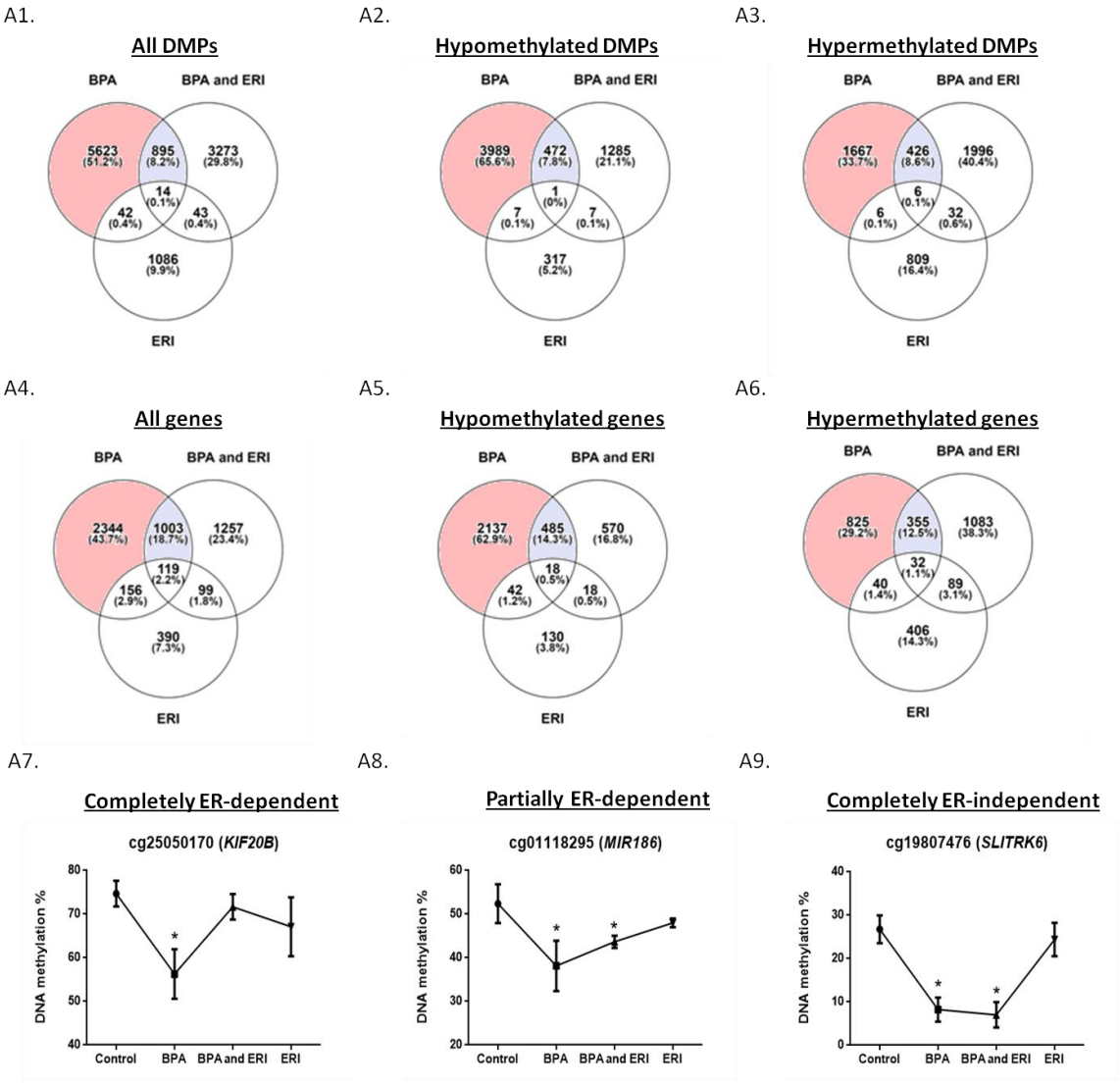
G. Most bisphenol-induced differentially methylated sites and regions were ER-dependent

Most of the revealed DMPs, DMRs and genes encompassing them were not associated with the bisphenols' treatments when combined with ERI and were hence dependent on ER (**Figure 24**, **Figure 25**). **Figures 24 and 25** show representative DMPs and DMRs (with the most significant and highest effect sizes) which were completely dependent on, partially dependent on or completely independent of ER. Interestingly, 25 out of 47pathways pertaining to DMP-derived genes of BPA were ER-dependent, including cAMP, MAPK, estrogen and Wnt signaling pathways. Moreover, all of the pathways pertaining to DMP-derived genes of BPS were ER-dependent (data not shown).

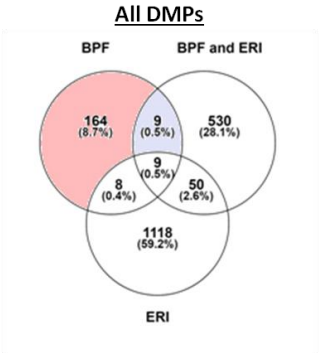
Figure 24. Differentially methylated probes (DMPs) and genes encompassing DMPs in MCF-7 cells treated for 48hrs with functional concentrations of BPA, BPF and BPS with or without ERI and ERI alone when compared to control

Venn diagrams of DMPs and genes encompassing DMPs of BPA ± ERI, BPF ± ERI and BPS ± ERI are shown in A1-A6, B1-B6 and C1-C6, respectively (**Rose: bisphenol-specific and ER-dependent**, **Blue: bisphenol-specific and ER-independent**)

Representative figures of DMPs (*genes*) altered by BPA, BPF or BPS treatment conditions and being dependent (A7, B7, C7), partially dependent (A8, B8, C8) or completely independent (A9, B9, C9) by ERI, respectively. Data are presented as mean ± SD of two independent trials. Comparisons were performed between each treatment condition and corresponding control using robust linear model (RLM) (*p-value < 0.05).



B1.



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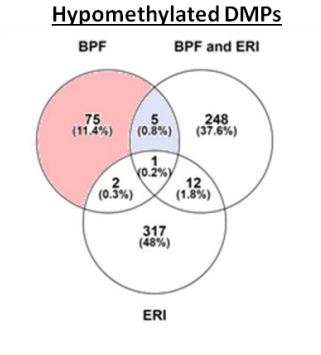

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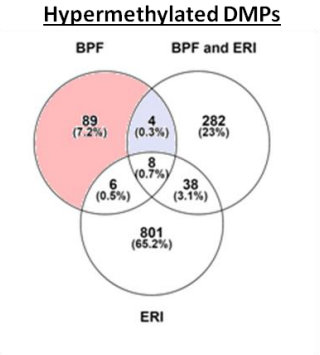

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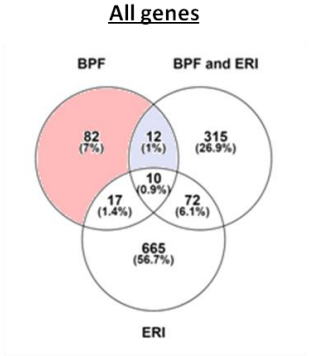

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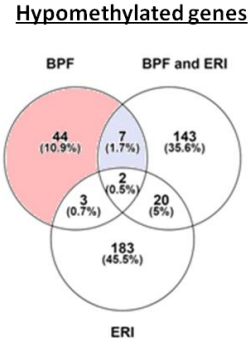

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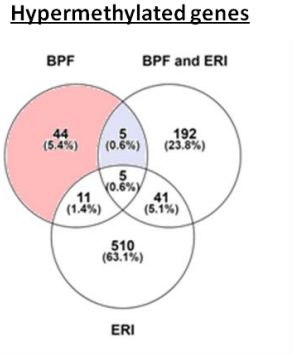

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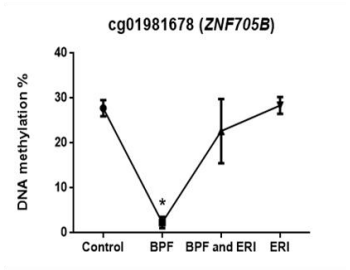

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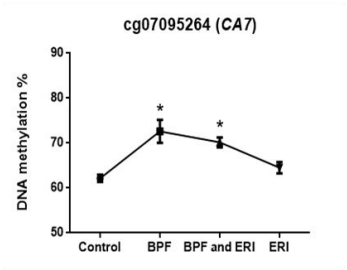
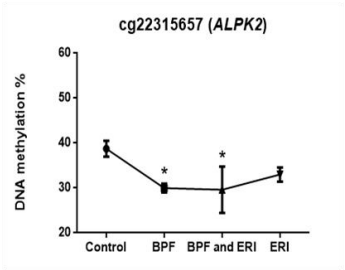
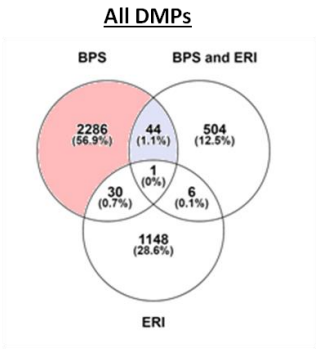


B9.



C1.



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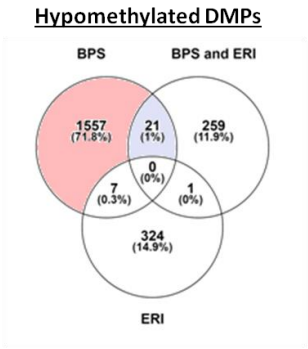
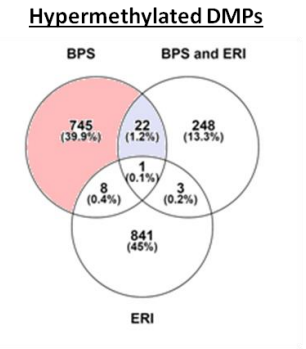
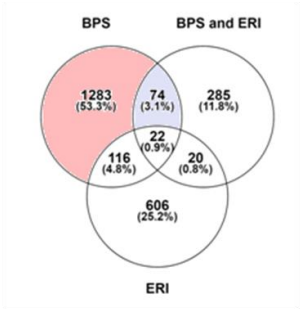


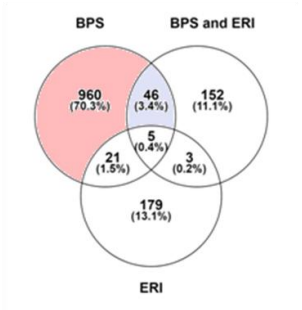
C3.



C4.



C5.



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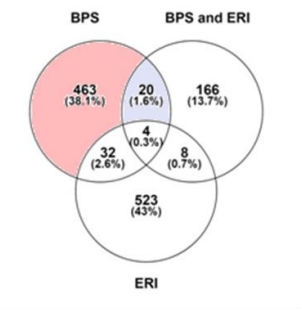

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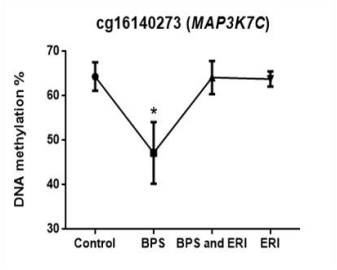

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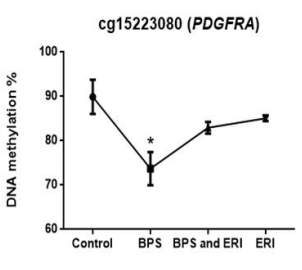


C9.

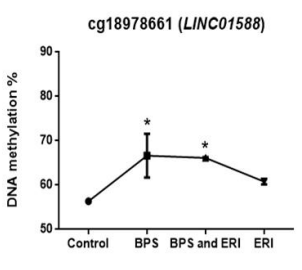
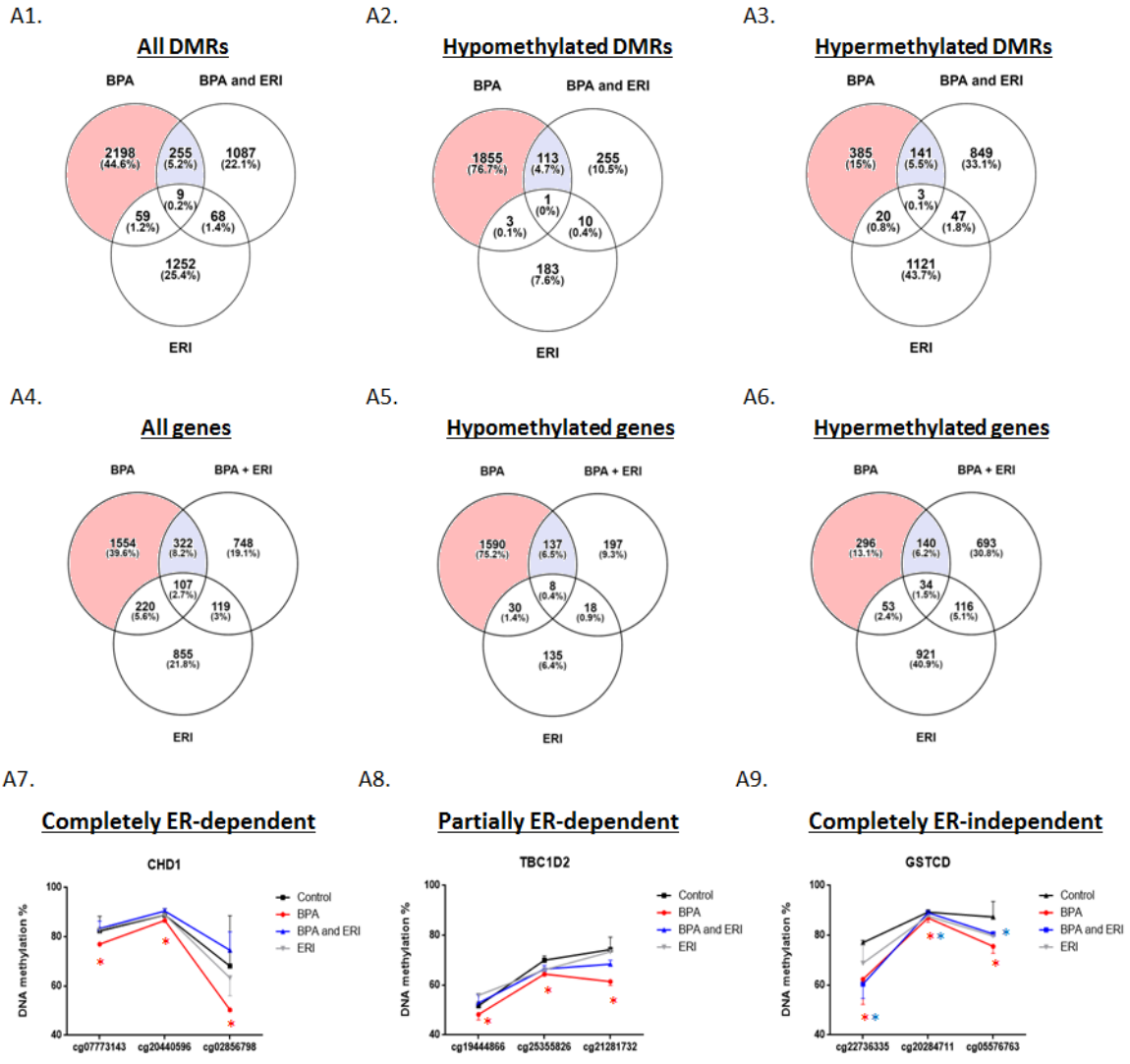


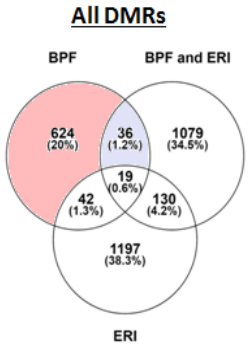
Figure 25. Differentially methylated regions (DMRs) and genes encompassing DMRs in MCF-7 cells treated for 48hrs with functional concentrations of BPA, BPF and BPS with or without ERI and ERI alone when compared to control

Venn diagrams of DMRs and genes encompassing DMRs of BPA ± ERI, BPF ± ERI and BPS ± ERI are shown in A1-A6, B1-B6 and C1-C6, respectively (**Rose: bisphenol-specific and ER-dependent**, **Blue: bisphenol-specific and ER-independent**)

Representative figures of DMRs (*genes*) altered by BPA, BPF or BPS treatment conditions and being dependent (A7, B7, C7), partially dependent (A8, B8, C8) or completely independent (A9, B9, C9) by ERI, respectively. Data are presented as mean ± SD of two independent trials. Comparisons were performed between each treatment condition and corresponding control using robust linear model (RLM) (*p-value < 0.05).



B1.



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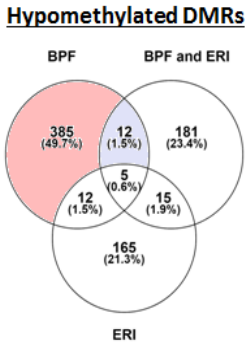
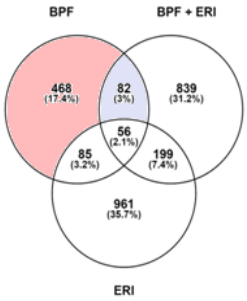


B3.

B4.



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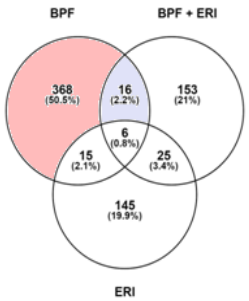
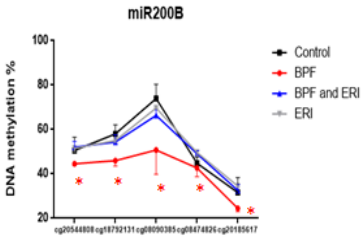


B6.

B7.



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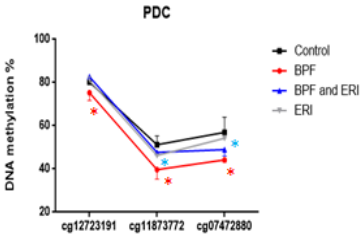
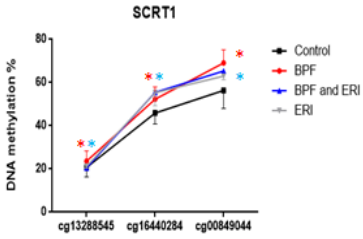
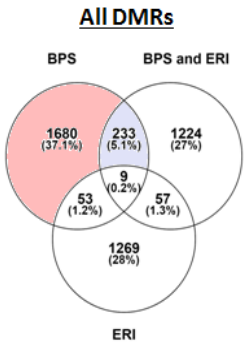


B9.



C1.



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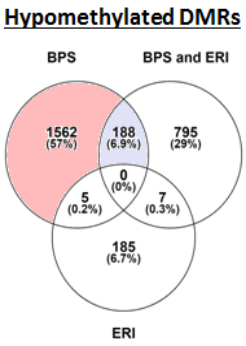
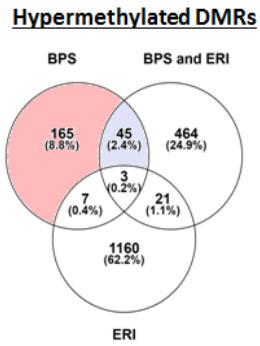
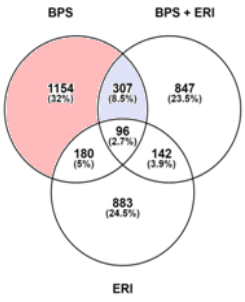


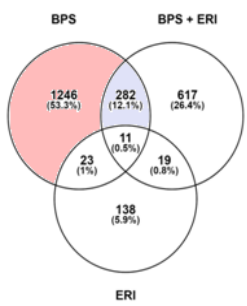
C3.



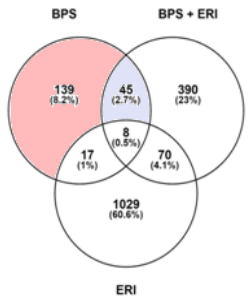
C4.



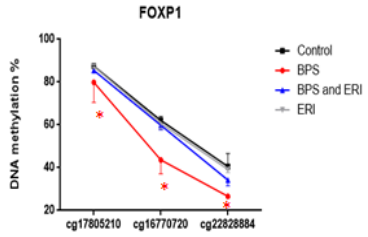
C5.



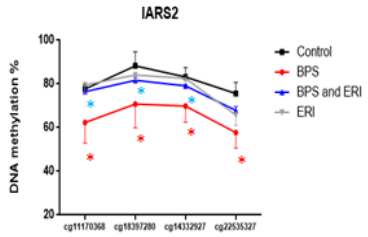
C6.



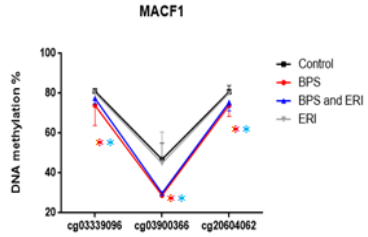
C7.



C8.



C9.

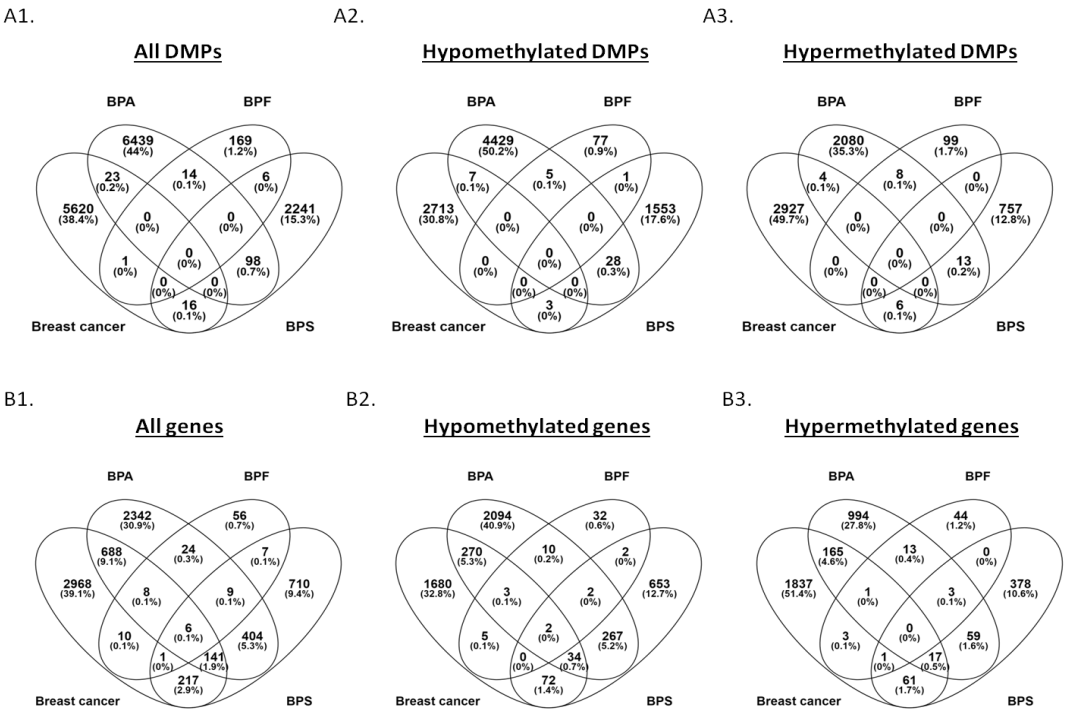


H. Some differentially methylated genes and cancer-related pathways induced by BPA and BPS were also dysregulated in ER-positive breast cancer patients

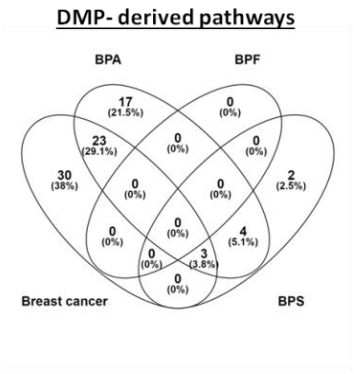
The methylation percentages of 5660 CpGs were statistically significantly different between ER-positive tumor and normal tissues. Aberrations in DNA methylation of 11 CpG sites (7 hypomethylated and 4 hypermethylated) were common with BPA and of 9 CpG sites (6 hypermethylated and 3 hypomethylated) were common with BPS (**Figure 26, Table 14**). There were no similarly dysregulated CpGs between BPF (having the least number of significant DMPs and DMRs) and breast cancer tissues. Of note, there were 309 common hypomethylated genes and 183 common hypermethylated genes with BPA, 108 common hypomethylated genes and 79 common hypermethylated genes with BPS, and only 10 common hypomethylated genes and 5 common hypermethylated genes with BPF. Interestingly, pathway analysis revealed the majority (26/47) of the BPA pathways to be common with those of breast cancer, with pathways in cancer, focal adhesion, cGMP-PKG signaling pathways being common between BPA, BPS and breast cancer **(Figure 26C)**.

Figure 26. Comparison between differentially methylated probes (DMPs) and genes encompassing DMPs in MCF-7 cells treated for 48hrs with functional concentrations of BPA, BPF and BPS in MCF-7 cells and those of 595 ER-positive tumor tissues when compared to 124 normal adjacent tissues of The Cancer Genome Atlas (TCGA) breast cancer patients

Venn diagrams of DMPs and genes encompassing DMPs of BPA, BPF, BPS and breast cancer are shown in A1-A3 and B1-B3, respectively Pathways of the genes encompassing DMPs of BPA, BPF, BPS and breast cancer were detected based on KEGG pathway database using Enrichr (http://amp.pharm.mssm.edu/Enrichr/) and shown in C1 and C2. BPF was not included in C2, because its DMP-derived genes were not significantly involved in any pathway (as shown in C1).



C1.


DMP- derived pathways
BPA
BPF
17 (21.5%)
0 (0%)
23 (29.1%)
0 (0%)
0 (0%)
30 (38%)
0 (0%)
0 (0%)
2 (2.5%)
0 (0%)
0 (0%)
4 (5.1%)
0 (0%)
3 (3.8%)
0 (0%)
Breast cancer
BPS

C2.

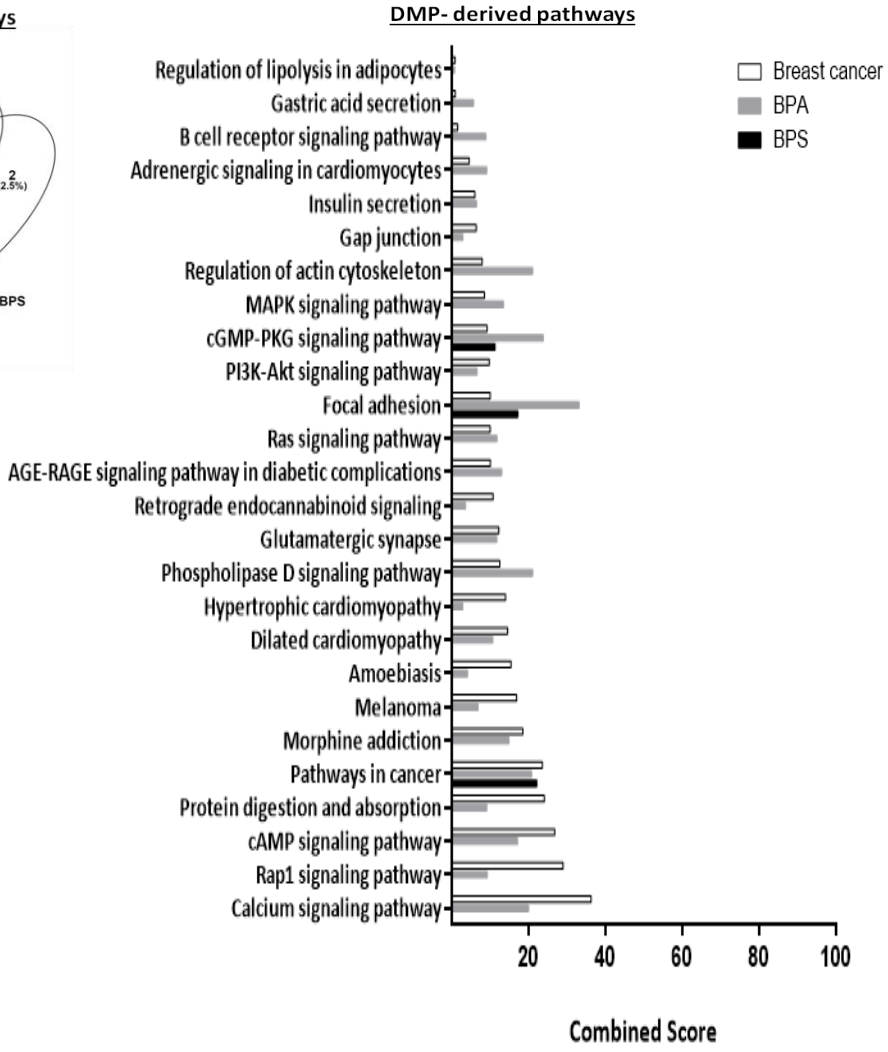

DMP- derived pathways
Breast cancer
BPA
BPS
Regulation of lipolysis in adipocytes
Gastric acid secretion
B cell receptor signaling pathway
Adrenergic signaling in cardiomyocytes
Insulin secretion
Gap junction
Regulation of actin cytoskeleton
MAPK signaling pathway
cGMP-PKG signaling pathway
PI3K-Akt signaling pathway
Focal adhesion
Ras signaling pathway
AGE-RAGE signaling pathway in diabetic complications
Retrograde endocannabinoid signaling
Glutamatergic synapse
Phospholipase D signaling pathway
Hypertrophic cardiomyopathy
Dilated cardiomyopathy
Amoebiasis
Melanoma
Morphine addiction
Pathways in cancer
Protein digestion and absorption
cAMP signaling pathway
Rap1 signaling pathway
Calcium signaling pathway
20
40
60
80
100
Combined Score

Table 14. CpG sites similarly dysregulated in MCF-7 cells treated for 48 hrs with functional concentrations of BPA and BPS when compared to control and in 595 ER-positive tumor tissues when compared to 124 normal adjacent tissues in breast cancer patients from The Cancer Genome Atlas (TCGA) database

CpG sites	$\Delta ß$ ($ß_{tumor}$ - $ß_{normal}$)	$\Delta ß$ ($ß_{BPA}$ - $ß_{control}$)	Methylation change	Gene	Gene name
Common CpG sites between BPA and breast cancer					
cg10861751	-21.53	-4.51	Hypo	*RGS1*	*Regulator of G protein signaling 1*
cg09147827	-18.09	-11.34	Hypo	*SERPINA6*	*Serpin Family A Member 6*
cg02097420	-16.85	-5.36	Hypo	*HRG*	*Histidine Rich Glycoprotein*
cg09448875	-16.85	-5.55	Hypo	*ABCC2*	*ATP Binding Cassette Subfamily C Member 2*
cg10818284	-9.62	-8.04	Hypo	*SYP*	*Synaptophysin*
cg00077877	-8.39	-6.11	Hypo	*ASAP1*	*ArfGAP With SH3 Domain, Ankyrin Repeat And PH Domain 1*
cg26775866	-7.11	-7.87	Hypo	*PTTG1*	*Pituitary Tumor-Transforming 1*
cg24719601	9.64	3.61	Hyper	*PHOX2B*	*Paired Like Homeobox 2b*
cg22411207	9.99	4.63	Hyper	*MOS*	*MOS Proto-Oncogene, Serine/Threonine Kinase*
cg18793806	15.34	4.34	Hyper	*ZNF514*	*Zinc Finger Protein 514*
cg17020834	34.34	3.26	Hyper	*GRIA1*	*Glutamate Ionotropic Receptor AMPA Type Subunit 1*
Common CpG sites between BPS and breast cancer					
cg01078434	-27.97	-3.80	Hypo	*MAS1L*	*MAS1 Proto-Oncogene Like, G Protein-Coupled Receptor*
cg09096383	-19.94	-13.02	Hypo	*CSN1S1*	*Casein Alpha S1*
cg00474004	-17.97	-3.95	Hypo	*IFNA14*	*Interferon Alpha 14*
cg06243556	7.02	4.32	Hyper	*ZSCAN18*	*Zinc Finger And SCAN Domain Containing 18*
cg00174901	15.77	7.10	Hyper	*PALM*	*Paralemmin*
cg21672276	18.40	9.71	Hyper	*ZNF502*	*Zinc Finger Protein 502*
cg12876594	20.06	3.28	Hyper	*NPR2*	*Natriuretic Peptide Receptor 2*
cg14614211	22.52	4.59	Hyper	*MKX*	*Mohawk Homeobox*
cg01580681	27.10	6.88	Hyper	*HAND2*	*Heart And Neural Crest Derivatives Expressed 2*

Hypo: hypomethylated; hyper: hypermethylated; ß: methylation percentage

Note that no CpG site was similarly dysregulated between BPF and breast cancer, so BPF is not included in the table.

CHAPTER V

DISCUSSION

PART I. EPIDEMIOLOGICAL STUDIES

A. BPA and RTL (H1)

We are the first to investigate the association between human BPA exposure and RTL alteration. We showed that higher urinary BPA levels adjusted for creatinine are associated with shorter RTL in peripheral blood of women. In the literature, several studies showed that BPA exposure was associated with DNA damage, increased telomerase activity, exacerbated oxidative stress and increase cell proliferation (Gassman, 2017; Macczak et al., 2017; Takahashi et al., 2004). As such, the direction of change in RTL seems to be determined by factors that increase telomere length such as increased telomerase expression, and by factors that decrease telomere length such as increased oxidative stress and cellular proliferation (von Zglinicki, 2002), with the dominating factors determining the direction of change in RTL. Our results together with the findings in the literature suggest that RTL may play a role in the BPA-associated health hazards. Nevertheless, more studies are required to confirm our results.

B. BPA and *LINE-1* methylation (H2)

Although there are several cell culture and animal studies investigating the DNA methylation effects of BPA, literature about the epigenetics of BPA in humans is limited (Luísa Camacho & Pogribny, 2017). To date, few studies investigated whether human BPA exposure is associated with DNA methylation alterations (Faulk et al., 2015; Hanna et al., 2012; J. H. Kim et al., 2013; Miao et al., 2014), one of which showed BPA exposure to be associated with that *LINE-1* hypomethylation in sperm but not blood samples of male factory workers (Miao et al., 2014). Hence, our study is the first to investigate the association between urinary BPA levels and *LINE-1* methylation in women. Similarly to the study performed on blood samples from men (Miao et al., 2014), our results showed that there is no association between BPA exposure and peripheral blood *LINE-1* methylation. Nevertheless data emanating from that same study (Miao et al., 2014) and another cell culture evaluation in breast cancer cells (MCF-7) (Mine Senyildiz, 2015) point to the possibility that BPA-related methylation effects are potentially tissue specific.

C. RTL and breast cancer (H3&H5)

Clinical studies on the association between RTL in blood and breast cancer showed inconsistent results, which may stem from differences in ethnic and genetic background, detection method and clinicopathologic characteristics of breast cancer patients (De Vivo et al., 2009; Martinez-Delgado et al., 2011; A.J. Pellatt et al., 2013; Y. L. Zheng et al., 2010). Essentially, to date there are four reports on the association between RTL in tissues and breast cancer risk. Similarly to our findings, three of these revealed an association between breast cancer and shorter telomeres, while the fourth study showed

that RTL was shorter in patients with early stage breast cancer and longer in those with advanced breast cancer (Looi et al., 2010; A.K. Meeker et al., 2004; Rashid-Kolvear et al., 2007; Thriveni et al., 2018). All these findings are consistent with ours, since all breast cancer patients in our study were non-metastatic and the majority had low stage and grade disease.

D. *LINE-1* methylation and breast cancer (H4&H6)

In the literature, several studies investigated the association between *LINE-1* methylation in blood and tissues with cancer risk and showed inconsistent results probably due to differences in study design and detection methods; hence, more studies are warranted in this field. The majority showed no association between *LINE-1* methylation and breast cancer (Brennan et al., 2012; Cho et al., 2010; Choi et al., 2009; Deroo et al., 2014; Kankava et al., 2018; Kitkumthorn, Tuangsintanakul, Rattanatanyong, Tiwawech, & Mutirangura, 2012; Tang et al., 2016; Wu et al., 2012; X. Xu et al., 2012). Our results showed that *LINE-1* hypermethylation is statistically significantly higher in peripheral blood of breast cancer patients when compared to controls, a finding that was not replicated in breast tissues. We also showed no association between *LINE-1* methylation in tissues and grade and stage of breast cancer patients similar to findings of Kankava *et al.* (2018) study (Kankava et al., 2018). Moreover, our results showed that *LINE-1* hypermethylation is associated with positive status of PR but not HER2. These results were in line with the reported strong association of PR positive status with higher methylation % of several promoters, and the modest association of HER2 receptor status with whole genome DNA methylation;

suggesting different molecular targets and pathways pertaining to different subtypes of breast cancer (Benevolenskaya et al., 2016; L. Li et al., 2010).

E. Limitations

Our data are cross-sectional and do not permit evaluation of causality. The study design is also limited by the potential variability of BPA levels over time; thus, it does not allow for longitudinal assessment of the association of BPA exposure with *LINE-1* methylation and RTL. A major limitation is the lack of BPA levels in breast cancer patients, so direct association between BPA and breast cancer development is not possible. Besides, our study suffers from its relatively small sample size, and it should hence be considered exploratory. Additional limitations include the drawback of using blood samples since telomere length and *LINE-1* methylation may depend on blood cellular composition (Lin et al., 2016; Wu et al., 2011). Had the methylome-wide data been available, these could have been used for prediction of the blood cell composition and we could have adjusted for them later in the analysis (Waite et al., 2016). Nevertheless, in the literature, there was a correlation in telomere length between different peripheral blood mononuclear cells within the same individual (Lin et al., 2016), and the likelihood that breast cancer cases had different blood counts than controls is minimal since blood was withdrawn prior to cytotoxic therapy. In addition, although we used pyrosequencing which is the gold standard for detection of methylation of candidate genes (Akika, Awada, Mogharbil, & Zgheib, 2017), *LINE-1* methylation results may depend on the detected CpG sites (Nelson, Marsit, & Kelsey, 2011). Finally, although we adjusted the results for BMI, smoking, and alcohol consumption, there may be additional relevant confounding factors that should have

been accounted for such as physical activity or supplements' intake such as folate intake (Marques-Rocha et al., 2016).

F. Conclusions

Despite its limitations, we are the first to show that non-occupational human BPA exposure is associated with shorter RTL and is not associated with *LINE-1* methylation in females. The association of *LINE-1* methylation and RTL with breast cancer development also adds to the growing literature, based on a target population in which the outcome is highly prevalent. Unlike *LINE-1* which was significantly hypermethylated only in blood of breast cancer patients, RTL was shorter in both blood and tumor tissues of breast cancer patients when compared to their corresponding controls. Hence, BPA exposure was associated with shorter RTL which was also linked to breast cancer, and shorter RTL could be predictive of both BPA exposure and breast cancer. Our results however should be validated in a larger cohort. Further investigations including longitudinal assessment of BPA exposure with breast cancer risk and changes in RTL and *LINE-1* methylation are also warranted.

PART II. CELL CULTURE STUDIES

Our results showed that BPA and its analogues induce ER-dependent increases in cell proliferation, migration and S-G2/M cell cycle proportions in MCF-7 cells, all of which represent phenotypic markers of cancer promotion. The functional and exposure concentrations at which these effects were observed were identical between BPA and

BPF but one order of magnitude higher for BPS, consistent with previous studies showing that the estrogenic potency of BPS is around 10 times less than BPA and BPF (Rochester & Bolden, 2015; Rosenmai et al., 2014). While only few cell culture studies showed that BPF (Pisapia et al., 2012; Stroheker et al., 2004) and BPS (J. Y. Kim et al., 2017) increase the proliferation of breast cancer cell lines (MCF-7 or MCF-7 CV), several showed that BPA increases the proliferation of these same cell lines (H. S. Lee et al., 2014; Pfeifer et al., 2015; Pisapia et al., 2012; Qin et al., 2012; Ricupito et al., 2009; H. Song et al., 2015; Stroheker et al., 2004; W. Zhang et al., 2012) and few investigated whether this increase is ER-dependent (J. Y. Kim et al., 2017; H. S. Lee et al., 2014). We also observed that the functional concentration of the three bisphenols increased the percentage of cells in the G2/M and S phases in MCF-7 cells after 24hrs, and this was also shown in the literature for BPA and BPF (Pisapia et al., 2012). Similar to our results, two studies showed that BPA increased the cell migration of ER-positive breast cancer cell lines (J. Y. Kim et al., 2017; G. A. Lee et al., 2017). However, in the literature, only one study tested the effects of BPF and BPS on cell migration of breast cancer cells and showed that, similarly to BPA, BPF and BPS increased cell migration and altered the expression of epithelial to mesenchymal transition markers in MCF-7 CV cells (J. Y. Kim et al., 2017). As expected, we did not observe any change in the cell metabolic activity, viability and migration of ER-negative MDA-MB-231 cells consistently with other studies which observed no effects of BPA on the proliferation of these cells (Stroheker et al., 2004; W. Zhang et al., 2012).

A. Telomerase-linked mechanisms

hTERT promoter contains an ERE that is activated by ligand-bound ER, so few investigators studied the effects of EDCs on *hTERT* expression; as such, BPA increased *hTERT* expression in human cervical, lung and breast cancer cells (Takahashi et al., 2004), and telomerase activity in hepatoblastoma cells (B. L. Xu et al., 2015). In the literature, the effects of BPF and BPS on telomerase expression and RTL were not studied. Our results showed that similarly to BPA, BPF and BPS increase telomerase activity in MCF-7 cells. However our results showed that the three bisphenols are not associated with prolongation in RTL. This may be attributed to the short exposure period or to other factors associated with telomere attrition, such as increased proliferation or enhanced oxidative stress (Macczak et al., 2017; von Zglinicki, 2002). Of interest, clinical studies showed that higher telomerase expression was associated with worse histopathological characteristics and poorer prognosis in breast cancer patients (Hoos et al., 1998; Mokbel et al., 2000). Hence, the increased telomerase expression may be a marker of enhanced cancer progression of MCF-7 cells.

B. DNA methylation

Our results showed that the functional concentrations of the three bisphenols are associated with a trend towards *LINE-1* hypomethylation in MCF-7 cells. However, taking into account that *LINE-1* is a repetitive sequence that comprises around 17% of the genome (Baba et al., 2018), a decrease of only 1% in *LINE-1* methylation is considered biologically relevant. Global DNA hypomethylation has been associated

with cancer development (Barchitta et al., 2014) including breast cancer (Delgado-Cruzata et al., 2012; Kuchiba et al., 2014). In the literature, global DNA hypomethylation was reported in MCF-7 cells after 48hrs of treatment with 10^{-7}M and 10^{-6}M BPA using 5-mC Elisa kit, but no changes were reported in the same cell line treated for 5 weeks with 10^{-5}M and 10^{-6}M BPA using the "gold standard" HPLC-MS assay (Mine Senyildiz, 2015; Wang et al., 2018). Discrepancies in results might be attributed to different treatment duration or measurement methods. Besides, a recent study showed hypermethylation in two transposons (*MaLR* and *Mariner-2*) out of eight analyzed in MCF-7 cells treated with 10^{-6}M BPS for 24hrs (Huang et al., 2019). However, no study measured *LINE-1* methylation in breast cell lines treated with any of BPA, BPF or BPS.

Genome-wide DNA methylation revealed that the three bisphenols significantly altered the DNA methylation of several CpG sites and CpG regions that were located mostly in promoters and exons, yet they showed minimal degree of overlap. In both DMP and DMR analyses, BPA was associated with the largest number of DMPs and DMRs followed by BPS then BPF; hence, BPA has the strongest effect on the DNA methylome compared to BPF and BPS. This may be due to concentration-dependent alterations in DNA methylation; hence, higher concentrations of BPF should also be evaluated. Noteworthy, the majority of DMPs and DMRs altered by bisphenols were ER-dependent. In the literature, only one methylome-wide analysis was performed with MCF-7 cells treated for 5 weeks with 10^{-5}M and 10^{-6}M BPA using the older generation Infinium Human Methylation450 BeadChip arrays, and showed that both concentrations of BPA induce hypermethylation in tumor suppressor genes and hypomethylation in oncogenes (Wang et al., 2018). Out of the reported 32

hypomethylated genes and 45 hypermethylated genes with both concentrations of BPA, three genes were similarly hypomethylated (*ZNF423*, *SYT4*, *IMMP2L*) and five genes were similarly hypermethylated (*THSD4*, *SETBP1*, *NTM*, *HLA-DRB1*, *AFF1*) with our 48hr-treatment with BPA. No epigenome-wide study has so far been reported for BPF and BPS in breast cells.

The potential effect of the three bisphenols on the expression of DNA methylation enzymes in breast cells has not been previously addressed in the literature. However, BPA showed tissue-specific alterations in *DNMT* gene expression in several tissue types other than the breast (Doshi et al., 2011; Patel, Raad, Sebag, & Chalifour, 2013). Our results showed that although the three bisphenols induce ER-dependent increase in *DNMT1* gene expression and induce differential ER-dependent effects on *TET (2 and 3)* gene expression levels, these alterations barely exceeded the commonly used two-fold change limit and were not translated into alterations in enzymatic activity. Hence, we propose that the DNA methylation aberrations induced by the three bisphenols in MCF-7 cells were potentially mediated by changes in signaling pathways (including the ER) without directly affecting enzymatic activities of DNMTs/TETs.

Pathway analysis revealed that the genes encompassing differentially methylated probes by BPA and BPS were involved in seven common pathways whereby focal adhesion, cGMP-PKG signaling and cancer pathways had the highest score. However, those of BPF were not statistically significantly involved in any pathway. All of the pathways dysregulated by BPS were ER-dependent; yet, roughly half of the pathways dysregulated by BPA were ER-dependent including cAMP, MAPK, estrogen and Wnt signaling pathways. Of note, two other tools (Go Biological and WikiPathways)

showed that genes differentially methylated by BPA and BPS were involved in Wnt signaling cascade (data not shown), a pathway that was also obtained by the KEGG database for DMRs of BPA. This pathway controls development and stemness, and has been tightly linked to cancer development, metastasis and telomerase expression (Ayyanan et al., 2011; Park et al., 2009; Zhan, Rindtorff, & Boutros, 2017).

When comparing the methylation data with those of clinical data, the overlap in the genes encompassing the DMPs was the highest with BPA and the least with BPF. As a matter of fact, pathway analysis using KEGG revealed that the discovered top three common pathways between BPA and BPS with the highest combined score, namely focal adhesion, cGMP - PKG signaling and cancer pathways, were also common with breast cancer.

C. Limitations

The study is limited by several factors related to the choice of cells and treatment duration. The major limitation was that our cell culture assays were performed on breast cells that are already cancerous, so we could not test for the carcinogenic effects of bisphenols in the breast. Hence, similar experiments on normal-like breast epithelial cells are warranted. Moreover, although we exposed the MCF-7 cells to human exposure concentrations of BPA, BPF and BPS, some of the molecular changes and epigenetic aberrations were observed at larger concentrations. However, treatment duration was only two days compared with a lifetime exposure to BPA, BPF and BPS. Additionally, the levels of BPA, BPF and BPS vary between different age groups and

different human compartments; for instance, BPA levels were higher in children than in adults and concentrated in human placental and fetal liver tissues (Becker et al., 2009; X. L. Cao et al., 2012). It is essential to take into consideration that many papers reported a high occupational BPA exposure, whereby BPA manufacturers had urinary and semen BPA levels approaching the functional concentration we used and sometimes even higher (Ribeiro et al., 2017). Notably, we fulfilled several of the recommended guidelines concluded in a recent review on epigenetics of BPA in regards of use of multiple concentrations, multiple time points and integration of epigenetic data with other molecular and phenotypic readouts (Luísa Camacho & Pogribny, 2017).

D. Conclusions

Despite these limitations, our results were the first to elucidate the telomerase and epigenetic-linked mechanisms of BPF and BPS in human carcinoma cells and compare them to those of BPA. Similarly to other studies, we showed ER-dependent cancer promoting effects of the three bisphenols in breast cancer cells, with BPS being 10 times less potent than BPA and BPF in its cell proliferation effects. However, at equipotent concentrations, the three bisphenols induced similar ER-dependent increase in the telomerase expression/activity, and showed differential DNA methylation alterations which were likely not mediated by effects on DNA (de)methylation enzymes. BPA had the strongest effect on the DNA methylome, followed by BPS then BPF, and the majority of bisphenol-induced DNA methylation alterations were dependent on ER pathway. Differentially methylated genes by BPA and BPS were

involved in focal adhesion, cGMP-PKG and cancer pathways (including several cancer pathway subsets), which were also dysregulated in ER-positive breast cancer tumor tissues. DNA methylation aberrations induced by BPA and BPS were also involved in Wnt signaling that is positively linked to telomerase expression. We conclude that the three bisphenols have important epigenetic and cancer promoting effects in breast cell lines, overlapping with cancer related pathways in clinical breast cancer models, hence, warranting further investigation regarding the safety of BPA derivatives.

CHAPTER VI

CONCLUSIONS

Despite that in the epidemiological part, our results showed that human BPA exposure was associated with shorter RTL in peripheral blood of non-breast cancer individuals, which was also associated with breast cancer risk in both blood and tissues of breast cancer patients, BPA was not associated with shorter RTL in MCF-7 breast cancer cell lines. This may be attributed to the short treatment duration in cell lines (24 and 48hrs) as compared to the lifetime exposure to BPA in humans.

As for *LINE-1* methylation, it was not associated with BPA exposure in both epidemiological and cell culture settings, and although it was hypermethylated in peripheral blood of breast cancer patients when compared to non-breast cancer patients, no statistically significant differences were observed between breast cancer and normal adjacent tissues.

Additionally, in the cell culture part, we showed that the three bisphenols induce ER-dependent cancer promoting effects in breast cancer cells, with BPS being 10 times less potent than BPA and BPF in its cell proliferation effects. At concentration s associated with similar cancer promoting effects (equipotent concentrations), the three bisphenols increased the expression and activity of telomerase that was also reported in breast cancer patients of high stage and grade in the literature. However, they differentially altered the DNA methylation of several genes with BPA exerting the strongest effect on the DNA methylome, followed by BPS then BPF. Comparisons of the DNA

methylome-wide results of bisphenol-treated MCF-7 cells with clinical data on ER-positive breast cancer patients revealed that those of BPA and BPS are also differentially methylated in ER-positive breast cancer tissues when compared to normal adjacent ones. The increase in the expression and activity of telomerase and the majority of DNA methylation changes were ER-dependent.

Our results went a step further in elucidating the epigenetic and telomerase-linked mechanisms of BPF and BPS in comparison to BPA, the contribution of ER pathway in these mechanisms, and their overlap with aberrations occurring in breast cancer patients. Further studies are required using multiple concentrations of BPF and BPS and longer time points in order to enhance our understanding of the safety of the three bisphenols. Similar assays on normal-like breast epithelial cells are also warranted to determine the carcinogenic potential and mechanisms of the BPA analogues in comparison to BPA. In order to determine the causality between bisphenols and breast cancer, more cell culture experiments performed on manipulated cell-line models (with knock-out of genes of interest), as well as animal and prospective epidemiological studies are warranted with particular focus on the genes whose DNA methylation was found dysregulated in the bisphenol-treated cells and TCGA breast cancer patients (such as the proto-oncogenes ASAP1 and PTTG1 and the tumor suppressor genes NPR2 and HAND2) in an attempt to determine biomarkers predictive of both bisphenol exposure and breast cancer. Moreover, studying the expression of telomere-binding proteins such as telomere repeat-binding factors (TRF1 and TRF2) and genetic variants in telomere pathway genes (*TERT* and *POT1*), and stratifying the results on the basis of the clinicopathological types and molecular subtypes of breast cancer might further unravel biomarkers specific to both bisphenol exposure and to particular breast cancer types.

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