

1 **Transcriptome profiling reveals bisphenol A alternatives**
2 **activate estrogen receptor alpha in human breast cancer cells**

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10

1 **Abstract**

2 **Background:** Plasticizers with estrogenic activity, such as bisphenol A (BPA), have been
3 reported to have potential adverse health effects in humans. Due to mounting evidence of
4 these health effects and public pressure, BPA is being phased out by the plastics
5 manufacturing industry and replaced by other bisphenol variants in “BPA-free” products.

6 **Objectives:** We have compared estrogenic activity of BPA to 6 bisphenol analogues
7 (bisphenol S, BPS; bisphenol F, BPF; bisphenol AP, BPAP; bisphenol AF, BPAF; bisphenol
8 Z, BPZ; bisphenol B, BPB) in three human breast cancer cell lines.

9 **Methods:** Estrogenicity was assessed by cell growth in an estrogen receptor (ER)-mediated
10 cell proliferation assay, and by the induction of estrogen response element (ERE)-mediated
11 transcription in a luciferase assay. Gene expression profiles were determined in MCF-7
12 human breast cancer cells by microarray analysis and confirmed by Illumina-based RNA
13 sequencing.

14 **Results:** All bisphenols showed estrogenic activity in promoting cell growth and inducing
15 ERE-mediated transcription. BPAF was the most potent bisphenol, followed by BPB > BPZ ~
16 BPA > BPF ~ BPAP > BPS. The addition of ICI 182,780 antagonized the activation of ERs
17 by bisphenols. Data mining of ToxCast high-throughput screening assays confirms our
18 results but also shows divergence in the sensitivities of the assays. The comparison of
19 transcriptome profile alterations resulting from BPA alternatives with an ER α gene
20 expression biomarker further indicates that all BPA alternatives act as ER α agonists in MCF-
21 7 cells. These results were confirmed by RNA sequencing.

22 **Conclusion:** In conclusion, BPA alternatives are not necessarily less estrogenic in a human
23 breast cancer cell model. Three bisphenols (BPAF, BPB, and BPZ) were more estrogenic
24 than BPA. The relevance of human exposure to BPA alternatives in hormone-dependent
25 breast cancer risk should be investigated.

1 Introduction

2 Plasticizers such as bisphenol A (BPA) have been reported to have potential adverse
3 health effects in humans, including reproductive endocrine disorders and neurobehavioral
4 problems (Rochester, 2013). BPA is one of the best-studied endocrine disrupting chemicals
5 (EDCs), with more than 75 out of 91 published studies showing associations between BPA
6 exposure and human health as of May 2013 (Rochester, 2013). The primary mode of action
7 of BPA is the activation of estrogen receptor (ER) mediated transcription (Shioda *et al.*,
8 2013). Biomonitoring studies suggest that the urine from a majority of individuals in
9 industrialized countries contain measurable BPA and metabolites (Gerona *et al.*, 2016). BPA
10 is detected in breast milk (Nakao *et al.*, 2015). A causal link between BPA and breast
11 cancer remains equivocal because epidemiological studies have reported conflicting results
12 (Rochester, 2013). Nonetheless, there is ample evidence from laboratory rodent and non-
13 human primate studies that prenatal exposure to BPA may increase the propensity to
14 develop breast cancer during adulthood (Mandrup *et al.*, 2016; Tharp *et al.*, 2012). In these
15 studies, low-doses of BPA has been shown to affect mammary gland development by
16 causing hyperplastic lesions in rats, which are parallel to early signs of breast neoplasia in
17 women (Mandrup *et al.*, 2016). These findings suggests that mammary tumors provoked by
18 BPA may be initiated in the womb, and thus more prospective studies measuring BPA in
19 utero are needed in order to conclude there is a carcinogenic potential for humans. Due to
20 mounting evidence of harm and public pressure, BPA is being phased out by plastics
21 manufacturers and is being replaced by other BP variants in “BPA-free” products (Rochester
22 and Bolden, 2015).

23 Bisphenol F (BPF), Bisphenol S (BPS), and Bisphenol AF (BPAF) are among the
24 main substitutes of BPA in polycarbonate plastics and epoxy resins (Chen *et al.*, 2016). They
25 are commonly found in baby feeding bottles, on thermal receipt papers, glues, dental
26 sealants, food packaging or personal care products. There is a strong negative correlation
27 between the levels of BPA and BPS on thermal paper, whereby the papers containing high
28 quantities of BPS have low quantities of BPA, suggesting that BPS has in part replaced BPA
29 in this context (Liao *et al.*, 2012). The handling of a thermal receipt paper before eating
30 French fries is sufficient to cause a rapid increase of serum BPA levels within 30 min
31 (Hormann *et al.*, 2014). Epidemiological data on the human body burden of different BPA
32 alternatives is very limited, but evidence suggests that they are generally widespread. In an
33 investigation of the presence of BPS and bisphenol F (BPF) in human urine, these
34 compounds were found to be present in 78% and 55% of respective samples in the United
35 States (Zhou *et al.*, 2014). Another investigation revealed that although the average urinary

1 concentration of BPA in 130 individuals from Saudi Arabia was 4.92 ng/mL, 7 other
2 bisphenols were found with the total bisphenol concentration reaching 19 ng/mL
3 (Asimakopoulos *et al.*, 2016), suggesting that BPA is only one of many BP alternatives that
4 are found in humans.

5 Some of these BPA replacements are structurally related to BPA and have endocrine
6 disrupting effects. Numerous studies have suggested that BPS and BPF have potencies
7 similar to that of BPA resulting in endocrine disrupting effects such as uterine growth in
8 rodents (Rochester and Bolden, 2015). A number of in vitro studies have looked at
9 endocrine mode of action of some BPA alternatives in nuclear hormone receptor interactions
10 (Chen *et al.*, 2016). The range of EC50 values for estrogen activity (0.02 μ M to >1000 μ M)
11 and androgen activity (0.29 μ M to >1000 μ M) of 20 BP congeners (5 of which are being
12 tested here) indicates remarkable differences (Kitamura *et al.*, 2005). Another study showed
13 that 5 BPA alternatives (3 studied here) exhibited potencies within the same range as BPA
14 on steroidogenesis, androgen receptor, aryl hydrocarbon receptor, and retinoic acid receptor
15 activity (Rosenmai *et al.*, 2014). Some BPA alternatives have also been tested in ER high-
16 throughput screening assays in the context of the US Environmental Protection Agency
17 (EPA) ToxCast program and shown in some cases to have estrogen-like effects (Judson *et*
18 *al.*, 2015).

19 Perturbation of the gene expression profile of a cell by chemicals results in unique
20 signatures, allowing comparisons between transcriptome alterations caused by drugs and
21 diseases to predict therapeutic and off-target effects (Iorio *et al.*, 2010). The clinical utility of
22 gene expression profiles is exemplified by studies showing that transcriptome signatures
23 may affect clinical decision-making in breast cancer (Rhodes and Chinnaiyan, 2005). Gene
24 expression have been shown to be a more powerful predictor of breast cancer survival than
25 standard systems based on clinical and histologic criteria (van de Vijver *et al.*, 2002).
26 Exposure of breast cancer cells to BPA present a gene expression profile of tumor
27 aggressiveness associated with poor clinical outcomes for breast cancer patients (Dairkee *et*
28 *al.*, 2008). While ER activation drives two-thirds of breast cancer (Green and Carroll, 2007),
29 there have been no studies aimed at elucidating transcriptome alterations underlying
30 estrogenic effects of a broad range of BPA alternatives to which human populations are
31 exposed. In order to address this deficiency, we studied alterations in gene expression
32 profiles of estrogen-dependent MCF-7 human breast cancer cells caused by BPA and 6
33 alternatives (Figure 1) using Affymetrix microarray and Illumina RNA sequencing platforms.
34 These BPA alternatives are detected in different categories of food items (Liao and Kannan,
35 2013) and are found in human biological fluids (Asimakopoulos *et al.*, 2016). The
36 transcriptome signatures obtained were compared to a gene expression biomarker, which

1 has been demonstrated to accurately predict ER modulation, including by “very weak”
2 agonists (Ryan *et al.*, 2016). We also used an E-screen assay (cell proliferation in estrogen-
3 responding cells) as well as an estrogen response element (ERE)-luciferase reporter gene
4 assay system. Our results clearly demonstrate that all 6 BPA alternative compounds are
5 estrogenic. Alterations of MCF-7 transcriptome profiles caused by BPA alternatives bear the
6 signature of ER activation with three bisphenols (BPAF, BPB, BPZ) having more estrogenic
7 potency than BPA.

8

9 **Methods**

10 **Cell culture and treatments**

11 All reagents and chemicals, unless otherwise specified, were of analytical grade and
12 purchased from Sigma-Aldrich (UK). MCF-7, MDA-MB-231 and T47D human breast cancer
13 cell lines were a gift from Prof. Joy Burchell (Research Oncology Department, King’s College
14 London, UK). The T47D-KBluc cells were purchased from the American Type Culture
15 Collection (ATCC, Teddington, UK) and harbour a stably integrated copy of a luciferase
16 reporter gene under control of a promoter containing EREs. All cells were grown at 37°C
17 (5% CO₂) in 75 cm² flasks (Corning, Tewksbury, USA) in a maintenance medium composed
18 of phenol red free DMEM (Life Technologies, Warrington, UK), 10% fetal bovine serum
19 (FBS; GE Healthcare Life Sciences, Buckinghamshire, UK), 2mM glutamine (GE Healthcare
20 Life Sciences) and 10 µg/ml penicillin/streptomycin (Life Technologies). Test substances
21 were diluted in 100% ethanol to prepare stock solutions. All treatments were prepared in a
22 test medium containing phenol red free DMEM, 5% charcoal stripped FBS (Life
23 Technologies), 2mM glutamine (GE Healthcare Life Sciences) and 10 µg/ml
24 penicillin/streptomycin (Life Technologies). The solvent control contained 0.1% (v/v) absolute
25 ethanol diluted in test medium. Cells were detached from the flask substrate using 0.05%
26 trypsin EDTA (Life Technologies) and counted using a haemocytometer prior to seeding.
27 After a 24 h recovery period in DMEM maintenance medium tests were applied.

28

29

30 **E-Screen assay**

31 The E-screen assay allows the determination of estrogenic effects by determining
32 estrogen receptor-mediated cell proliferation in hormone-responsive cells. This assay was
33 performed as originally described (Soto *et al.*, 1995), except that the bioassay was
34 terminated using a MTT assay, indirectly measuring cell number by testing the activity of
35 mitochondrial succinate dehydrogenase, in order to assess cell proliferation. Briefly, cells

1 were seeded into 48-well plates (Dutscher Scientific, Brentwood, UK) at a density of 20,000
2 cells per well in 250 μ l maintenance medium. Following a 24-hour incubation to allow cell
3 attachment, medium was changed to include various concentrations of treatment
4 substances. The test medium was refreshed after 3 days. After another 3 day incubation, an
5 MTT assay was performed as follows. Cells were incubated with 250 μ l of MTT solution (1
6 mg/ml) for 2 hours and the test terminated by lysing the cells with dimethyl sulfoxide
7 (DMSO). The optical density of the cell lysate was measured at 570 nm using the
8 SPECTROstar Nano plate reader (BMG Labtech, Ortenberg, Germany). The cell
9 proliferative effect was expressed as a percentage of the control, untreated samples.

10

11 **ERE-mediated luciferase reporter gene assay**

12 The ERE-mediated transcription of a luciferase reporter gene (3xERE) was
13 determined in the T47D-KBluc cells (Wilson *et al.*, 2004) using the Steady-Glo[®] luciferase
14 assay system following the manufacturer's instructions (Promega, Southampton, UK). The
15 T47D-KBluc cells were seeded in white 96 well plates (Greiner Bio-One, Germany) at a
16 density of 20,000 cells per well in 50 μ l of maintenance medium and allowed to attach
17 overnight. An initial 24 h incubation was performed in the absence of test substances to
18 purge cells of hormone residues in order to improve estrogen deprivation. Test substances
19 were added and plates were incubated for another 24 hours before the addition of 50 μ l
20 Steady-Glo[®] luciferase reagent. The plates were left to stand for 10 minutes in the dark at
21 room temperature to allow cell lysis and establishment of the luciferase reaction.
22 Luminescence was measured using the Orion II microplate luminometer (Berthold Detection
23 Systems, Bad Wildbad, Germany). Estrogen receptor modulation was confirmed by
24 measuring the inhibitory effect of ICI 182,780 on the concentration of BPA resulting in a 20-
25 fold increase in luciferase activity.

26

27 **Microarray gene expression profiling**

28 MCF-7 cells were seeded into 96-well plates with maintenance medium at a density
29 of 20,000 cells per well. After 24 h of steroid hormone deprivation in hormone-free medium,
30 the cells were stimulated with test substances for 48 h in triplicate in three independent
31 experiments. RNA extraction was performed using the Agencourt RNAdvance Cell V2 kit
32 according to the manufacturer's instructions (Beckman Coulter Ltd, High Wycombe, UK).
33 The samples were checked for RNA quality using the Agilent 2100 Bioanalyzer (Agilent
34 Technologies LDA UK Limited, Stockport, UK) and quantified using the Nanodrop ND-1000
35 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The RNA integrity number
36 (RIN) was 9.7 ± 0.3 . Subsequently, triplicate samples which passed quality control

1 (QC) criteria were pooled appropriately such that the final input amount of each sample was
2 3 ng.

3
4 The gene expression profiles were determined using the Affymetrix Human
5 Transcriptome 2.0 Array as follows. Single Primer Isothermal Amplified (SPIA) cDNA was
6 generated using the Ovation Pico WTA System V2 kit (Nugen, AC Leek, The Netherlands)
7 following the manufacturer's instructions. In addition, the SPIA cDNA was subjected to a QC
8 check to assess quality (Agilent 2100 Bioanalyzer) and quantity (Nanodrop ND-1000
9 Spectrophotometer) in preparation for the next stage. The SPIA cDNA was fragmented and
10 biotin-labelled using the Encore Biotin Module (Nugen) according to the manufacturer's
11 instructions. The fragmented and biotin-labelled cDNA was subjected to a further round of
12 QC checks to assess fragmentation size (Agilent 2100 Bioanalyzer). Hybridisation cocktails
13 were prepared from the fragmented labelled-cDNA according to Nugen's recommendations
14 and hybridised to the microarrays at 45°C overnight. The arrays were washed and stained
15 using the wash protocol FS450_0001 recommended for Affymetrix Human Transcriptome
16 2.0 Arrays on the GeneChip Fluidics 450 station. Ultimately, the arrays were scanned using
17 the Affymetrix GeneChip Scanner. CEL files were QC assessed in the Expression Console
18 software package (Affymetrix) by using standard metrics and guidelines for the Affymetrix
19 microarray system. Data was imported and normalised together in Omics Explorer 3.0
20 (Qlucore, New York, NY, USA), using the Robust Multi-array Average (RMA) sketch
21 algorithm. These microarray data have been submitted to Gene Omnibus and are accessible
22 through accession number GSE85350.

23

24 **Gene expression profiling by RNA- sequencing**

25 RNA-sequencing was performed by applying Illumina sequencing by synthesis
26 technology as follows. The amount of RNA for each library (100 ng) was a pool made up of
27 33 ng of RNA from each of the replicate wells for each sample. The preparation of the library
28 was done by NEBNext Ultra Directional RNA (New England Biolabs, Hitchin, UK) following
29 the manufacturer's protocol. The amplified library was assessed using the Agilent
30 2100 Bioanalyzer for size and presence of adapter/primers/dimers – sized at ~400bp
31 (including ~130bp adapter). The rRNAs were removed using the rRNA depletion module
32 (New England Biolabs) following the manufacturer's protocol. Libraries were pooled together
33 and sequenced on a HiSeq2500 using a Rapid Run v2 flowcell with on-board clustering in a
34 2x100 paired-end (PE) configuration. BCL files were processed and deconvoluted using
35 standard techniques. The sequencing output FASTQ files contain the sequences for each
36 read and also a quality score. We have analysed the quality scores and other metrics using

1 FASTQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Contamination from
2 rRNA was measured using an alignment script
3 (<http://genomespot.blogspot.co.uk/2015/08/screen-for-rna-contamination-in-rna.html>).
4 Adapter sequences (standard TruSeq LT adapter seq) were removed/trimmed using
5 cutadapt (Martin, 2011). Sequences were then aligned to the genome (hg38 database) using
6 the hierarchical indexing for spliced alignment of transcripts program HISAT2 (Kim *et al.*,
7 2015). BAM files were imported to Qlucore omics explorer, along with the GTF file for known
8 genes in hg38 (downloaded from UCSC). Qlucore normalises data using a method similar to
9 the trimmed mean of M-values normalization method (TMM), which corrects for transcript
10 length and applies a log transformation (Robinson and Oshlack, 2010) and gives values
11 similar to the quantification of transcript levels in reads per kilobase of exon model per
12 million mapped reads (RPKM) (Robinson and Oshlack, 2010), but also incorporates the
13 TMM normalization factor for an improved between-sample normalization.

14 A total of 376.6 million raw reads were obtained (25.1 ± 5.4 million reads per
15 sample). The average value of Q30, representing the probability of an incorrect base call 1 in
16 1,000 times, was above 96%. The GC content (%GC) of the reads was on average 49%. A
17 total of $90.92 \pm 6.54\%$ of the clean reads were mapped onto the human reference genome
18 hg38. Among them, an average of $71.01 \pm 5.82\%$ and $18.25 \pm 3.79\%$ reads align
19 concordantly exactly one time and more than one time, respectively. These RNA-seq data
20 have been submitted to Gene Omnibus and are accessible through accession number
21 GSE87701.

22

23 **ToxCast data mining**

24 We analyzed publically available data from the ToxCast program using the iCSS
25 ToxCast Dashboard (<http://actor.epa.gov/dashboard/>). In the ToxCast program, out of 18 ER
26 assays, 5 measured ERE-mediated transcription and gave results comparable to those of
27 our T47D-Kluc assay. One assay was a single-readout fluorescent protein induction system
28 measuring interaction of ER with ERE at two time points by microscopy technology
29 (OT_ERa_EREGFP_0120 and _0048). Two other assays were reporter gene assays
30 measuring mRNA in HepG2 cells (ATG_ERa_TRANS_up and ATG_ERE_CIS_up). The last
31 two assays measured reporter protein levels in HEK293T (Tox21_ERa_BLA_Agonist_ratio)
32 and BG-1 (Tox21_ERa_LUC_BG1_Agonist) cell lines. Other assays of protein
33 complementation that measure formation of ER dimers and test for activity against ER α
34 (OT_ER_ERaERa_0480 and _1440). Finally, NVS_NR_bER and NVS_NR_hER are
35 radioligand receptor binding assays. Not all assays were performed on all bisphenols. For

1 instance, BPAP was only tested in Attagene assays. In this study, the AC50 value was used
2 as a quantitative measure to reflect the potency of BP alternatives.

3

4 **Comparison of the microarray and RNA-Seq data to the ER biomarker**

5 A rank-based nonparametric analysis strategy called the Running Fisher test
6 implemented within the NextBio database (Illumina, San Diego, CA) was used to compare
7 gene lists derived from the microarray and RNA-Seq data to the ER biomarker characterized
8 earlier (Ryan et al, 2016). The Running Fisher test is a normalized ranking approach which
9 enables comparability across data from different studies, platforms, and analysis methods by
10 removing dependence on absolute values of fold-change, and minimizing some of the
11 effects of normalization methods used, while accounting for the level of genome coverage by
12 the different platforms. The Running Fisher algorithm computes statistical significance of
13 similarity between ranked fold-change values of two gene lists using a Fisher exact test
14 (Kupershmidt *et al.*, 2010). A p-value .was selected as our cutoff for significance based on
15 prior evaluation of the cutoff as predictive of activation of ER (Ryan et al., 2016).

16

17 **Statistical analysis**

18 The concentration required to elicit a 50% response (AC50) was determined using a
19 nonlinear regression fit using a sigmoid (5-parameters) equation calculated with GraphPad
20 software (GraphPad Software, Inc., La Jolla, CA, USA). For the transcriptome analysis, pair-
21 wise comparisons of each tested substance to the negative control were performed using a
22 t-test controlling for batch effects in Omics Explorer 3.0 (Qlucore). Data used for the
23 functional analysis were selected at cut-off p-values < 0.05 with fold-change > 1.2 to study
24 the ER activation signature as previously described (Ryan et al., 2016). Gene and disease
25 ontology were analyzed using the Thomson Reuters MetaCore Analytical Suite version 6.28
26 recognizing network objects (proteins, protein complexes or groups, peptides, RNA species,
27 compounds among others). The p-values are determined by hypergeometric calculation and
28 adjusted using a Benjamini & Hochberg approach. All experiments were performed 3 times
29 in triplicate (n=3).

30

31

32

1 Results

2 *E-screen and ERE-luciferase reporter gene assays*

3 We have studied the estrogenic activities of 7 BP found in foodstuffs and human
4 biological fluids in three human breast cancer cell lines. As an initial investigative step of
5 estrogenic potential, an E-Screen assay was performed using two hormone dependent
6 (MCF-7, T47D) and one hormone independent (MDA-MB-231) human breast cancer cell
7 lines (Figure 2A). The positive control 17 β -estradiol was very potent in inducing the
8 proliferation of MCF-7 cells (AC₅₀ = 8 pM). All BP derivatives were also able to promote cell
9 growth at concentrations 10,000 to 100,000 higher than 17 β -estradiol (Figure 2A, top panel).
10 BPAF was the most potent BP (AC₅₀=0.03 μ M) followed by BPZ (0.11 μ M) > BPB (0.24 μ M)
11 > BPA (0.36 μ M) > BPAP (0.39 μ M) > BPF (0.55 μ M) > BPS (1.33 μ M). The same overall
12 trend was observed with the T47-D cell line (Figure 2A, middle panel), albeit to a lesser
13 extent which can be accounted for by the fact that T47-D cells express lower levels of ER.
14 As expected no cell proliferative effects were observed with the hormone independent, ER-
15 negative MDA-MB-231 cell line suggesting that proliferative effects were mediated by ER
16 (Figure 2A, bottom panel).

17 Since the observed BP-derivative induced proliferation of MCF-7 and T47D cells
18 (Figure 2A and 2B) could be mediated by different hormones, estrogenic effects of BPA
19 alternatives was then investigated employing a luciferase reporter gene assay allowing
20 measurement of ERE-mediated transcription. The results (Figure 2B, upper panel) were very
21 similar to those obtained from the E-Screen assay: BPAF was the most potent bisphenol
22 (AC₅₀ = 0.08 μ M) at stimulating ERE-luciferase reporter gene expression followed by BPB
23 (0.3 μ M) > BPZ (0.4 μ M) ~ BPA (0.4 μ M) > BPF (1 μ M) ~ BPAP (1 μ M) > BPS (1.5 μ M)
24 having estrogenic effects in the same range as BPA. Moreover, the addition of ICI 182,780
25 (100 nM) antagonized the activation of ER by estradiol and bisphenols confirming reporter
26 gene expression via this receptor (Figure 2C). However, estrogenic effects of some BP
27 derivatives, such as BPB and BPZ, were not completely antagonized by 100 nM ICI addition,
28 suggesting estrogenic activation mechanisms independent of ER.

29 ToxCast data mining (Table 1) confirms our results but also reveals some
30 discrepancy with the results of the ToxCast assays. BPF, BPZ and BPB were classified as
31 inactive in the ToxCast gene reporter ERa_BLA_Agonist_ratio assay, because they did not
32 reach the response threshold, possibly due to the lower sensitivity of this assay. BPF was
33 classified as inactive in the majority of ToxCast assays although it has been found to be
34 positive in the EDSP Tier 1 uterotrophic assay (Judson et al., 2015).

1

2 *Transcriptome profiling*

3 MCF-7 cells treated for a period of 48h with BPA, 6 BP alternatives, or 17 β -estradiol,
4 were then subjected to full transcriptome profiling using the Affymetrix microarray platform.
5 The length of exposure (48h) and the concentration of test agent were selected, because
6 previous studies showed that these conditions produce robust gene expression changes
7 (Shioda *et al.*, 2006). Supplementary File 1 shows the statistical significance of differential
8 transcript cluster expression with respective fold-changes by volcano plots. The set of genes
9 altered by treatment with BPA and BP alternatives (Supplementary File 2) were highly
10 enriched in genes involved in the regulation of the cell cycle (Figure 4A), as well as in genes
11 regulated by steroid hormones (Figure 4B). Additionally, an enrichment analysis of MeSH
12 terms reveals that exposure to both BPA or the 6 BP alternatives are significantly enriched in
13 genes involved in the etiology of breast cancer (Figure 4C) such as *ALDH1A3*, *CAMK1D*,
14 *PPARG* or *PGR*. Genes having the highest fold-changes were similar and indicative of a
15 hormone-induced proliferative effect (Table 2). The gene encoding the progesterone
16 receptor (*PGR*) consistently exhibited the highest fold-change after stimulation with BPA or
17 all the BP alternatives. ER binding motifs were overrepresented in the promoter of the
18 differentially expressed genes (DEGs). There were 4.8 to 6.4 more ER binding sequences
19 (EREs) in the promoters of the DEGs than what would be expected by chance (Figure 3D).

20 Statistical values derived from gene ontology analysis can have limited reliability due
21 to the background set including only the genes that are likely to be expressed in the
22 experiment (Tipney and Hunter, 2010). Genes disturbed by chance have a higher probability
23 to be associated with endocrine ontologies in an endocrine sensitive tissue such as the
24 breast. This could result in an increased false positive rate. In order to circumvent this
25 problem, we applied a biomarker approach to predict ER modulation. The gene expression
26 biomarker consists of 46 genes which exhibit consistent expression patterns after exposure
27 to 7 agonists and three antagonists of ER determined using MCF-7 microarray data derived
28 from the CMAP 2.0 dataset (Ryan *et al.*, 2016). This gene expression biomarker has a
29 balanced accuracy for prediction of ER activation of 94%. Gene expression profiles from the
30 cells treated with BPA and BP alternatives were compared to the ER biomarker using the
31 Running Fisher algorithm as previously described (Ryan *et al.*, 2016). The cut-off value for
32 statistical significance was p-value \leq 0.0001 after a Benjamini Hochberg correction of $\alpha =$
33 0.001. All transcriptome profile alterations resulting from exposure to BPA and all 6 BP
34 alternatives (Figure 3E) were highly statistically significant resulting from expression of
35 biomarker genes in a pattern highly similar to that of the biomarker (Figure 3F).

1 In order to confirm estrogenic effects provoked by BPA and BP alternatives, RNAs
2 extracted from MCF-7 cells treated with BPAF (0.08 μ M), BPA (0.36 μ M) and estradiol (1
3 nM) were subjected to total transcriptome RNA sequencing (RNA-seq) analysis using the
4 Illumina HiSeq 2500 system. This also allowed us to compare the sensitivity of RNA-seq and
5 microarrays to determine estrogenic effects. We have performed pairwise comparisons to
6 determine the list of DEGs following the same criteria used for the microarray analysis. Only
7 12-21% of the DEGs identified by RNA-seq were also found to be altered on the microarrays
8 (Figure 4B). The fold changes of these genes found commonly altered in both the microarray
9 and the RNA-seq analysis in comparison to their respective controls were well correlated
10 (Figure 4A) for 17 β -estradiol (Pearson $r = 0.81$), BPA ($r = 0.86$) and BPAF ($r = 0.81$). Overall,
11 the RNA-seq method was more sensitive and identified 2-3 times more significantly altered
12 genes compared to the microarray method (Figure 4B). A total of 5091, 2930 and 3093
13 genes were significantly altered by estradiol, BPA and BPAF, respectively. Genes commonly
14 found altered after the application of both transcriptome profiling methods generally had a
15 higher fold change by the RNA-seq platform (Supplementary File 3). The ontology analysis
16 based on RNA-seq data gave similar results to our previous calculations based on the
17 microarray data and confirms estrogenic effects of BPA and BPAF (Figure 4C). Although
18 more DEGs related to cell cycle function were found by RNA seq than by microarray, the –
19 log₁₀ p-value for BPA was lower because the total number of DEGs found by RNA seq was
20 higher than by microarray. We similarly applied the ER gene expression biomarker to detect
21 ER agonists after analysis with the Illumina sequencing platform (Figure 4D and 4E). The
22 RNA-seq platform gave results similar to that of Affymetrix microarrays confirming estrogenic
23 effects of BPA alternatives.

24

25 Discussion

26 Although human populations are exposed to a wide range of BPA alternatives, their
27 estrogenic effects have never been thoroughly determined. We present here the first
28 comprehensive, side-by-side comparison of the estrogenic effects of BPA and 6 BP
29 alternatives (BPZ, BPAF, BPAP, BPB, BPF, BPS) found in foodstuffs and human fluids using
30 both cell proliferation and gene expression profile assays. Our study revealed that the 6
31 bisphenols introduced in “BPA-free” plastics displayed estrogenic effects with BPAF, BPB
32 and BPZ being more potent than BPA, although their estrogenic potential remained in the
33 same range as BPA. The comparison of transcriptome profiles revealed a signature
34 demonstrating that these effects were mediated via ER (Figure 3). Our results highlight the
35 need to conduct studies to examine possible pathological effects in laboratory animals at

1 concentrations relevant for human exposures. Similar transcriptome alterations should also
2 be confirmed in other cell lines or primary cells. In addition, combined effects of these
3 bisphenols should be explored in further studies to determine the relevance of these
4 estrogenic effects in terms of human health risk assessment. In particular, our results call for
5 the relevance of human exposure to BPA alternatives in hormone-dependent breast cancer
6 progression to be investigated.

7 The structure of BPA alternatives (Figure 1) provide insights into the possible
8 mechanisms of action toward ER. BPA binding to ER is mainly driven by van der Waals'
9 forces and hydrogen bond interactions (Li *et al.*, 2015). BPA and 17 β -estradiol bind in a
10 similar manner, with two phenol rings pointing to the two ends of the ER hydrophobic pocket.
11 Differences in estrogenic activities between the different BPA alternatives may be due to the
12 different groups present at the bridge between the two phenol rings. Their hydrophobicity is
13 driving their affinity, since the matching of the group carried by the methylene bridge is
14 known to determine binding affinity towards the hydrophobic surface of the ER binding site
15 (Endo *et al.*, 2005). Such a mechanism of ER interaction is supported by the hydrophobicity
16 ranking of bisphenols (logP values BPZ > BPAF > BPAP > BPB > BPA > BPF > BPS), which
17 closely corresponds to their AC50 toxicity score.

18 Future studies need to explore effects at lower doses, since EDCs can elicit a
19 nonmonotonic response that significantly deviates from the usual sigmoidal curve
20 (Vandenberg *et al.*, 2012). In another transcriptomics study in which MCF-7 cells were
21 exposed to varying concentrations of BPA, a weak gene activation peak at a very low
22 concentration range (~0.1 nM) was observed in addition to the main peak of gene activation
23 (Shioda *et al.*, 2013). Additionally, an investigation of the combinatorial effects of bisphenols
24 would reflect real-world exposure conditions even if estrogenic effects of chemical mixtures
25 *in vitro* are predicted by the use of concentration addition models (Evans *et al.*, 2012).

26 Our data allows a direct comparison of the sensitivity of RNA-seq and microarray
27 hybridisation in the determination of the transcriptome signature of endocrine disrupting
28 effects. Overall, the application of RNA-seq resulted in the detection of more statistically
29 significant alterations in gene expression than the microarray method. These genes
30 generally had a higher fold change leading to p-values associated with gene expression
31 biomarkers being lower. Overall, RNA sequencing appears to be more sensitive than
32 microarray analysis. This was also the conclusion of studies performed in other cell lines
33 (Perkins *et al.*, 2014), mesenchymal stem cells (Li *et al.*, 2016) and rat tissues (Perkins *et al.*,
34 2014). In a study of 498 primary neuroblastomas, RNA-seq outperformed microarrays in
35 determining the transcriptomic characteristics of these cancers, while RNA-seq and

1 microarray performed similarly in clinical endpoint prediction (Zhang *et al.*, 2015). Both
2 technologies are suitable to detect estrogenic effects of BPA alternatives using the gene
3 expression biomarker of ER α activation. Although the genes weakly expressed were
4 differently detected by the two platforms, the biomarker signatures were comparable.
5 However, it is perhaps noteworthy that the microarray processing and analysis pipelines are
6 better standardized and could thus be more reliable in comparing experiments performed by
7 different laboratories. Further intra- and inter-experimental comparisons would be necessary
8 to conclude which method is the most sensitive and reliable to determine transcriptome
9 signatures.

10 The mechanisms of action resulting in toxic effects from BPA at low levels of
11 exposure have long remained elusive due to its relatively low affinity to ER. Higher affinities
12 in the circulating exposure range for BPA have been reported on membrane estrogen
13 receptor GPR30 / GPER at a concentration of 1 nM (Sheng *et al.*, 2012). BPA is very potent
14 at inducing rapid non-genomic responses from membrane surface receptors at low
15 concentrations (1-100 pM) (Wozniak *et al.*, 2005). For example, BPA administration inhibited
16 meiotic maturation of Zebrafish oocytes through a G protein-coupled ER-dependent
17 epidermal growth factor receptor pathway (Fitzgerald *et al.*, 2015). BPA can also induce
18 adipogenesis through other receptors such as peroxisome proliferator-activated receptor
19 gamma (PPAR γ) (Ahmed and Atlas, 2016). Surprisingly, this latest study provided evidence
20 that BPS is a more potent adipogen than BPA in inducing 3T3-L1 adipocyte differentiation
21 and lipid accumulation. Overall, effects of BPA alternatives have been poorly investigated.
22 Recent studies have revealed that less studied endocrine-related systems such as
23 glucocorticoid, PPAR, monoamine, noradrenaline and serotonin pathways could be targets
24 of endocrine disruption (Filer *et al.*, 2014).

25 In adult humans, BPA is considered to be rapidly metabolized by the liver, with
26 elimination virtually complete within 24 h of exposure, although chronic exposures could
27 result in accumulation if BPA distributes within tissues that slowly release BPA (Stahlhut *et al.*,
28 2009). In an investigation of concentrations of BPA metabolites in the urine of 112
29 pregnant women (ethnically and racially diverse), total BPA consisted of 71% BPA
30 glucuronide, 15% BPA sulfate and 14% unconjugated BPA (Gerona *et al.*, 2016). These
31 metabolites have long been believed to be biologically inactive because they are not thought
32 to be ER agonists. However, this does not preclude effects on other receptor systems or the
33 creation of equilibrium between the metabolite and the parent if the metabolite is long-lived.
34 For instance, a recent study showed that BPA glucuronide can induce lipid accumulation and
35 differentiation of pre-adipocytes (Boucher *et al.*, 2015). Metabolites of the main BPA
36 alternatives have not been routinely measured in human biomonitoring studies, which may

1 greatly underestimate real world exposures. Moreover, BPA metabolites may be
2 deconjugated by β -glucuronidase, which is particularly active in the placenta and fetal liver
3 resulting in increased fetal exposure (Ginsberg and Rice, 2009). Overall, the assessment of
4 potential health effects arising from exposure to bisphenols would be greatly facilitated if
5 biomonitoring studies examining reference populations like those of the U.S. CDC's
6 NHANES programme, were to begin routine examination of human fluids for BPA
7 alternatives as well as their metabolites.

8 Even though disruption of ER function is one of the most investigated endocrine-
9 related targets in the ToxCast data (18 assays) (Judson et al., 2015), our analysis of the high
10 throughput system (HTS) employed has revealed discrepancies between our results and
11 those of this screen with some of these ToxCast assays failing to detect estrogenic effects
12 (Table 1). This was especially the case for BPF which was found negative in 9 out of the 11
13 assays scrutinized. The two systems of analysis detecting the estrogenic capability of BPF
14 were the Attagene assays. BPF was found to be less potent in these assays than in the
15 present study (Table 1). Our results are in agreement with previously published estrogenic
16 effects of BPF reporting an EC₅₀ of 0.82 μ M in a ER luciferase assay (Rosenmai et al.,
17 2014). BPAP on the other hand was found to be more potent in the ToxCast assays,
18 although the difference within the 2 current assays was minimal and could be explained by
19 different grades of chemical purity. Another reason for these differences could be the
20 different sensitivities of HTS assays. These are frequently based on heterologous
21 expression of reporter genes in non-hormone-responsive cell lines that possibly do not
22 possess all the co-factors necessary to transduce the hormone signal, thus resulting in a
23 decreased sensitivity to detect relevant signals.

24 The US EPA has proposed to substitute the Endocrine Disruptor Screening Program
25 Tier 1 assays with *in vitro* data in order to reduce the cost and time of toxicity testing, as well
26 as animal use (Browne *et al.*, 2015). One way forward to comply with these objectives would
27 be to integrate gene expression profiling into the HTS system. Transcriptome profiles
28 resulting from exposure to a given chemical could be correlated to signatures of a wide
29 range of chemicals using the Library of Integrated Network-based Cellular Signatures
30 (LINCS) database, which contains signatures from around 4000 chemicals screened in
31 approximately 17 cell lines (<http://www.lincsproject.org>). This could be used as a "Tier 0"
32 assay to further prioritize targeted *in vitro* testing in the context of toxicity testing
33 programmes (Ryan et al., 2016).

34

35

1 **Conclusion**

2 We have detected estrogenic effects of BPA alternatives by the application of a gene
3 expression biomarker of ER α activation, whose results are corroborated by functional
4 cellular assays. Our comparison of microarray and RNA-Seq technologies showed that both
5 platforms are suitable for the use of this ER α gene expression biomarker.

6 Recently, the plastics manufacturing industry have turned to alternative bisphenols to
7 produce their “BPA-free” products, often with little toxicology testing. Our data show that
8 some of these BPA alternatives are even more potent ER activators than BPA, suggesting
9 that “bisphenol-free” labels on consumer products may need to be more informative.

10

1

2 **References**

- 3 Ahmed, S., and Atlas, E. (2016). Bisphenol S- and bisphenol A-induced adipogenesis of
4 murine preadipocytes occurs through direct peroxisome proliferator-activated receptor
5 gamma activation. *Int J Obes (Lond)* **40**(10), 1566-1573.
6
- 7 Asimakopoulos, A. G., Xue, J., De Carvalho, B. P., Iyer, A., Abualnaja, K. O., Yaghmoor, S.
8 S., Kumosani, T. A., and Kannan, K. (2016). Urinary biomarkers of exposure to 57
9 xenobiotics and its association with oxidative stress in a population in Jeddah, Saudi Arabia.
10 *Environ Res* **150**, 573-81
11
- 12 Boucher, J. G., Boudreau, A., Ahmed, S., and Atlas, E. (2015). In Vitro Effects of Bisphenol
13 A beta-D-Glucuronide (BPA-G) on Adipogenesis in Human and Murine Preadipocytes.
14 *Environ Health Perspect* **123**(12), 1287-93.
15
- 16 Browne, P., Judson, R. S., Casey, W. M., Kleinstreuer, N. C., and Thomas, R. S. (2015).
17 Screening Chemicals for Estrogen Receptor Bioactivity Using a Computational Model.
18 *Environ Sci Technol* **49**(14), 8804-14
19
- 20 Chen, D., Kannan, K., Tan, H., Zheng, Z., Feng, Y. L., Wu, Y., and Widelka, M. (2016).
21 Bisphenol Analogues Other Than BPA: Environmental Occurrence, Human Exposure, and
22 Toxicity-A Review. *Environ Sci Technol* **50**(11), 5438-53.
23
- 24 Dairkee, S. H., Seok, J., Champion, S., Sayeed, A., Mindrinos, M., Xiao, W., Davis, R. W.,
25 and Goodson, W. H. (2008). Bisphenol A induces a profile of tumor aggressiveness in high-
26 risk cells from breast cancer patients. *Cancer Res* **68**(7), 2076-80.
27
- 28 Endo, Y., Yoshimi, T., Ohta, K., Suzuki, T., and Ohta, S. (2005). Potent estrogen receptor
29 ligands based on bisphenols with a globular hydrophobic core. *J Med Chem* **48**(12), 3941-4
30
- 31 Evans, R. M., Scholze, M., and Kortenkamp, A. (2012). Additive Mixture Effects of
32 Estrogenic Chemicals in Human Cell-Based Assays Can Be Influenced by Inclusion of
33 Chemicals with Differing Effect Profiles. *PLoS ONE* **7**(8), e43606
34
- 35 Filer, D., Patisaul, H. B., Schug, T., Reif, D., and Thayer, K. (2014). Test driving ToxCast:
36 endocrine profiling for 1858 chemicals included in phase II. *Current Opinion in*
37 *Pharmacology* **19**, 145-152
38
- 39 Fitzgerald, A. C., Peyton, C., Dong, J., and Thomas, P. (2015). Bisphenol A and Related
40 Alkylphenols Exert Nongenomic Estrogenic Actions Through a G Protein-Coupled Estrogen
41 Receptor 1 (Gper)/Epidermal Growth Factor Receptor (Egfr) Pathway to Inhibit Meiotic
42 Maturation of Zebrafish Oocytes. *Biol Reprod* **93**(6), 135.
43
- 44 Gerona, R. R., Pan, J., Zota, A. R., Schwartz, J. M., Friesen, M., Taylor, J. A., Hunt, P. A.,
45 and Woodruff, T. J. (2016). Direct measurement of Bisphenol A (BPA), BPA glucuronide and
46 BPA sulfate in a diverse and low-income population of pregnant women reveals high
47 exposure, with potential implications for previous exposure estimates: a cross-sectional
48 study. *Environ Health* **15**, 50.
49
- 50 Ginsberg, G., and Rice, D. C. (2009). Does rapid metabolism ensure negligible risk from
51 bisphenol A? *Environ Health Perspect* **117**(11), 1639-43

- 1 Green, K. A., and Carroll, J. S. (2007). Oestrogen-receptor-mediated transcription and the
2 influence of co-factors and chromatin state. *Nat Rev Cancer* **7**(9), 713-22
3
- 4 Hormann, A. M., Vom Saal, F. S., Nagel, S. C., Stahlhut, R. W., Moyer, C. L., Ellersieck, M.
5 R., Welshons, W. V., Toutain, P. L., and Taylor, J. A. (2014). Holding thermal receipt paper
6 and eating food after using hand sanitizer results in high serum bioactive and urine total
7 levels of bisphenol A (BPA). *PLoS One* **9**(10), e110509
8
- 9 Iorio, F., Bosotti, R., Scacheri, E., Belcastro, V., Mithbaokar, P., Ferriero, R., Murino, L.,
10 Tagliaferri, R., Brunetti-Pierri, N., Isacchi, A., and di Bernardo, D. (2010). Discovery of drug
11 mode of action and drug repositioning from transcriptional responses. *Proc Natl Acad Sci U*
12 *S A* **107**(33), 14621-6
13
- 14 Judson, R. S., Magpantay, F. M., Chickarmane, V., Haskell, C., Tania, N., Taylor, J., Xia, M.,
15 Huang, R., Rotroff, D. M., Filer, D. L., Houck, K. A., Martin, M. T., Sipes, N., Richard, A. M.,
16 Mansouri, K., Setzer, R. W., Knudsen, T. B., Crofton, K. M., and Thomas, R. S. (2015).
17 Integrated Model of Chemical Perturbations of a Biological Pathway Using 18 In Vitro High-
18 Throughput Screening Assays for the Estrogen Receptor. *Toxicol Sci* **148**(1), 137-54
19
- 20 Kim, D., Langmead, B., and Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low
21 memory requirements. *Nature methods* **12**(4), 357-60
22
- 23 Kitamura, S., Suzuki, T., Sanoh, S., Kohta, R., Jinno, N., Sugihara, K., Yoshihara, S.,
24 Fujimoto, N., Watanabe, H., and Ohta, S. (2005). Comparative study of the endocrine-
25 disrupting activity of bisphenol A and 19 related compounds. *Toxicol Sci* **84**(2), 249-59
26
- 27 Kupershmidt, I., Su, Q. J., Grewal, A., Sundaresh, S., Halperin, I., Flynn, J., Shekar, M.,
28 Wang, H., Park, J., Cui, W., Wall, G. D., Wisotzkey, R., Alag, S., Akhtari, S., and Ronaghi,
29 M. (2010). Ontology-based meta-analysis of global collections of high-throughput public
30 data. *PLoS One* **5**(9), 10.1371/journal.pone.0013066.
31
- 32 Li, J., Hou, R., Niu, X., Liu, R., Wang, Q., Wang, C., Li, X., Hao, Z., Yin, G., and Zhang, K.
33 (2016). Comparison of microarray and RNA-Seq analysis of mRNA expression in dermal
34 mesenchymal stem cells. *Biotechnology letters* **38**(1), 33-41
35
- 36 Li, L., Wang, Q., Zhang, Y., Niu, Y., Yao, X., and Liu, H. (2015). The Molecular Mechanism
37 of Bisphenol A (BPA) as an Endocrine Disruptor by Interacting with Nuclear Receptors:
38 Insights from Molecular Dynamics (MD) Simulations. *PLoS ONE* **10**(3), e0120330
39
- 40 Liao, C., and Kannan, K. (2013). Concentrations and profiles of bisphenol A and other
41 bisphenol analogues in foodstuffs from the United States and their implications for human
42 exposure. *J Agric Food Chem* **61**(19), 4655-62
43
- 44 Liao, C., Liu, F., and Kannan, K. (2012). Bisphenol s, a new bisphenol analogue, in paper
45 products and currency bills and its association with bisphenol a residues. *Environ Sci*
46 *Technol* **46**(12), 6515-22
47
- 48 Mandrup, K., Boberg, J., Isling, L. K., Christiansen, S., and Hass, U. (2016). Low-dose
49 effects of bisphenol A on mammary gland development in rats. *Andrology* **4**(4), 673-83
50
- 51 Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing
52 reads. *2011* **17**(1)
- 53 Nakao, T., Akiyama, E., Kakutani, H., Mizuno, A., Aozasa, O., Akai, Y., and Ohta, S. (2015).
54 Levels of tetrabromobisphenol A, tribromobisphenol A, dibromobisphenol A,

- 1 monobromobisphenol A, and bisphenol a in Japanese breast milk. *Chem Res Toxicol* **28**(4),
2 722-8
- 3 Perkins, J. R., Antunes-Martins, A., Calvo, M., Grist, J., Rust, W., Schmid, R., Hildebrandt,
4 T., Kohl, M., Orengo, C., McMahon, S. B., and Bennett, D. L. H. (2014). A comparison of
5 RNA-seq and exon arrays for whole genome transcription profiling of the L5 spinal nerve
6 transection model of neuropathic pain in the rat. *Molecular Pain* **10**, 7-7, 10.1186/1744-8069-
7 10-7.
- 8 Rhodes, D. R., and Chinnaiyan, A. M. (2005). Integrative analysis of the cancer
9 transcriptome. *Nat Genet* **37 Suppl**, S31-7
- 10 Robinson, M. D., and Oshlack, A. (2010). A scaling normalization method for differential
11 expression analysis of RNA-seq data. *Genome Biol* **11**(3), R25
- 12 Rochester, J. R. (2013). Bisphenol A and human health: a review of the literature. *Reprod*
13 *Toxicol* **42**, 132-55
- 14
- 15 Rochester, J. R., and Bolden, A. L. (2015). Bisphenol S and F: A Systematic Review and
16 Comparison of the Hormonal Activity of Bisphenol A Substitutes. *Environ Health Perspect*
17 **123**(7), 643-50
- 18
- 19 Rosenmai, A. K., Dybdahl, M., Pedersen, M., Alice van Vugt-Lussenburg, B. M., Wedebye,
20 E. B., Taxvig, C., and Vinggaard, A. M. (2014). Are structural analogues to bisphenol a safe
21 alternatives? *Toxicol Sci* **139**(1), 35-47
- 22
- 23 Ryan, N., Chorley, B., Tice, R. R., Judson, R., and Corton, J. C. (2016). Moving Toward
24 Integrating Gene Expression Profiling Into High-Throughput Testing: A Gene Expression
25 Biomarker Accurately Predicts Estrogen Receptor alpha Modulation in a Microarray
26 Compendium. *Toxicol Sci* **151**(1), 88-103
- 27
- 28 Sheng ZG1, Huang W, Liu YX, Zhu BZ. (2012) Bisphenol A at a low concentration boosts
29 mouse spermatogonial cell proliferation by inducing the G protein-coupled receptor 30
30 expression. *Toxicol Appl Pharmacol.* **267**(1):88-94.
- 31
- 32 Shioda, T., Chesnes, J., Coser, K. R., Zou, L., Hur, J., Dean, K. L., Sonnenschein, C., Soto,
33 A. M., and Isselbacher, K. J. (2006). Importance of dosage standardization for interpreting
34 transcriptomal signature profiles: evidence from studies of xenoestrogens. *Proc Natl Acad*
35 *Sci U S A* **103**(32), 12033-8
- 36 Shioda, T., Rosenthal, N. F., Coser, K. R., Suto, M., Phatak, M., Medvedovic, M., Carey, V.
37 J., and Isselbacher, K. J. (2013). Expressomal approach for comprehensive analysis and
38 visualization of ligand sensitivities of xenoestrogen responsive genes. *Proc Natl Acad Sci U*
39 *S A* **110**(41), 16508-13
- 40
- 41 Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., and Serrano, F. O.
42 (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic
43 environmental pollutants. *Environ Health Perspect* **103 Suppl 7**, 113-22.
- 44
- 45 Stahlhut, R. W., Welshons, W. V., and Swan, S. H. (2009). Bisphenol A data in NHANES
46 suggest longer than expected half-life, substantial nonfood exposure, or both. *Environ Health*
47 *Perspect* **117**(5), 784-9
- 48
- 49 Tharp, A. P., Maffini, M. V., Hunt, P. A., VandeVoort, C. A., Sonnenschein, C., and Soto, A.
50 M. (2012). Bisphenol A alters the development of the rhesus monkey mammary gland. *Proc*
51 *Natl Acad Sci U S A* **109**(21), 8190-5

- 1
2 Tipney, H., and Hunter, L. (2010). An introduction to effective use of enrichment analysis
3 software. *Hum Genomics* **4**(3), 202-6.
4
5 van de Vijver, M. J., He, Y. D., van't Veer, L. J., Dai, H., Hart, A. A., Voskuil, D. W.,
6 Schreiber, G. J., Peterse, J. L., Roberts, C., Marton, M. J., Parrish, M., Atsma, D., Witteveen,
7 A., Glas, A., Delahaye, L., van der Velde, T., Bartelink, H., Rodenhuis, S., Rutgers, E. T.,
8 Friend, S. H., and Bernards, R. (2002). A gene-expression signature as a predictor of
9 survival in breast cancer. *N Engl J Med* **347**(25), 1999-2009
10
11 Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs, D. R., Lee, D. H.,
12 Shioda, T., Soto, A. M., Vom Saal, F. S., Welshons, W. V., Zoeller, R. T., and Myers, J. P.
13 (2012). Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic
14 dose responses. *Endocr Rev* **33**, 10.1210/er.2011-1050.
15
16 Wilson, V. S., Bobseine, K., and Gray, L. E., Jr. (2004). Development and characterization of
17 a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection
18 of estrogen receptor agonist and antagonists. *Toxicol Sci* **81**(1), 69-77
19
20 Wozniak, A. L., Bulayeva, N. N., and Watson, C. S. (2005). Xenoestrogens at picomolar to
21 nanomolar concentrations trigger membrane estrogen receptor-alpha-mediated Ca²⁺ fluxes
22 and prolactin release in GH3/B6 pituitary tumor cells. *Environ Health Perspect* **113**(4), 431-9
23
24 Zhang, W., Yu, Y., Hertwig, F., Thierry-Mieg, J., Zhang, W., Thierry-Mieg, D., Wang, J.,
25 Furlanello, C., Devanarayan, V., Cheng, J., Deng, Y., Hero, B., Hong, H., Jia, M., Li, L., Lin,
26 S. M., Nikolsky, Y., Oberthuer, A., Qing, T., Su, Z., Volland, R., Wang, C., Wang, M. D., Ai,
27 J., Albanese, D., Asgharzadeh, S., Avigad, S., Bao, W., Bessarabova, M., Brilliant, M. H.,
28 Brors, B., Chierici, M., Chu, T. M., Zhang, J., Grundy, R. G., He, M. M., Hebring, S.,
29 Kaufman, H. L., Lababidi, S., Lancashire, L. J., Li, Y., Lu, X. X., Luo, H., Ma, X., Ning, B.,
30 Noguera, R., Peifer, M., Phan, J. H., Roels, F., Rosswog, C., Shao, S., Shen, J., Theissen,
31 J., Tonini, G. P., Vandesompele, J., Wu, P. Y., Xiao, W., Xu, J., Xu, W., Xuan, J., Yang, Y.,
32 Ye, Z., Dong, Z., Zhang, K. K., Yin, Y., Zhao, C., Zheng, Y., Wolfinger, R. D., Shi, T., Malkas,
33 L. H., Berthold, F., Wang, J., Tong, W., Shi, L., Peng, Z., and Fischer, M. (2015).
34 Comparison of RNA-seq and microarray-based models for clinical endpoint prediction.
35 *Genome Biol* **16**, 133, 10.1186/s13059-015-0694-1.
36
37 Zhou, X., Kramer, J. P., Calafat, A. M., and Ye, X. (2014). Automated on-line column-
38 switching high performance liquid chromatography isotope dilution tandem mass
39 spectrometry method for the quantification of bisphenol A, bisphenol F, bisphenol S, and 11
40 other phenols in urine. *J Chromatogr B Analyt Technol Biomed Life Sci* **944**, 152-6

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1 **Table 1. Comparison to ToxCast data.** Publically available data was analysed from the
 2 ToxCast program using the iCSS ToxCast Dashboard. AC50 values (μM) for ToxCast
 3 assays relative to ESR1 activation are compared to the results presented here. Negative
 4 results are displayed by grey boxes. White boxes shows assays in which bisphenols have
 5 not been tested.

6

CASRN	Name	ATG_ERa_TRANS_up	ATG_ERE_CIS_up	NVS_NR_bER	NVS_NR_hER	OT_ER_ERaERa_0480	OT_ER_ERaERa_1440	OT_ERa_EREFGFP_0120	OT_ERa_EREFGFP_0480	TOX21_ERa_BLA_Agonist_ratio	TOX21_ERa_LUC_BG1_Agonist	AC50 MCF7 this study	AC50 LUC this study
1478-61-1	Bisphenol AF	0.36	0.09	0.07	0.03	1.05	0.712	0.1	0.11	0.36	0.05	0.03	0.08
843-55-0	Bisphenol Z									####	0.31	0.11	0.4
77-40-7	Bisphenol B	0.13	0.17	0.21	0.27	1.82	1.41	0.18	0.22	####	0.11	0.24	0.3
80-05-7	Bisphenol A	0.12	0.1	0.42	0.23	5.73	4.33	0.42	0.63	1.01	0.36	0.36	0.4
1571-75-1	Bisphenol AP	0.12	0.29									0.39	1
2467-02-9	Bisphenol F	34.9	30.3	####	####	####	####	####	####	####	####	0.55	1
80-09-1	Bisphenol S	0.8	1.42	17.7	5.45	43.8	56.1	59.2	26.6	9.29	1.87	1.33	1.5

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2 **Table 2. List of the 5 most up- or down-regulated genes after treatment with BPA,**
 3 **BPAF or estradiol (E2).** The transcriptome profiling was performed using the Affymetrix
 4 microarray platform.

5

E2 (10 pM)		E2 (100 pM)		E2 (1 nM)		BPA		BPAF	
ALDH1A3	0.4	ALDH1A3	0.2	ALDH1A3	0.2	SEMA5A	0.3	ALDH1A3	0.3
SEMA5A	0.4	PRICKLE2-AS3	0.2	PSCA	0.2	ALDH1A3	0.3	PSCA	0.4
PPARG	0.5	SEMA5A	0.3	TFPI	0.2	PSCA	0.3	RP11-706C16.7	0.4
PSCA	0.5	PSCA	0.3	PRICKLE2-AS3	0.2	PRICKLE2-AS3	0.3	SEMA5A	0.4
IGFBPL1	0.5	EPAS1	0.3	EPAS1	0.3	GABBR2	0.3	EPAS1	0.4
AC005534.8	2.6	MYBL1	5.5	AC106875.1	6.4	AC106875.1	3.3	AGR3	3.2
MYB	3.0	AC106875.1	6.3	MYBL1	6.5	MYB	3.7	MYB-AS1	3.4
AC106875.1	3.2	GREB1	7.1	GREB1	7.1	GREB1	3.8	MGP	3.5
GREB1	3.7	PGR	10.0	PGR	9.2	MGP	4.2	MYB	4.1
PGR	4.6	MGP	12.4	MGP	15.0	PGR	6.2	PGR	5.7
BPB		BPF		BPS		BPZ		BPAP	
ALDH1A3	0.3	ALDH1A3	0.3	ALDH1A3	0.2	ALDH1A3	0.3	ALDH1A3	0.3
GABBR2	0.3	SEMA5A	0.3	PSCA	0.3	TFPI	0.3	SEMA5A	0.4
PSCA	0.3	EPAS1	0.4	SEMA5A	0.3	GABBR2	0.3	GABBR2	0.4
PRICKLE2-AS3	0.4	PSCA	0.4	TFPI	0.3	PSCA	0.4	TFPI	0.4
EPAS1	0.4	HCAR3	0.4	PRICKLE2-AS3	0.3	EPAS1	0.4	EPAS1	0.4
AGR3	3.3	MYB	3.9	AC106875.1	5.4	STC1	2.8	STC1	3.1
GREB1	3.8	MYB-AS1	4.4	MYB	5.7	ASCL1	2.9	AL121578.2	3.1
MYB	4.1	GREB1	4.5	GREB1	6.0	AGR3	3.3	MYB	3.8
MGP	4.4	MGP	5.0	MGP	7.8	MYB-AS1	3.4	AGR3	3.8
PGR	6.8	PGR	6.5	PGR	9.5	PGR	5.8	PGR	5.8

6

7

1

2 **Figures Legends**

3

4 **Figure 1. Molecular structures of BPA and bisphenol alternatives in comparison to the**
5 **natural hormone 17 β -estradiol.**

6

7 **Figure 2. Bisphenol A alternatives can effectively substitute for estradiol in promoting**
8 **growth through estrogen receptors of human breast cancer cells. (A)** Proliferative effect
9 of BPA and bisphenol alternatives on mammary cells in an E-Screen bioassay. After 24h of
10 steroid hormone starvation cells were treated for 6 days with the test compounds. Numbers
11 of cells was measured by an MTT colorimetric assay. Results are expressed as proliferative
12 effect percentage relative to the proliferation of cells under hormone-free conditions. Data
13 are the mean +/- SE of three independent experiments, with each performed in triplicate. **(B)**
14 Bisphenol A alternatives stimulate estrogen response element-mediated transcription. After
15 24 hours of steroid hormone starvation, T47D-Kluc cells were treated with the test
16 compounds for 24 hours. Cells were then lysed and subjected to a bioluminescence
17 luciferase reporter gene assay. **(C)** Luciferase assays of T47D-Kluc cells treated with
18 bisphenol A alternatives in the absence (black) or presence (red) of ICI 182,780, an estrogen
19 receptor antagonist. Results show that ICI 182,780 represses ERE-mediated transcription
20 induced by bisphenol A alternatives.

21

22

23 **Figure 3. Alteration of MCF-7 transcriptome profiles caused by BPA alternatives bear**
24 **the signature of ER activation.** Transcriptome analysis of MCF-7 cells treated with BPA
25 and alternative bisphenols reflect cell cycle changes **(A)**, response to hormone **(B)**, as well
26 as an association to breast cancer **(C)**. The p-values are determined by hypergeometric
27 calculation and adjusted using a Benjamini & Hochberg approach. Ratios show the total
28 number of network objects belonging to each term in comparison to those disturbed by the
29 treatments. The total of network objects (that is, all DEG) recognized by Metacore are
30 indicated in parentheses **(D)**. Overrepresentation of ER binding motifs in the promoters of
31 the differentially expressed genes. The analysis conducted with the transcription factor
32 analysis tool of Metacore. A total of 1262 ER binding sites are found in the 42909 protein-
33 based objects in the Metacore background list. A, number of targets in the activated dataset
34 regulated by ESR1; E, expected mean of hypergeometric distribution; p-value calculated

1 using hypergeometric distribution. **(E)** A gene expression biomarker confirms that BPA and
2 bisphenol alternatives are ER agonists. Lists of statistically significant genes from MCF-7
3 cells treated with BPA and bisphenol alternatives or the natural hormone 17β -estradiol were
4 examined against an ER gene expression biomarker signature consisting of 46 genes. The
5 heat map shows the expression of genes in the biomarker after exposure to the indicated
6 compound. Fold-change values for the ER biomarker are the average across 7 agonist
7 treatments. **(F)** Bar plots showing the significance of the correlation by their $-\log_{10}$ p-values.
8 Classification of activation or suppression required $p < 0.0001$. The number of genes
9 overlapping the ER biomarker is indicated at the top of each bar.

10

11 **Figure 4. Comparison of RNA-seq and microarray platforms in determining endocrine**
12 **disrupting effects of BPA and bisphenol alternatives.** RNA extracted from MCF-7 cells
13 was subjected to a full transcriptome profiling using the Illumina RNA sequencing or the
14 microarray technology under similar conditions. **(A)** Pearson correlation coefficients between
15 the RNA-seq and microarray data. The fold changes in gene function having an altered
16 expression by the two methods are presented. **(B)** Venn diagrams showing the number of
17 genes uniquely or commonly disturbed. **(C)** Gene ontology analysis of terms associated with
18 MCF-7 hormone induced proliferation in the transcriptome profiles obtained by RNA-seq or
19 microarray analysis. Ratios are showing the total number of network objects belonging to
20 each term in comparison to those disturbed by the treatments. The total of network objects
21 (that is, all DEG) recognized by Metacore are indicated in parentheses. **(D)** Heat map of
22 genes whose expression was statistically significantly altered examined against an ER gene
23 expression biomarker. **(E)** Bar plots showing the significance of the correlation by their $-\log_{10}$
24 p-values.

25

26 **Supplementary files**

27 **Supplementary File 1. Volcano plots of the MCF7 transcriptome profile changes**
28 **induced by BPA and bisphenol alternatives.** Alterations in MCF7 transcriptome profiles
29 induced by a 48h exposure to three concentrations of 17β -estradiol and the AC(50) of 7
30 bisphenols were determined using the Affymetrix GeneChip® Human Transcriptome Array
31 2.0. The volcano plots show the \log_2 fold changes and the $-\log_{10}$ p-values in transcript
32 cluster expression compared to the negative control. Data selected at the cut off values $p <$
33 0.05 and fold change > 1.2 for the functional analysis were represented by red dots.

34

1 **Supplementary File 2. Alterations of the MCF7 transcriptome induced by BPA and**
2 **bisphenol alternatives.** MCF-7 cells were subjected to a full transcriptome profiling using
3 the Affymetrix microarray platform. The list of genes having their expression altered by the
4 treatment with the BPA alternatives or three concentrations of 17 β -estradiol is provided with
5 their fold-changes and the p-values. Data were selected at the cut off values $p < 0.05$ and
6 fold change > 1.2 .

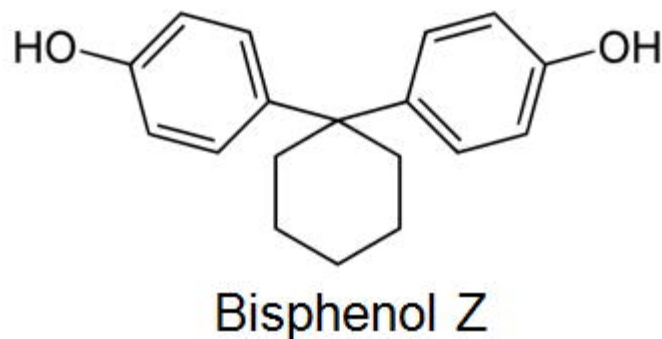
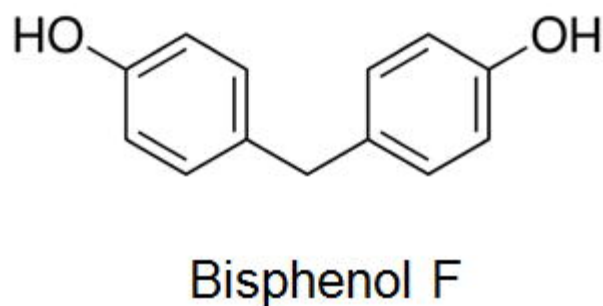
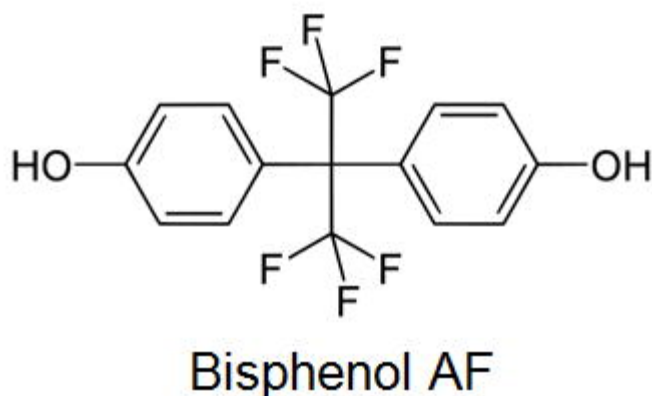
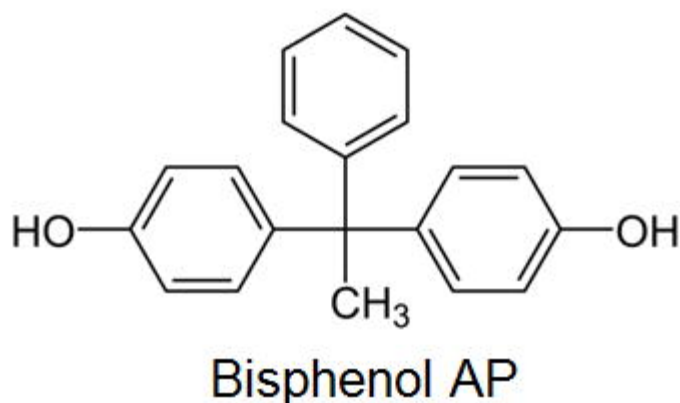
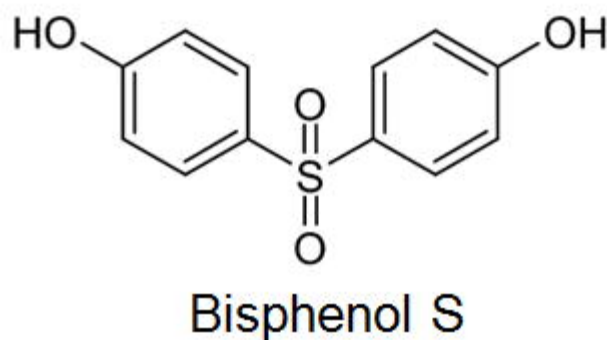
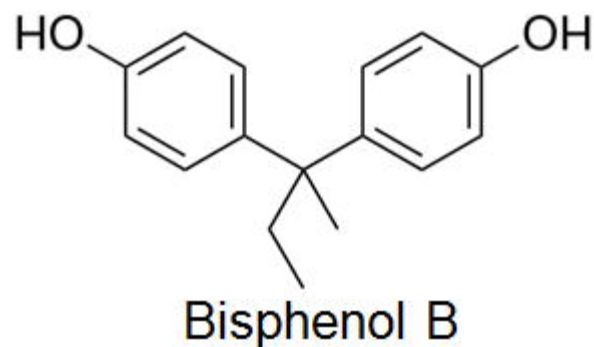
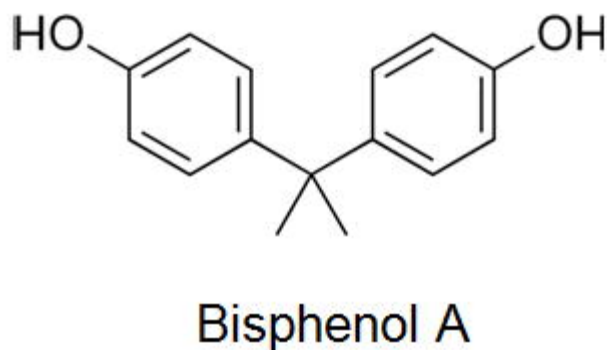
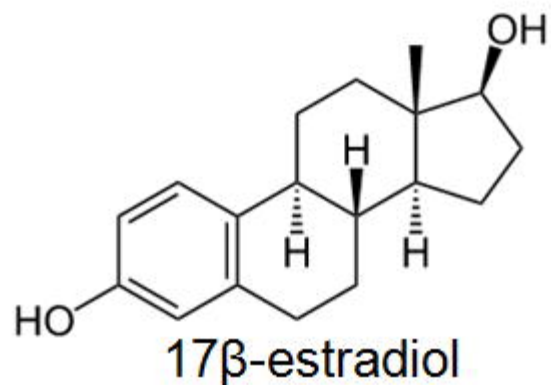
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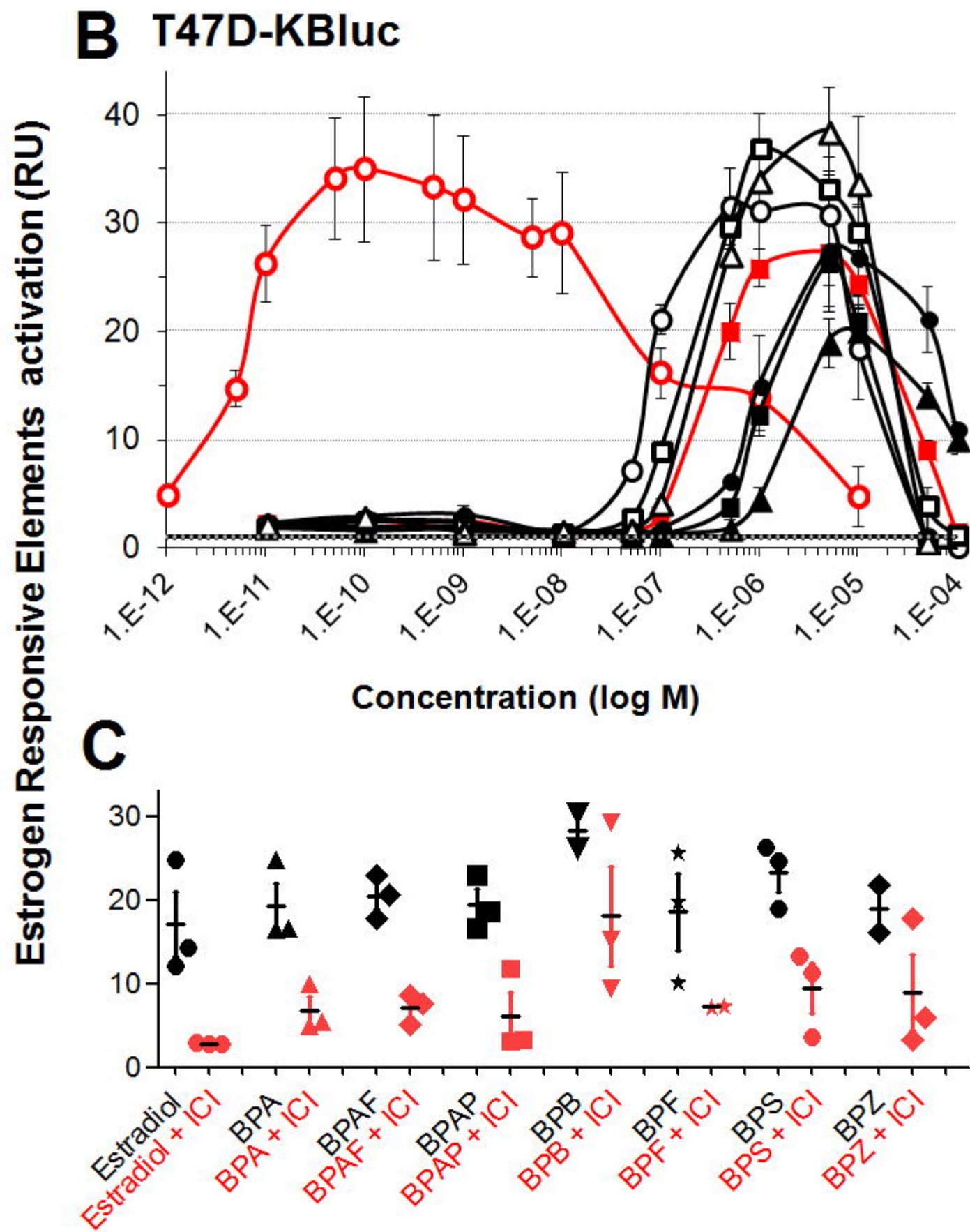
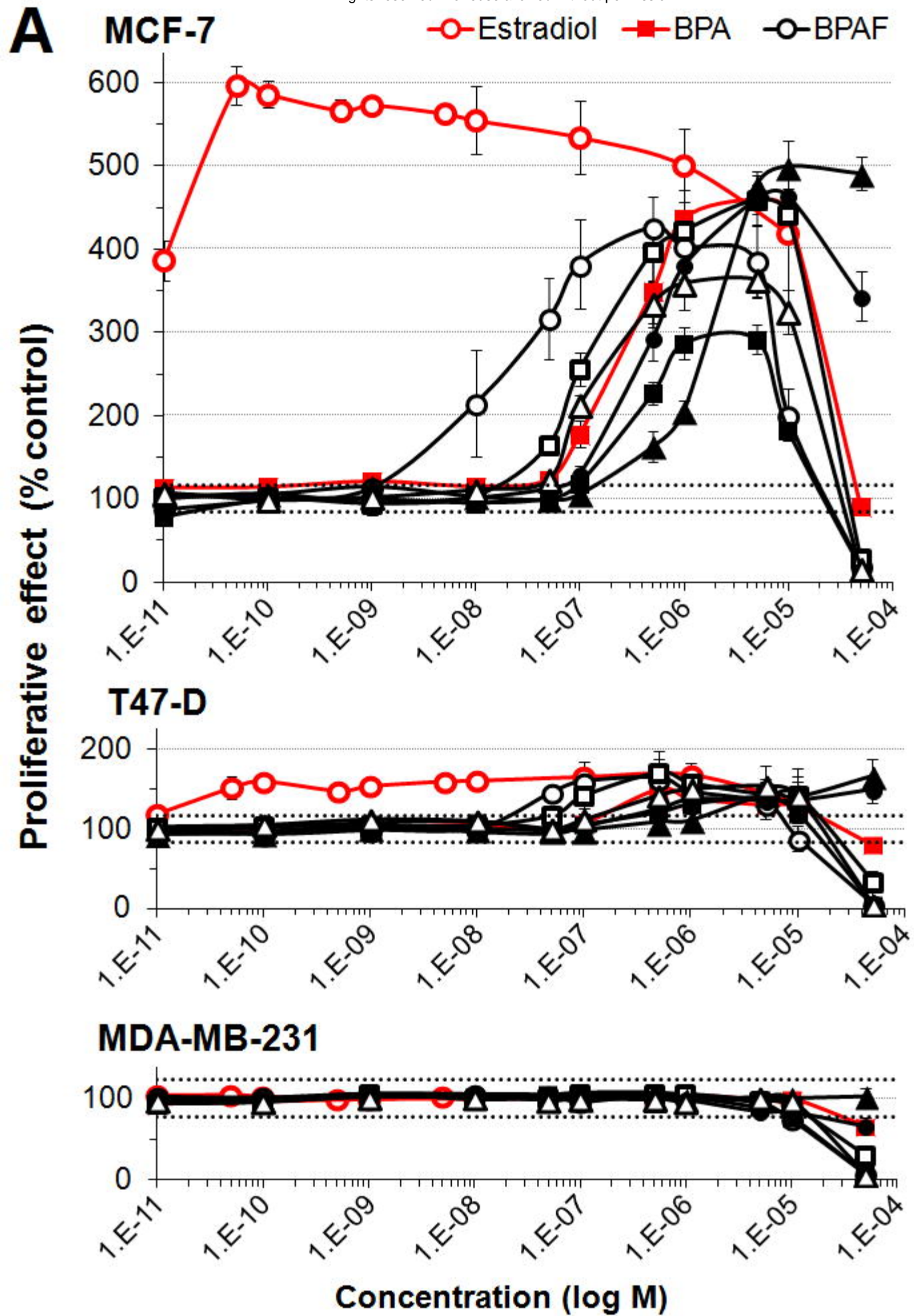
8 **Supplementary File 3. Alterations of the MCF7 transcriptome induced by BPA, BPAF,**
9 **and 17 β -estradiol.** MCF-7 cells were subjected to a full transcriptome profiling using the
10 Illumina RNA sequencing technology. The list of genes having their expression altered by
11 the treatment with BPA, BPAF, or 17 β -estradiol is provided with their fold-changes and the
12 p-values. Data were selected at the cut off values $p < 0.05$ and fold change > 1.2 .

13

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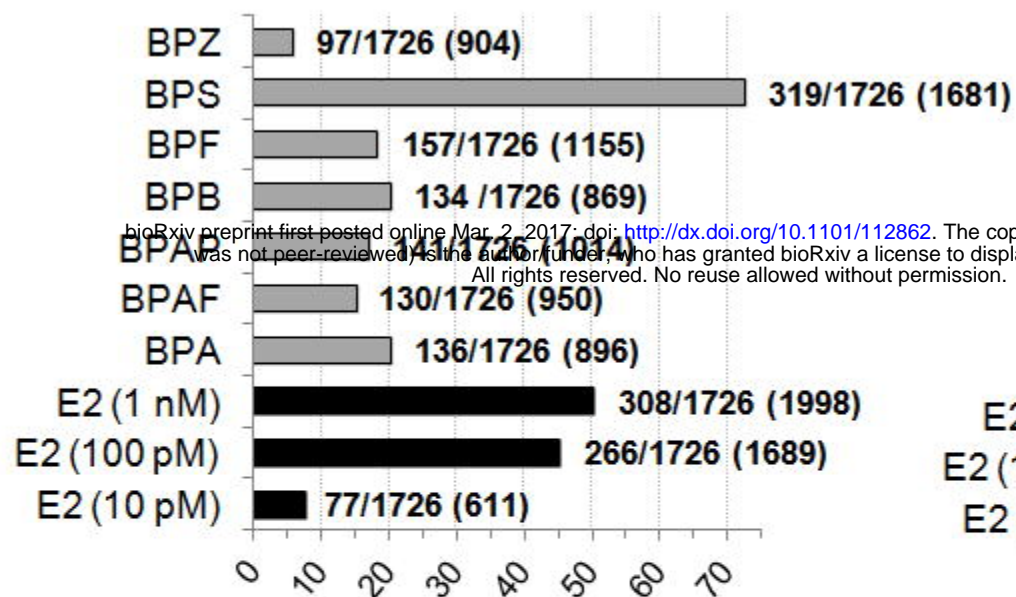
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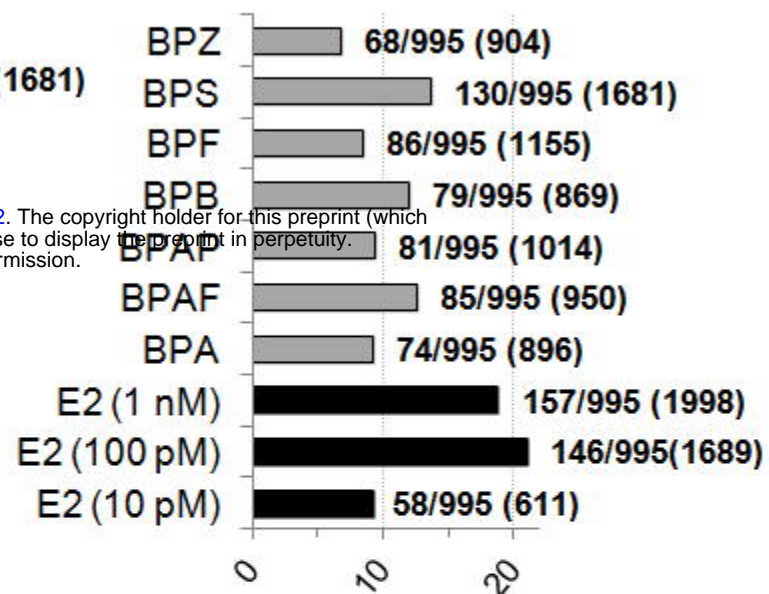


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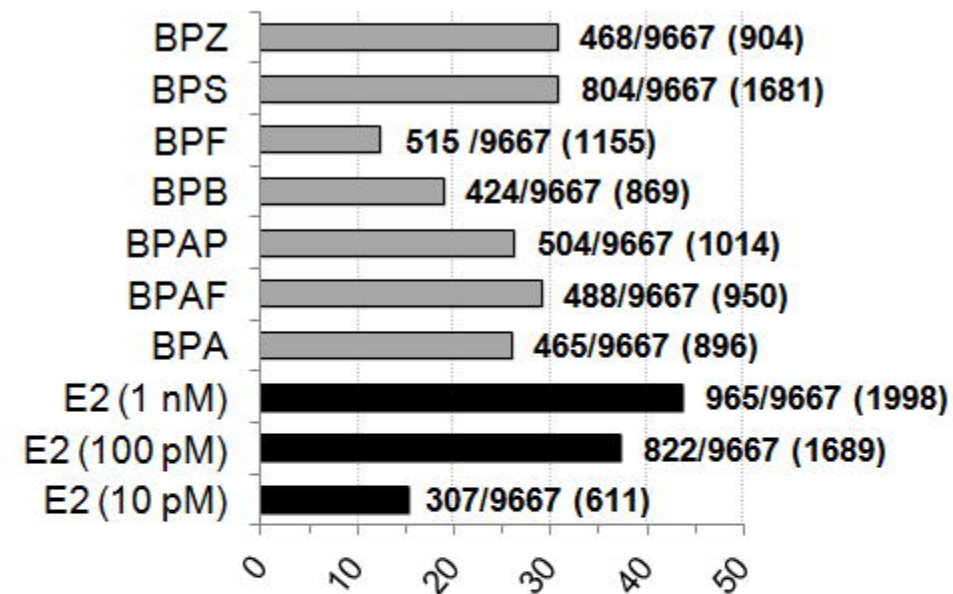
cell cycle (GO:0007049)

- log₁₀ FDR corrected p-values**B**

response to steroid hormone (GO:0048545)

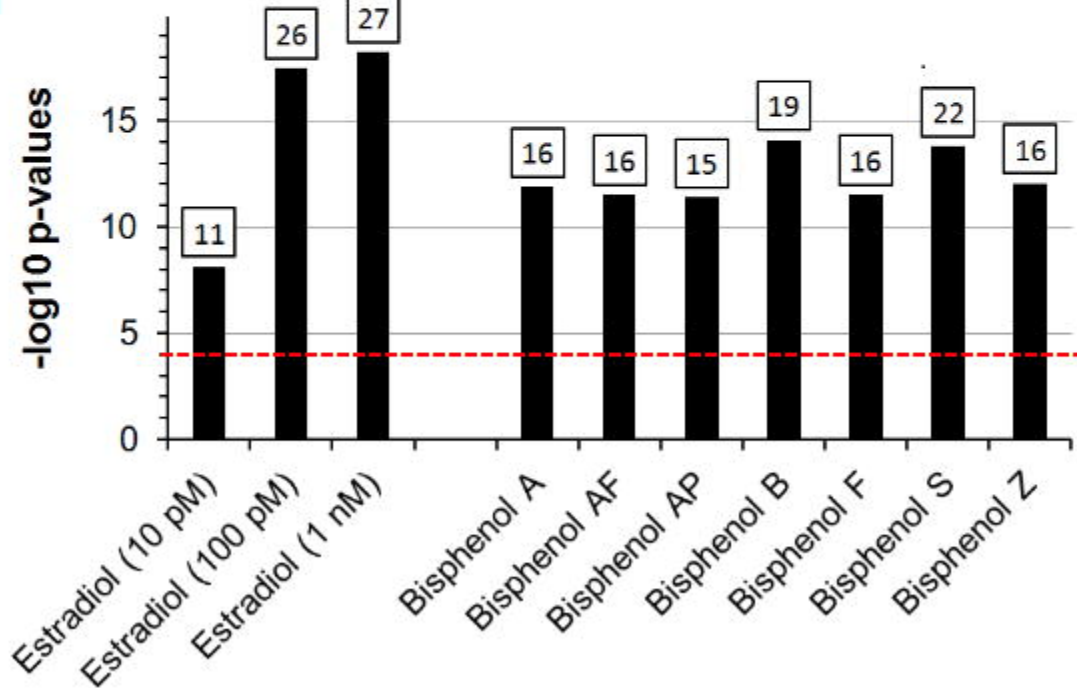
- log₁₀ FDR corrected p-values**C**

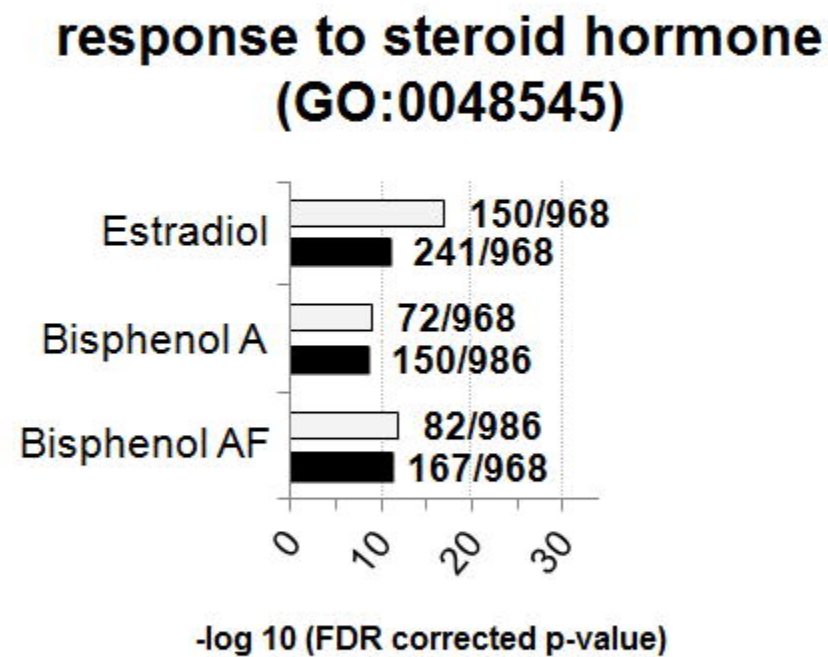
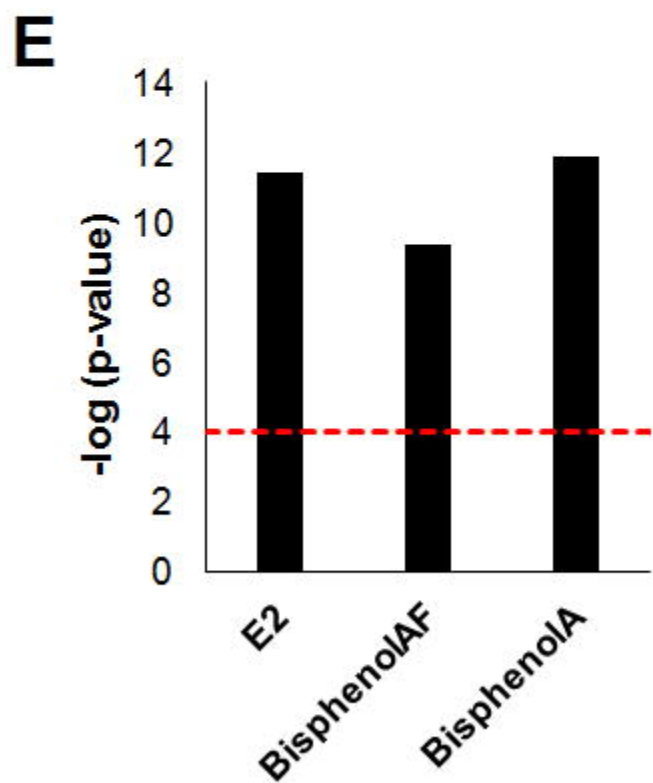
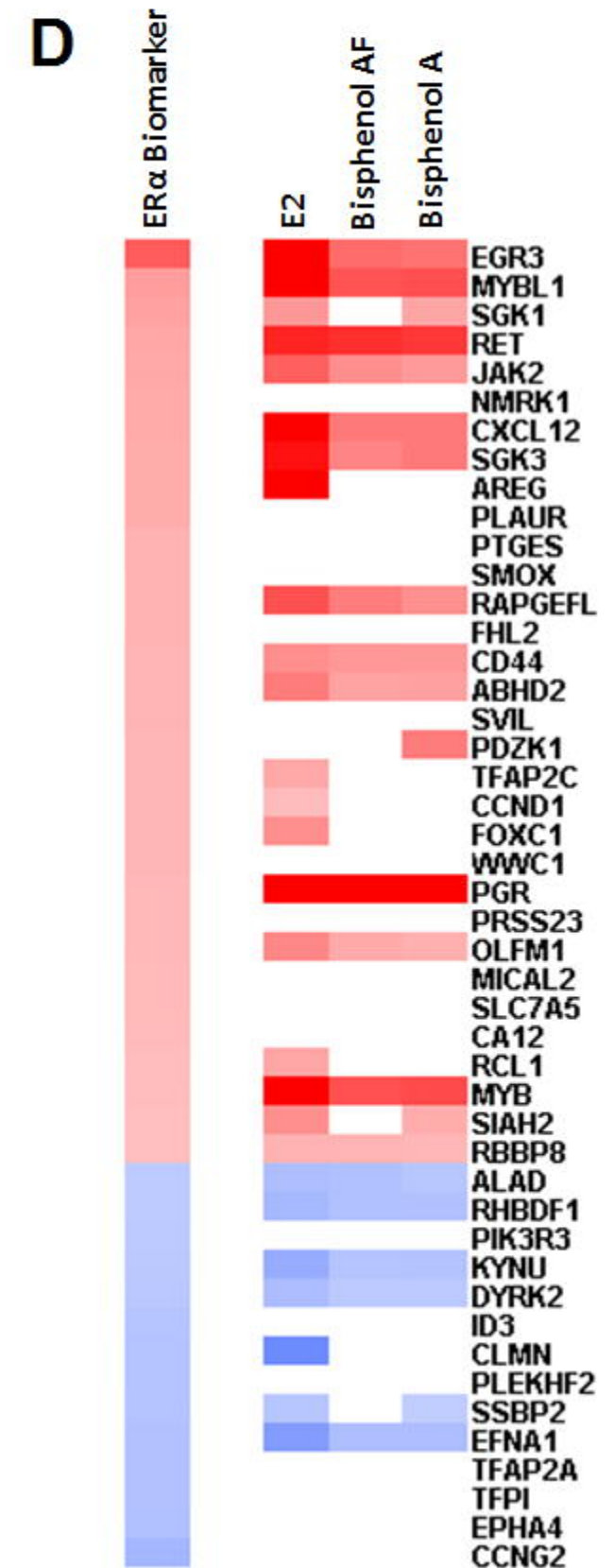
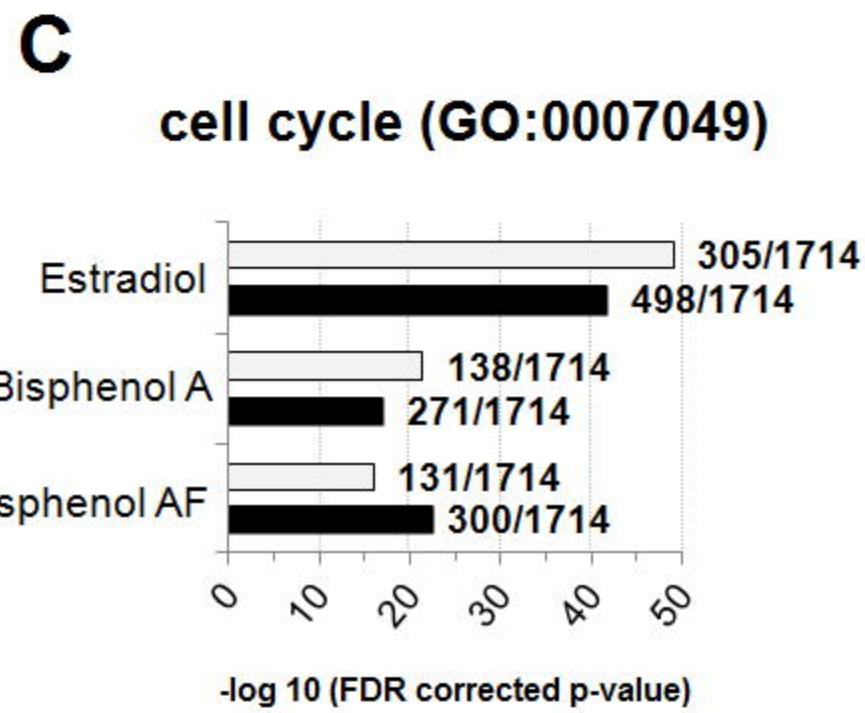
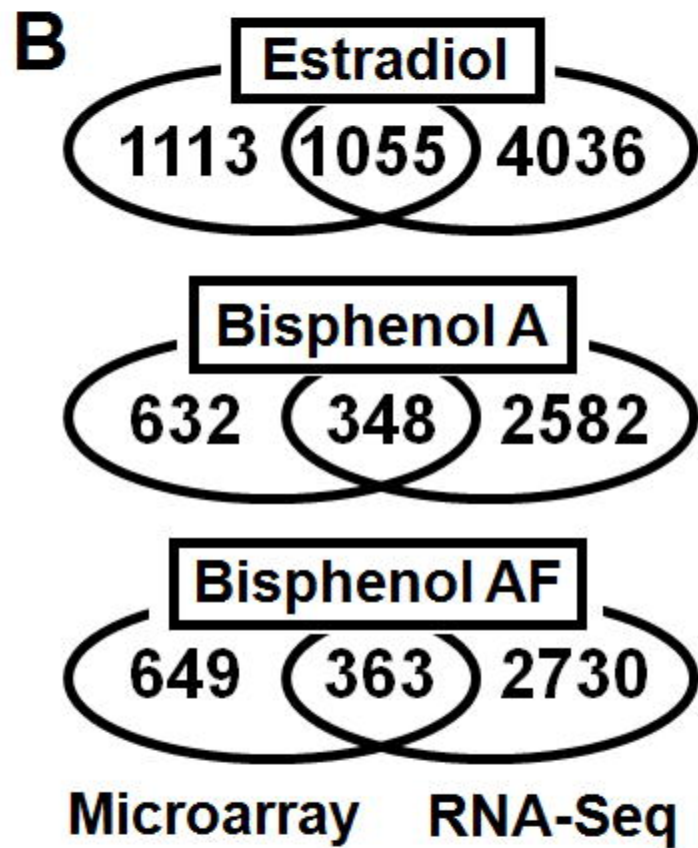
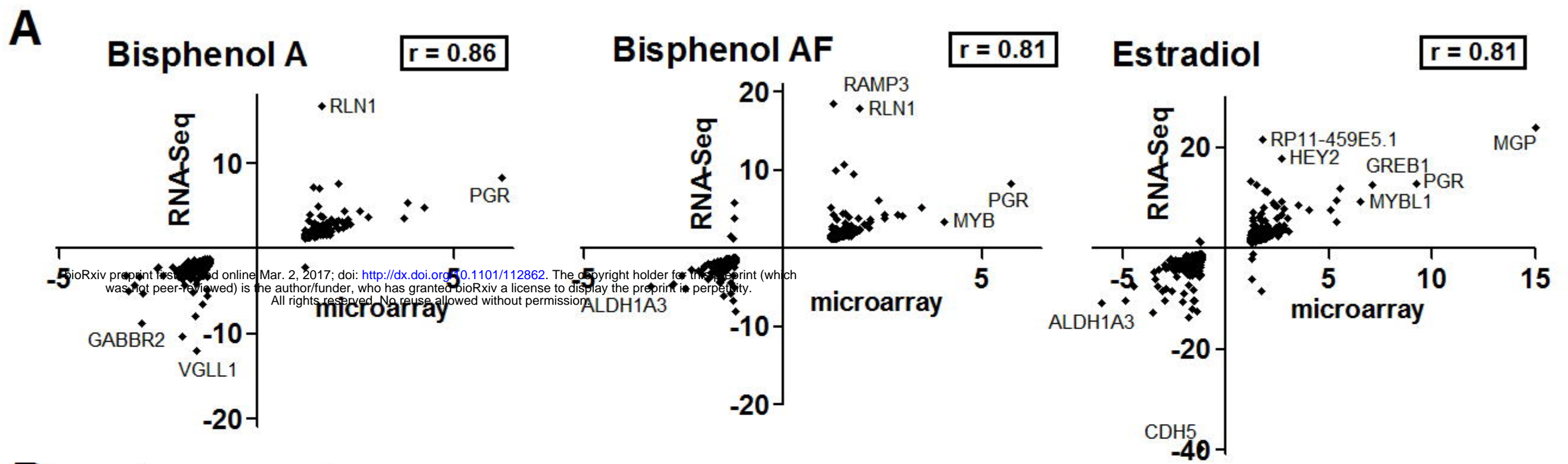
breast neoplasms (MeSH ID001943)

- log₁₀ FDR corrected p-values**D**

ESR1 transcription factor binding sites

	A	E	p-value
Estradiol (10 pM)	95	16.1	8.1e-45
Estradiol (100 pM)	226	44.6	4.1e-94
Estradiol (1nM)	276	52.8	7.8e-120
Bisphenol A	150	23.4	4.9e-76
Bisphenol AF	134	24.6	8.3e-59
Bisphenol AP	158	26.7	9.0e-75
Bisphenol B	141	22.7	1.7e-69
Bisphenol F	151	30	1.3e-61
Bisphenol S	217	44.8	2.3e-86
Bisphenol Z	124	22.8	2.3e-54

F**E**



▬ Microarray ■ RNA-Seq