

Associations between plasma levels of brominated flame retardants and methylation of DNA from peripheral blood: a cross-sectional study in a cohort of French women.

Authors: Hanane Omichessan(1), Vittorio Perduca(2,1), Silvia Polidoro(3), Marina Kvaskoff(1), Thérèse Truong(1), German Cano-Sancho(4), Jean-Philippe Antignac(4), Laura Baglietto(5), Francesca Romana Mancini(1), Gianluca Severi*(1,6)

1. Université Paris-Saclay, UVSQ, INSERM, CESP U1018, “Exposome, Heredity, Cancer and Health” Team, Gustave Roussy, Villejuif, France
2. Laboratoire MAP5 (UMR CNRS 8145), Université de Paris, Paris, France
3. Italian Institute for Genomic Medicine (IIGM), Candiolo, Italy
4. LABERCA, Oniris, INRAE, 44307, Nantes, France
5. Department of Clinical and Experimental Medicine, University of Pisa, Italy
6. Department of Statistics, Computer Science, Applications “G. Parenti”, University of Florence, Italy

***Corresponding Author:**

Gianluca Severi

Head - Group “Exposome, Heredity, Cancer and Health”

CESP (UMR Inserm 1018)

Université Paris-Saclay, UVSQ

Gustave Roussy

Villejuif, France

ph: +33 1 42 11 41 48

Abstract

Background: Brominated flame retardants (BFRs) are organic compounds that are widespread in the environment. Because of their persistence, they are able to bioaccumulate with major impacts on human health. It has been hypothesized that the effect of BFRs on human health is mediated by alterations of DNA methylation.

Objective: The aim of this study was to examine the association between methylation of DNA extracted from peripheral blood and circulating levels of BFRs measured in plasma.

Methods: We conducted a methylation wide association study on 336 blood samples from a study within the E3N (Etude Epidémiologique auprès de femmes de l'Education Nationale) cohort, a long-term longitudinal cohort of French women. DNA methylation at more than 850,000 cytosine-guanine dinucleotide (CpG) sites was measured with the Illumina Infinium HumanMethylation - EPIC BeadChip. Circulating levels of seven BFRs (BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154 and PBB-153) were measured by gas chromatography coupled to high-resolution mass spectrometry in plasma samples. The association between DNA methylation and BFRs plasma levels was assessed through linear mixed-effects models followed by gene-set enrichment analyses (GSEA).

Results: We identified 253 CpG sites whose methylation levels were significantly associated with exposure to BFRs after Bonferroni correction. For 50 of these CpGs the p-values were less than 2.2×10^{-9} with the strongest association being between BDE-154 and cg23619365 (4.32×10^{-13}). GSEA of CpG sites associated with exposure to BFRs identified significant enrichment of genes involved in hypoxia, glycolysis and adipogenesis.

Conclusions: Exposure to BFRs appears to be related to numerous alterations in DNA methylation. These findings, if replicated in

independent studies, provide insights into the biological and health effects of BFRs.

Key words

Endocrine disrupting chemicals, environmental exposure, epigenome, EWAS.

Highlights

- We analysed 336 blood samples from a French cohort of adult women to assess the association between circulating levels of brominated flame retardants (BFRs) and epigenome-wide methylation levels.
- Circulating levels of BFRs are associated with changes in methylation levels in at least 253 CpGs.
- Alterations in methylation levels associated with circulating levels of BFRs appear in particular in genomic regions involved in hypoxia, glycolysis and adipogenesis.
- Our findings suggest that altered methylation may be one of the mechanisms of biological action of exposure to BFRs in adults.

Introduction

Endocrine disrupting chemicals (EDCs) are “exogenous substances or mixtures that alter the function(s) of the endocrine system, causing adverse health effects in an intact organism, its progeny, or (sub)populations” (Gore et al., 2015; Kassotis et al., 2020). The effect of EDCs on biological systems and their widespread presence in the environment, including in food, have led to growing concerns about

their impact on human health. Of particular concern are persistent organic pollutants (POPs), a class of EDCs characterised by long-range transport, persistence in the environment, and the ability to biomagnify and bioaccumulate in ecosystems. Flame retardants are a group of chemicals used to reduce the flammability of combustible materials. The most common flame retardants contain bromine and are therefore known as brominated flame retardants (BFRs). These compounds are added to a wide variety of consumer goods, including electronics, furniture, building materials, and automobiles components, to make them less flammable. Widely produced in the past, the international community has called for actions to reduce and eliminate their production, use and release through the Stockholm Convention, an international legally binding instrument that entered into force on 17 May 2004 (<http://chm.pops.int/>). However, after being widely used for decades, these compounds have migrated from consumer products into the environment and human tissues due to their persistence and bioaccumulation properties.

BFRs are lipophilic compounds classified as POPs that accumulate in fat tissues and disrupt the endocrine system (Dishaw et al., 2014). They can be found in breastmilk and several other routes of exposures have been reported such as indoor air inhalation, dermal contact, dust ingestion and diet (Geyer et al., 2004). Health effects of BFRs have been mostly investigated for polybrominated diphenyl ethers (PBDEs) and polybrominated biphenyls (PBBs). PBDEs congeners have been suspected to be involved in the disruption of thyroid hormones (Shrestha et al., 2015; Liu et al., 2017; Byrne et al., 2018). Due to the similarity in chemical structures of PBDEs and thyroid hormones triiodothyronine (T3) and thyroxine (T4), it is believed that PBDEs have the potential to mimic and disrupt homeostatic conditions (Costa et al., 2014). Moreover studies have reported an association between PBDEs exposure and neurotoxicity (He et al., 2011; Hendriks and

Westerink, 2015), reproductive dysfunctions (Khalil et al., 2017), and diabetes (Ongono et al., 2019).

An epidemiological study conducted on the Michigan PBB Registry based on a population accidentally exposed to high levels of PBBs found an increased risk of lymphomas and cancers of the digestive tract, including esophagus, stomach, liver, and pancreas cancers, as well as breast cancer (Greeson et al., 2020). Evidence of the association between exogenous chemical compounds and epigenetic modifications like DNA methylation is increasing, being acknowledged as one of the key mechanisms of action of EDCs (La Merrill et al., 2020). DNA methylation is a biochemical process that refers to the catalytic addition of a methyl (-CH₃) group to the fifth carbon position of a DNA base. It is a critical epigenetic mechanism for gene expression whose alteration may contribute to the development of various conditions and diseases (Handy et al., 2011).

To date, little is known about the effects of BFRs on DNA methylation. Findings suggest that the joint exposure to the pesticide dichlorobiphenyl trichloroethane (DDT) and PBDEs is associated with global hypomethylation (Huen et al., 2014; Kim et al., 2010). While a study reported no association between BDE-47 and global DNA methylation (Karimi et al., 2011), a study conducted within the British Birth Cohort suggested that high maternal BDE-47 exposure was associated with decreased cord blood methylation levels in the promoter region of tumor necrosis factor (TNF) alpha, a proinflammatory gene (Dao et al., 2015). Additionally, blood levels of PBBs have been associated with 1890 CpGs related to immune function and endocrine-related auto-immune disease (Curtis et al., 2019).

We conducted a methylome-wide association study (MWAS) using a case-control study on breast cancer nested in the French E3N cohort of women to investigate the association between plasma levels of

BFRs and methylation in individual CpGs using DNA extracted from peripheral blood samples. We conducted the MWAS for each of the seven measured BFRs belonging to the PBDEs and PBB-153. Our hypothesis was that alterations in DNA methylation could be used as markers of exposure to BFRs.

Materials and Methods

Study population and sample

We used data from a case-control study on breast cancer nested in the E3N (*Etude Epidémiologique auprès de femmes de l'Education Nationale*) prospective cohort involving approximately 100,000 French women born between 1925 and 1950, living in metropolitan France at inclusion in 1990, and affiliated to the French national health insurance for workers of the Educational System and their families, the Mutuelle Générale de l'Education Nationale (MGEN). Women were enrolled in the cohort after returning a baseline self-administered questionnaire on their lifestyle and medical history, along with informed and written consent. Follow-up questionnaires were sent every 2-3 years thereafter to update information about lifestyle, diet, medical history, use of drugs and other treatments. Average response rate at each follow-up round was about 83%, and the overall loss to follow-up since 1990 was 3%. Details about the E3N cohort have been published previously (Clavel-Chapelon et al., 1997; Clavel-Chapelon and E3N Study Group, 2015). The study received ethical approval from the French Commission Nationale de l'Informatique et des Libertés (CNIL).

The case-control study nested in the E3N cohort aimed at evaluating the association between plasma levels of BFRs and breast cancer risk

included 197 incident cases of breast cancer (aged between 47 and 72 years at baseline) and 197 controls selected using density sampling and matched to cases by age (± 2 years), menopausal status (premenopausal or postmenopausal), body mass index (BMI) (< 25 or ≥ 25 kg/m²), all at blood collection, and year of blood collection (period 1994-1999). Details about the case-control study have been published previously (Mancini et al., 2020). The present MWAS was conducted in a subset of 168 breast cancer cases and 168 controls for which both BFRs and methylation data were available.

Assessment of BFRs plasma levels

Plasma circulating levels of BFRs have been measured by the LABERCA laboratory (Oniris Nantes, FRANCE; ISO 17025:2005 accredited laboratory). The methods used to isolate, detect, and quantify the PBDE congeners (BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154) and PBB-153 have been previously validated and published elsewhere (Cariou et al., 2005; Mancini et al., 2020). Briefly, plasma samples were first submitted to a liquid/liquid extraction with pentane and the resulting extracts were weighed to measure fat content using an enzymatic method (Biolabo; Maizy, France) before reconstitution in hexane for further purification. Then, determinations were performed using gas chromatography (Agilent 7890A) coupled to high-resolution mass spectrometry (GC-HRMS) on double sector instruments (JEOL MS 700D and 800D) after electron impact ionization (70 eV), operating at 10000 resolutions (10% valley) and in the single ion monitoring (SIM) acquisition mode. Finally, as described by Akins and colleagues (Akins et al., 1989), the total plasma lipid (TPL) levels were calculated by combining the concentration of phospholipids (PHO), triacylglycerides (TAG), total cholesterol (t.CHO) and free cholesterol (f.CHO) as follows: $TPL = 1.677 * (t.CHO - f.CHO) + f.CHO + TAG + PHO$.

Due to their lipophilic nature and their tendency to bioaccumulate in tissues, we have chosen to contrast the amount of BFRs circulating levels measured in nanograms with the amount of lipids in plasma measured in grams. For BDE-47, BDE-99, BDE-100, BDE-153, PBB-153, all samples have been quantified – i.e. none was below the limit of detection (LOD). The limits of detection (LOD) were 0.03 ng/L for BDE-28, 0.02 ng/L for BDE-154, and 0.1 ng/L for BDE-47, BDE-99, BDE-100, BDE-153 and PBB-153. For those samples for which levels of BDE-28 or BDE-154 were below the LOD (1 and 99 samples respectively) the measure has been replaced by half of the LOD.

To ensure the quality of the analysis, besides the appropriate use of internal standards in each sample, $^{13}\text{C}_{12}$ labeled standards were added at the end of each clean-up process in order to calculate recoveries that ranged between 60 and 120%. The accuracy was determined according the ISO 17025 requirements, setting up the lower spiking level at 0.5 ng/L ($n = 5$), being the trueness within the range between 3% (BDE-99) and 37% (BDE-28), and the precision within the range between 13% (BDE-47) and 28% (PBB153). The analytical performance was considered acceptable for all congeners; however, it should be noted the analytical uncertainty associated to lower concentrations for BDE28. Cleaned laboratory glassware was rinsed with dichloromethane prior to use and the analyses were carried out in an over-pressurized room to minimize environmental contamination followed by a continuous monitoring of the analytical procedure implemented through procedural blanks (Udesky et al., 2019). The blank controls were not deducted because of the absence of analytical contamination.

DNA methylation measurement and data pre-processing

The Infinium Human Methylation EPIC array has been used on circulating DNA to quantify methylation in more than 850,000 CpG sites. DNA was extracted from archived buffy coats.

DNA extraction, bisulfite conversion, quality control analyses, running of the methylation assays as well as the methylation data pre-processing were performed at the Italian Institute of Genomic Medicine (IIGM) in Turin, Italy, according to manufacturers' protocols and procedures developed by IIGM for previous studies on DNA methylation (Fasanelli et al., 2019, 2015).

Genomic DNA was extracted from buffy coats using the QIAasympy DNA Midi Kit (Qiagen, Hilden, Germany). Five hundred nanograms (1 microgram for a few samples) of DNA were bisulfite-converted using the EZ-96 DNA Methylation-Gold™ Kit (Zymo, California, USA) and hybridized to Infinium Human Methylation EPIC BeadChips (Illumina, California, USA). Each chip was subsequently scanned using the Illumina HiScanSQ system, and sample quality was assessed using control probes on the microarrays. Raw intensity data were finally exported from Illumina GenomeStudio (version 2011.1).

Samples were distributed into 96-well plates and processed in chips of 12 arrays (8 chips per plate) with case–control pairs arranged randomly on the same chip. Data pre-processing was carried out using in-house software written in the R statistical computing environment (Fasanelli et al., 2015).

For each sample and each probe, measurements were set to missing if obtained by averaging intensities over less than three beads, or if averaged intensities were below detection thresholds estimated from negative control probes. Background subtraction (to remove background noise) and dye bias correction (for probes using the Infinium II design) were also performed. The resulting subset of 867

867 CpG loci was selected for further analyses, and among these, probes with missing values in more than 5% of the samples were excluded from the analyses, leaving 805 837 probes. The initial set includes 380 samples with those including more than 5% of non-detected probes and unmatched pairs excluded from the analysis.

The final dataset included 168 case-control pairs that passed the pre-processing step and methylation measures for 805 837 CpGs.

For each CpG site we calculated a beta value, defined as the ratio of the corresponding methylated probe intensity over the overall intensity (sum of methylated and unmethylated probe intensities). We considered the log₂ ratio of beta values to conduct the association studies.

Cellular heterogeneity

Methylation profile varies with cell type, depending on the individual; adjustment for the proportion of cell types was then necessary. Based on the method developed by Houseman and colleagues (Houseman et al., 2012), we estimated from the methylation data the proportions of cell types in each blood sample, including B cells, CD4⁺ T cells, CD8⁺ T cells, granulocytes, monocytes and natural killer cells.

Statistical analysis

Main analyses

We assessed the association between DNA methylation and individual BFRs plasma levels through linear mixed-effects models with DNA methylation as response variable and median value within quartiles of BFR levels as exposure; plate and chips were included into the models as random effects, thus making possible to account for the random variability of methylation levels due to technical sources. Also, we fitted models adjusting for age at blood collection

(below or above the median, that is 56.1), parity and total breastfeeding duration (no children and no breastfeeding, at least 1 child and ≤ 6 months breastfeeding, at least 1 child and > 6 months breastfeeding), BMI (≤ 25 kg/m², > 25 kg/m²), and cell type proportions. Using the same model, we also evaluated whether global and regional methylation (average level over all CpG sites across the whole genome and across specific regions) may be associated with exposure to BFRs.

Because the women in our dataset were selected conditionally on their breast cancer status, the breast cancer probability in our dataset (50% by design) was much larger than in the general population. In the linear mixed regression analyses, to account for the over-representation of breast cancer cases in the data set compared to the general population, all models were weighted according to the method proposed by van der Laan (van der Laan, 2008; VanderWeele and Vansteelandt, 2010). Specifically, each case and control was given a weight of $p/0.5$ and $(1-p)/0.5$ respectively, where p was set to 0.08 to reflect the proportion of breast cancer cases in the general population of women aged more than 40 years. We adjusted for multiple testing by controlling the Family Wise Error Rate (FWER) through Bonferroni adjusted p-values. We also controlled the False Discovery Rate (FDR) by computing q-values using the qvalue R package (Storey, 2011) and calling all tests with q-values below 5% significant.

Sensitivity analysis

As an alternative correction of the aforementioned selection bias, we have also fitted models on controls only (i.e. on women that have not been diagnosed with breast cancer at the date of diagnosis of the matched case).

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) (Subramanian et al., 2005) is a computational method that determines whether members of *a priori-defined* gene set are over-represented at the top (or bottom) of a ranked list of markers which have been ordered by their correlation with a specific phenotype, in our case the levels of each specific BFR. For each BFR, we performed a GSEA using CpG sites that are located in promoter regions and are significantly associated with the BFR circulating levels (unadjusted p-value < 5%). GSEA produces a ranked list of gene sets based on an enrichment score and this score was considered ‘significant’ when the FDR < 25% (see the [GSEA user guide](#)¹ for more details). To explore the target gene sets associated with levels of BFRs we used the hallmark gene set v7.4 (Liberzon et al., 2015), a collection of 50 gene sets representing well-defined biological states. We conducted our analysis using GSEA_4.1² which includes a function to compute the corresponding q-values. The parameter “Enrichment statistics” was set to the “classic” item, and the parameter “Metric for ranking genes” was set to “Pearson”. 1000 permutations were carried out to evaluate the FDR and the p-value of the enrichment score with permutation type set to “gene_set”.

Results

Baseline characteristics of the study participants

The baseline characteristics of the 336 study participants are summarized in Table 1. Median age of the study participants was 56.1 years and most of them had a healthy body mass index with only one quarter of them being overweight or obese. About 39% of them were nulliparous or never breastfed, 40% had at least one child but

¹<https://www.gsea-msigdb.org/gsea/doc/GSEAUUserGuideFrame.html>

²https://software.broadinstitute.org/cancer/software/gsea/wiki/index.php/GSEA_v4.1.x_Release_Notes

breastfed for less than 6 months while the remaining 20% had breastfed for more than 6 months.

	N= 336
Age (years), median [IQ range]	56.10 [51.08, 60.60]
BMI (kg/m ²), mean (SD)	23.66 (3.40)
Parity, N (%)	
nulliparous and no breastfeeding	133 (39.6)
parous with less than 6 months of lactation	136 (40.5)
parous with more than 6 months of lactation	67 (19.9)
BDE-28(ng/g of lipids), median [IQ range]	0.04 [0.03, 0.06]
BDE-47(ng/g of lipids), median [IQ range]	0.61 [0.40, 0.87]
BDE-99(ng/g of lipids), median [IQ range]	0.13 [0.10, 0.21]
BDE-100(ng/g of lipids), median [IQ range]	0.17 [0.12, 0.26]
BDE-153(ng/g of lipids), median [IQ range]	0.53 [0.42, 0.67]
BDE-154(ng/g of lipids), median [IQ range]	0.03 [0.02, 0.05]
PBB-153(ng/g of lipids), median [IQ range]	0.31 [0.24, 0.40]

Table 1. Baseline characteristics of the studied population (N = 336).

Regarding PBDEs levels, tables 1 and 2 show that BDE-47 was the predominant congener with a median concentration of 0.61 ng/g of lipids and a large variation in levels across women (min-max: 0.07 to 11.4 ng/g of lipids). High concentration was also observed for BDE-99. For the only PBB studied (PBB-153), we found relatively high concentrations with a median level of 0.31 ng/g of lipids (min-max: 0.11 and 11.12 ng/g of lipids).

	Min.	Mean	Max.
BDE-28(ng/g of lipids)	0.01	0.06	0.71
BDE-47(ng/g of lipids)	0.07	0.85	11.4
BDE-99(ng/g of lipids)	0.02	0.21	4.12
BDE-100(ng/g of lipids)	0.03	0.24	2.84
BDE-153(ng/g of lipids)	0.22	0.58	2.32
BDE-154(ng/g of lipids)	0	0.04	0.28
PBB-153(ng/g of lipids)	0.11	0.42	11.12

Table 2. Summary statistics of selected BFRs (ng/g of lipids).

Figure 1 presents the correlation between PBDEs that was particularly high for BDE-47, BDE-99 and BDE-100.

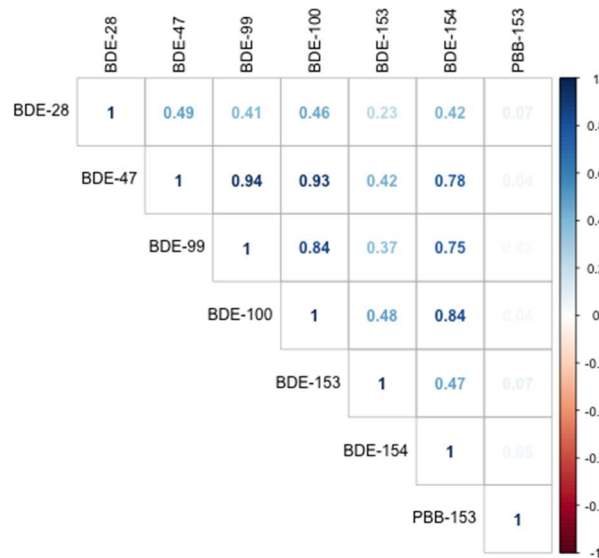
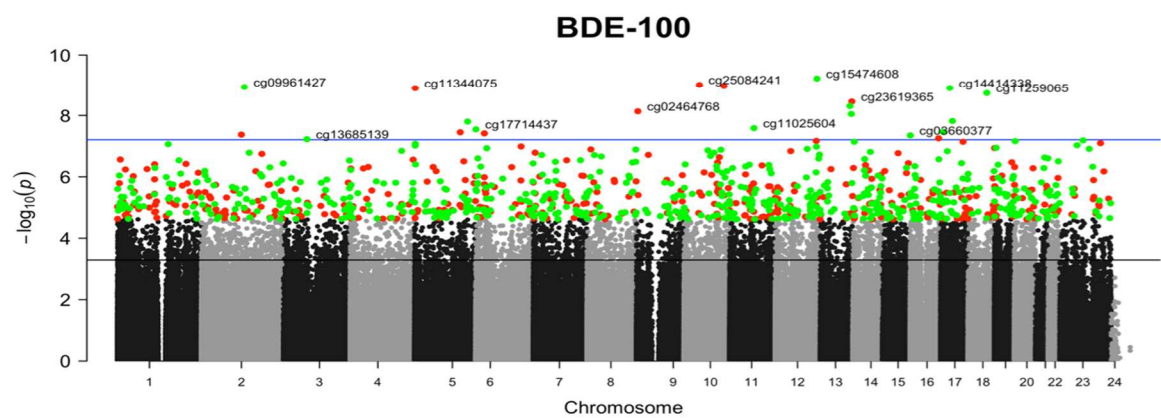
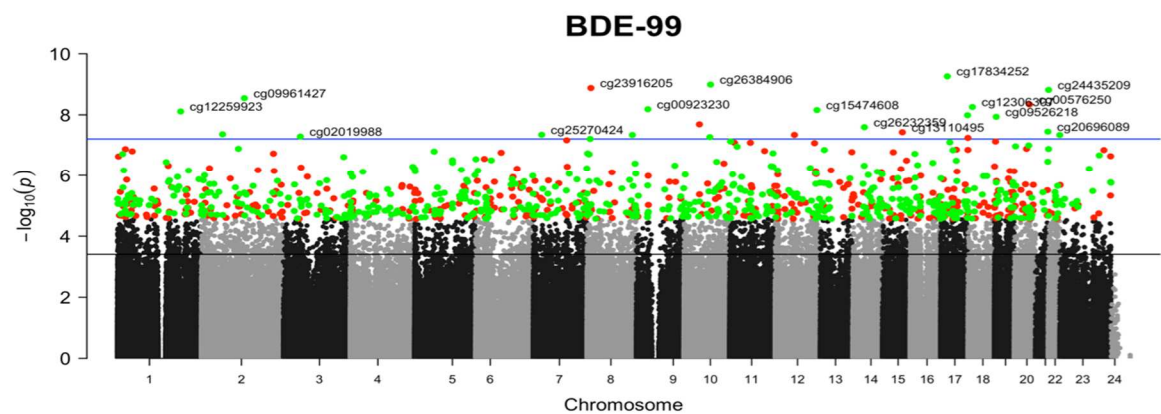
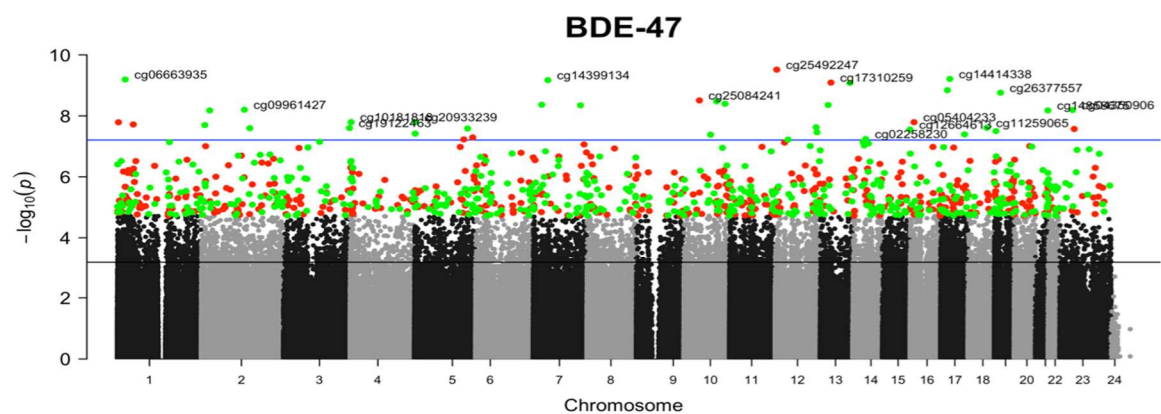
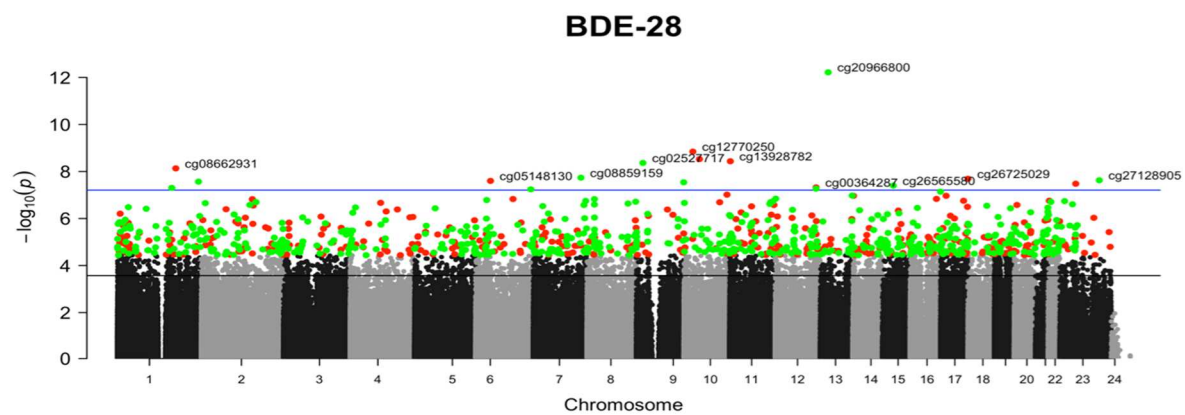


Figure 1. Correlation between the circulating levels of BFRs congeners.

Association between plasma levels of BFRs and DNA methylation

We identified 253 CpGs sites where DNA methylation levels were associated with circulating levels of BFRs at a genome-wide level of significance, see Figure 2. For each congener, the top CpG sites ranked according to their p-values are shown in Supplementary tables 1-7. All of them showed strong associations even after multiple testing Bonferroni correction. For most of the considered BFRs, the majority of CpG sites that were significantly associated with exposure after Bonferroni correction showed hypermethylation, i.e. positive correlation with exposure. Only for BDE-153 and BDE-154 the majority of the CpGs in the top list showed hypomethylation, i.e. negative correlation with exposure (Supplementary table 8).

(a)



(b)

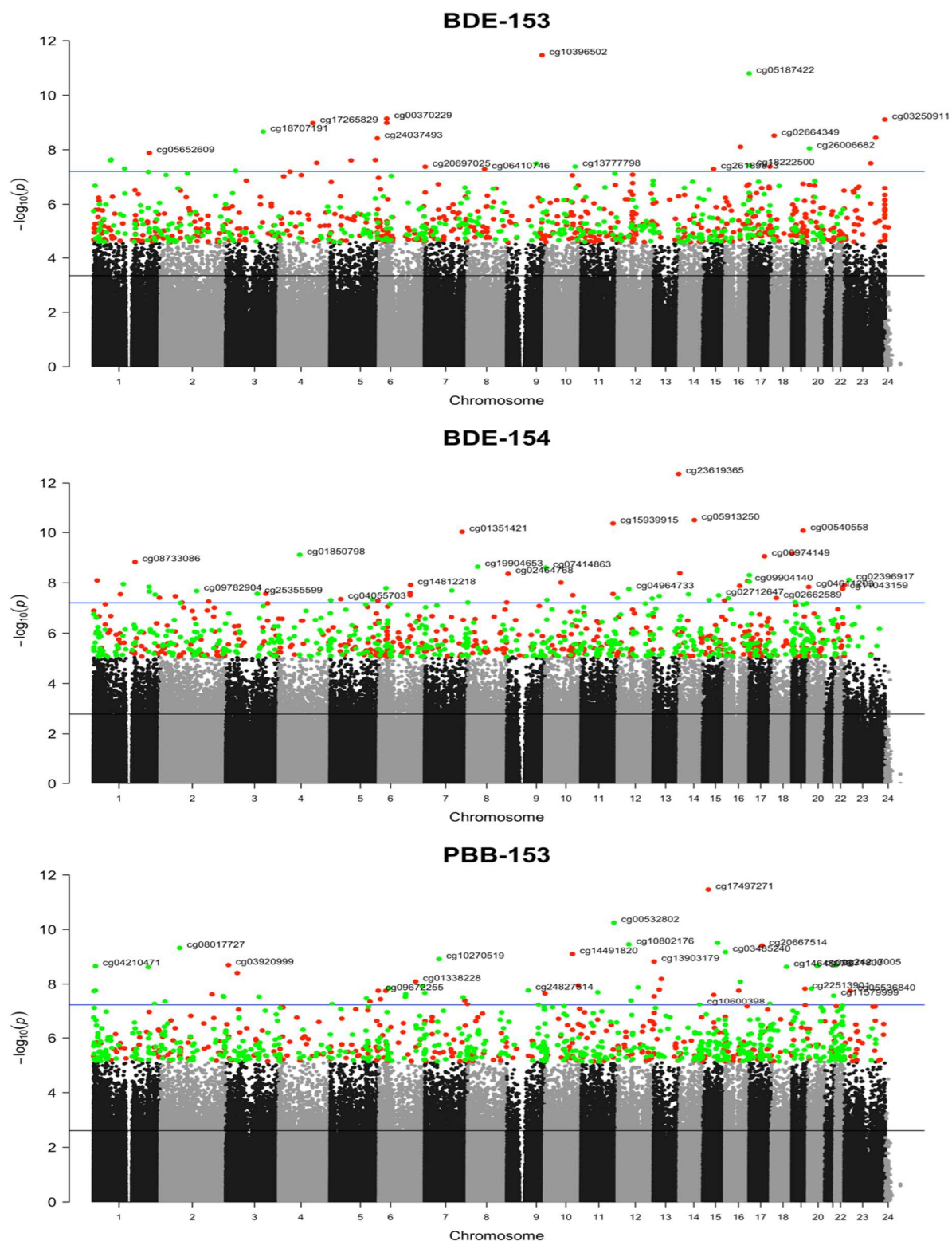


Figure 2 (a)-(b). Manhattan plots of the association between BFR congeners and 805 837 CpG sites. The y-axis shows the $-\log_{10}$ p-

values of the tests of associations between DNA methylation and circulating levels of BFRs from our linear mixed model analyses. The black line corresponds to a FDR threshold of 5% while the blue line to the FWER 5% threshold. Green and red colors represent the top 1000 respectively hyper- and hypo-methylated CpGs.

As shown in Supplementary figure 1, the estimates of BFR coefficients in our main models fitted on breast cancer cases and controls were consistent with the estimates of the coefficients of the models fitted on controls only.

At genome wide level, we did not find significant association with considered BFRs (Table 3). At regional level (Table 4), BDE-154 was significantly associated with hypomethylation within TSS1500/TSS200 and promoter regions. BDE-47 and BDE-99 were also associated with hypomethylation within promoter regions.

	<i>Coefficients</i>	<i>Std</i>	<i>p</i>
BDE-28	0.001	0.002	0.652
BDE-47	-0.004	0.003	0.210
BDE-99	-0.001	0.004	0.805
BDE-100	-0.001	0.003	0.789
BDE-153	-0.000	0.002	0.945
BDE-154	0.001	0.002	0.558
PBB-153	0.005	0.007	0.481

Table 3. Association between average methylation across the whole genome and circulating levels of BFRs.

	Island or Shore			Shelf or None			Promoter			Other		
	<i>Coefficients</i>	<i>Std</i>	<i>p</i>	<i>Coefficients</i>	<i>Std</i>	<i>p</i>	<i>Coefficients</i>	<i>Std</i>	<i>p</i>	<i>Coefficients</i>	<i>Std</i>	<i>p</i>
BDE-28	0.000	0.003	0.873	0.002	0.004	0.696	-0.002	0.006	0.678	0.002	0.003	0.629
BDE-47	-0.005	0.003	0.094	-0.004	0.005	0.441	-0.010	0.007	0.127	-0.004	0.004	0.278
BDE-99	-0.006	0.005	0.189	0.001	0.008	0.850	-0.014	0.01	0.152	-0.001	0.005	0.914
BDE-100	-0.005	0.003	0.099	0.001	0.005	0.805	-0.008	0.007	0.195	-0.000	0.004	0.903
BDE-153	-0.003	0.002	0.159	0.001	0.003	0.646	-0.003	0.004	0.480	0.000	0.002	0.943
BDE-154	-0.005	0.002	0.019	0.004	0.003	0.205	-0.012	0.004	0.006	0.002	0.002	0.375
PBB-153	-0.013	0.008	0.092	0.023	0.012	0.061	-0.020	0.016	0.233	0.008	0.009	0.390

(a)

	TSS1500 or TSS200						Promoter					
	Island or Shore			Shelf or None			Island and Shore			Shelf or None		
	<i>Coefficients</i>	<i>Std</i>	<i>p</i>	<i>Coefficients</i>	<i>Std</i>	<i>p</i>	<i>Coefficients</i>	<i>Std</i>	<i>p</i>	<i>Coefficients</i>	<i>Std</i>	<i>p</i>
BDE-28	-0.000	0.003	0.971	0.000	0.003	0.892	-0.001	0.006	0.828	-0.004	0.003	0.192
BDE-47	-0.006	0.004	0.139	-0.005	0.004	0.158	-0.009	0.007	0.148	-0.008	0.004	0.026
BDE-99	-0.007	0.006	0.223	-0.002	0.006	0.709	-0.012	0.01	0.198	-0.011	0.005	0.043
BDE-100	-0.005	0.004	0.159	-0.002	0.004	0.671	-0.008	0.006	0.226	-0.006	0.004	0.087
BDE-153	-0.003	0.002	0.244	-0.000	0.002	0.921	-0.002	0.004	0.572	-0.002	0.002	0.243
BDE-154	-0.007	0.003	0.011	0.001	0.003	0.656	-0.012	0.004	0.007	-0.007	0.002	0.006
PBB-153	-0.017	0.01	0.069	0.019	0.009	0.046	-0.02	0.016	0.213	0.001	0.009	0.89

(b)

Table 4. Association between average methylation across specific regions and circulating levels of BFRs.

GSEA

A GSEA identified seven gene sets positively enriched at a FDR level of 0.25 (Table 5), of which four are positively enriched, meaning that the levels of DNA methylation of CpG sites included in the gene set are positively correlated with the plasma concentrations

of the corresponding BFRs. These results suggest that plasma concentrations of BFRs may be associated with methylation levels in genes involved in processes such as adipogenesis (BDE-99), glycolysis (BDE-100) and hypoxia (BDE-154).

BFRs congeners	Gene set	# genes	Enrichment Score	Negative normalized Enrichment Score	p-value	q-value
BDE-47	HALLMARK_IL2_STAT5_SIGNALING	76	0.17	1.73	0.03	0.22
BDE-99	HALLMARK_ADIPOGENESIS	88	-0.2	-2.19	0	0.02
BDE-100	HALLMARK_APICAL_JUNCTION	49	-0.23	-1.89	0.01	0.23
	HALLMARK_GLYCOLYSIS	80	-0.18	-1.87	0.01	0.12
BDE-153	HALLMARK_UV_RESPONSE_DN	45	0.28	2.24	0	0.03
BDE-154	HALLMARK_HYPOXIA	97	0.18	02.09	0	0.07
	HALLMARK_IL2_STAT5_SIGNALING	91	0.18	02.05	0.01	0.04
	HALLMARK_TNFA_SIGNALING_VIA_NFKB	105	0.16	1.95	0.01	0.05

Table 5. Gene set enrichment analysis results for gene sets with FDR q-value < 0.25. For each gene set, the number of genes in the set with CpG sites associated with a BFR is given, together with enrichment statistics.

Discussion

BFRs exposure has become an increasingly important global public health problem given the contamination of the environment, their tendency to bioaccumulate in human tissue and their effects on biological systems that are yet to be fully elucidated. Our underlying hypothesis is that BFRs may alter methylation levels in human DNA.

Through such alterations, BFRs would eventually exert multiple actions on human health.

Our analyses, based on a sample of 336 women from the French E3N cohort, provided convincing evidence of association between BFRs plasma concentrations and methylation levels of blood DNA at multiple CpG sites and in pathways of gene sets involved in hypoxia, glycolysis and adipogenesis.

To the best of our knowledge, this is the first epigenome-wide association study which involves multiple BFRs and DNA methylation in blood samples from adults. Previous studies showed that endocrine disruptors such as phthalates (Chen et al., 2018) or bisphenols (Laing et al., 2016; Liu et al., 2016) were associated with hypomethylation. Most of these studies analysed repetitive genomic elements as markers of global methylation (i.e. Alu and LINE-1) or used cell lines or animal models, mostly rodents or birth cohorts. Focus on early-life is explained by the fact that the epigenome undergoes extensive reprogramming throughout fetal development at gametogenesis and early embryo preimplantation, representing vulnerable stages to environmental exposure (Alvarado-Cruz et al., 2018).

In our data, we found no significant associations between global methylation and BFR levels in contrast with some previous studies that reported global hypomethylation or hypomethylation in target genes or genomic regions in relation to perinatal exposure to BDE-47 in brain tissue from murine models or in murine neuroblastoma cells (Bastos Sales et al., 2013; Byun et al., 2015; Woods et al., 2012). These findings are consistent with our observation of hypomethylation in some regions in relation to BFR levels (e.g. BDE-154 and average hypomethylation in gene promoters). The lack of association with average global methylation is consistent with the

findings from a study in a Korean population that observed no association between exposure to BDE-47 and BDE-99 and global methylation using LINE-1 and Alu assays (Kim et al., 2010).

In studies conducted in the placenta, an increase in DNA methylation as assessed by LUMA of imprinting genes (IGF2/H19) was observed in relation to PBDEs and other POPs congeners (Kappil et al., 2016). Moreover, low-level increases in methylation of CpG islands was observed in an in-vitro primary villous cytotrophoblasts model in response to exposure to BDE-47 (Robinson et al., 2019).

More recently, microarray technology was used to investigate the association between exposure to specific BFRs and methylation at CpG level in human populations. In particular, an epigenome-wide study in DNA from placenta identified 133 CpG sites with altered methylation levels in relation to exposure to BDE-47 (Ouidir et al. 2020). A study based on the Michigan PBB Registry found that methylation levels at 1890 CpG sites were associated with total PBB serum levels in a highly exposed population (Curtis et al., 2019). Using the same methylation array, which covers almost 1 million individual CpGs (more than 90% of all CpGs), we assessed methylation of DNA from peripheral blood in relation to serum levels of a broader range of BFR congeners in women from the general population. The levels of BFRs observed in our cohort of French women (Mancini et al., 2020) are similar to those observed in a recent French biomonitoring study³ but substantially lower than levels observed in studies from the U.S. (Alaee, 2003; Mancini et al., 2020). Our study can therefore provide useful insights about the potential sub-clinical effects of BFRs at low doses.

³<https://www.santepubliquefrance.fr/determinants-de-sante/exposition-a-des-substances-chimiques/perturbateurs-endocriniens/documents/rapport-synthese/impregnation-de-la-population-francaise-par-les-retardateurs-de-flamme-bromes-programme-national-de-biosurveillance-esteban-2014-2016>

Our study has some limitations. The main one is the cross-sectional design with BFRs plasma concentrations and DNA methylation levels assessed at the same time point preventing causal inference from the observed associations. For example, we cannot exclude that change in methylation of specific genes involved in the endocrine pathways may affect circulating levels of BFRs. Secondly, our study has a relatively small sample size and is limited to women only. As there are gender-based differences in DNA methylation levels (Kappil et al., 2016; Singmann et al., 2015) our results may not be generalizable to both genders.

While it is established that epigenetic changes including global hypomethylation are observed in tumor DNA, the secondary use of a case-control study on breast cancer is unlikely to have influenced our results for at least two reasons. First, studies on methylation in DNA from blood, including some from our group, have essentially failed to identify clear alterations associated with breast cancer (Bodelon et al., 2019). Second, the case-control study was nested within a longitudinal cohort with cases diagnosed years after the blood collection.

Finally, we cannot exclude that BFRs influence DNA methylation in other target tissues that were not available for this study as well as the possibility that a variety of POP mixtures may contribute to the observed findings.

Conclusion

Our study found evidence of association between BFRs exposure and moderate or strong alterations in circulating DNA methylation at the level of single CpGs and gene sets. These findings provide significant data on the effects of PBDE congeners on epigenetic mechanisms that

in population studies are still scant. Even if toxicity of these compounds in early development has been demonstrated by previous research, the suggestive evidence of association between BFRs exposure and blood DNA methylation alterations in specific gene pathways is intriguing as it might reflect a more complex and wider action of this class of substances.

Credit author statement

Hanane Omichessan: Formal analysis, Software, Conceptualization, Writing original draft; Vittorio Perduca: Conceptualization, Writing original draft, Supervision; Silvia Polidoro: Investigation, Resources; Marina Kvaskoff: Conceptualization, Funding acquisition; Thérèse Truong: Conceptualization; German Cano-Sancho: Investigation, Resources, Conceptualization; Jean-Philippe Antignac: Investigation, Resources; Laura Baglietto: Conceptualization, Writing original draft; Francesca Romana Mancini: Conceptualization, Funding acquisition; Gianluca Severi: Supervision, Conceptualization, Writing original draft, Resources, Funding acquisition.

Acknowledgements

The research was carried out using data from the Inserm (French National Institutes for Health and Medical Research) E3N cohort, which was established and maintained with the support of the Mutuelle Générale de l'Éducation Nationale (MGEN), Gustave Roussy, and the French League against Cancer (LNCC). E3N-E4N is also supported by the French National Research Agency (ANR) under the Investment for the future Program (PIA) (ANR-10-COHO-0006) and by the French Ministry of Higher Education, Research and Innovation (subsidy for public service charges #2102 918823). We acknowledge the support of a grant from La Ligue contre le Cancer. HO was supported by a PhD fellowship from the French Institut

National du Cancer (INCa_11330). We are grateful to the E3N-E4N study participants for their continued participation. We also thank all members of the E3N-E4N study group, in particular Rafika Chaït, Ghizlane Esselma, Marie Fangon, Pascale Gerbouin-Rérolle, Lyan Hoang, Roselyn Gomes and the data-management team, and Amandine Gelot for data management and/or technical assistance.

References

Akins, J.R., Waldrep, K., Bernert, J.T., 1989. The estimation of total serum lipids by a completely enzymatic ‘summation’ method. *Clin. Chim. Acta* 184, 219–226. [https://doi.org/10.1016/0009-8981\(89\)90054-5](https://doi.org/10.1016/0009-8981(89)90054-5)

Alaee, M., 2003. An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. *Environ. Int.* 29, 683–689. [https://doi.org/10.1016/S0160-4120\(03\)00121-1](https://doi.org/10.1016/S0160-4120(03)00121-1)

Alvarado-Cruz, I., Alegría-Torres, J.A., Montes-Castro, N., Jiménez-Garza, O., Quintanilla-Vega, B., 2018. Environmental Epigenetic Changes, as Risk Factors for the Development of Diseases in Children: A Systematic Review. *Ann. Glob. Health* 84, 212–224. <https://doi.org/10.29024/aogh.909>

Bastos Sales, L., Kamstra, J.H., Cenijn, P.H., van Rijt, L.S., Hamers, T., Legler, J., 2013. Effects of endocrine disrupting chemicals on in vitro global DNA methylation and adipocyte differentiation. *Toxicol. In Vitro* 27, 1634–1643. <https://doi.org/10.1016/j.tiv.2013.04.005>

Bodelon, C., Ambatipudi, S., Dugué, P.-A., Johansson, A., Sampson, J.N., Hicks, B., Karlins, E., Hutchinson, A., Cuenin, C., Chajès, V., Southey, M.C., Romieu, I., Giles, G.G., English, D., Polidoro, S., Assumma, M., Baglietto, L., Vineis, P., Severi, G., Herceg, Z., Flanagan, J.M., Milne, R.L., Garcia-Closas, M., 2019. Blood DNA methylation and breast cancer risk: a meta-analysis of four

prospective cohort studies. *Breast Cancer Res.* 21, 62.
<https://doi.org/10.1186/s13058-019-1145-9>

Byrne, S.C., Miller, P., Seguinot-Medina, S., Waghiyi, V., Buck, C.L., von Hippel, F.A., Carpenter, D.O., 2018. Associations between serum polybrominated diphenyl ethers and thyroid hormones in a cross sectional study of a remote Alaska Native population. *Sci. Rep.* 8, 2198. <https://doi.org/10.1038/s41598-018-20443-9>

Byun, H.-M., Benachour, N., Zalko, D., Frisardi, M.C., Colicino, E., Takser, L., Baccarelli, A.A., 2015. Epigenetic effects of low perinatal doses of flame retardant BDE-47 on mitochondrial and nuclear genes in rat offspring. *Toxicology* 328, 152–159.
<https://doi.org/10.1016/j.tox.2014.12.019>

Cariou, R., Antignac, J.-P., Marchand, P., Berrebi, A., Zalko, D., Andre, F., Le Bizec, B., 2005. New multiresidue analytical method dedicated to trace level measurement of brominated flame retardants in human biological matrices. *J. Chromatogr. A* 1100, 144–152.
<https://doi.org/10.1016/j.chroma.2005.09.040>

Chen, C.-H., Jiang, S.S., Chang, I.-S., Wen, H.-J., Sun, C.-W., Wang, S.-L., 2018. Association between fetal exposure to phthalate endocrine disruptor and genome-wide DNA methylation at birth. *Environ. Res.* 162, 261–270.
<https://doi.org/10.1016/j.envres.2018.01.009>

Clavel-Chapelon, F., E3N Study Group, 2015. Cohort Profile: The French E3N Cohort Study. *Int. J. Epidemiol.* 44, 801–809.
<https://doi.org/10.1093/ije/dyu184>

Clavel-Chapelon, F., van Liere, M.J., Giubout, C., Niravong, M.Y., Goulard, H., Le Corre, C., Hoang, L.A., Amoyel, J., Auquier, A., Duquesnel, E., 1997. E3N, a French cohort study on cancer risk factors. E3N Group. *Etude Epidémiologique auprès de femmes de l'Education Nationale. Eur. J. Cancer Prev. Off. J. Eur. Cancer Prev. Organ. ECP* 6, 473–478.

Costa, L.G., de Laat, R., Tagliaferri, S., Pellacani, C., 2014. A mechanistic view of polybrominated diphenyl ether (PBDE)

developmental neurotoxicity. *Toxicol. Lett.* 230, 282–294.
<https://doi.org/10.1016/j.toxlet.2013.11.011>

Curtis, S.W., Cobb, D.O., Kilaru, V., Terrell, M.L., Kennedy, E.M., Marder, M.E., Barr, D.B., Marsit, C.J., Marcus, M., Conneely, K.N., Smith, A.K., 2019. Exposure to polybrominated biphenyl (PBB) associates with genome-wide DNA methylation differences in peripheral blood. *Epigenetics* 14, 52–66.
<https://doi.org/10.1080/15592294.2019.1565590>

Dao, T., Hong, X., Wang, X., Tang, W.-Y., 2015. Aberrant 5'-CpG Methylation of Cord Blood TNF α Associated with Maternal Exposure to Polybrominated Diphenyl Ethers. *PLOS ONE* 10, e0138815.
<https://doi.org/10.1371/journal.pone.0138815>

Dishaw, L.V., J Macaulay, L., Roberts, S.C., Stapleton, H.M., 2014. Exposures, mechanisms, and impacts of endocrine-active flame retardants. *Curr. Opin. Pharmacol.* 19, 125–133.
<https://doi.org/10.1016/j.coph.2014.09.018>

Fasanelli, F., Baglietto, L., Ponzi, E., Guida, F., Campanella, G., Johansson, Mattias, Grankvist, K., Johansson, Mikael, Assumma, M.B., Naccarati, A., Chadeau-Hyam, M., Ala, U., Faltus, C., Kaaks, R., Risch, A., De Stavola, B., Hodge, A., Giles, G.G., Southey, M.C., Relton, C.L., Haycock, P.C., Lund, E., Polidoro, S., Sandanger, T.M., Severi, G., Vineis, P., 2015. Hypomethylation of smoking-related genes is associated with future lung cancer in four prospective cohorts. *Nat. Commun.* 6, 10192.
<https://doi.org/10.1038/ncomms10192>

Fasanelli, F., Giraudo, M.T., Vineis, P., Fiano, V., Fiorito, G., Grasso, C., Polidoro, S., Trevisan, M., Grioni, S., Krogh, V., Mattiello, A., Panico, S., Giurdanella, M.C., Tumino, R., De Marco, L., Ricceri, F., Sacerdote, C., 2019. DNA methylation, colon cancer and Mediterranean diet: results from the EPIC-Italy cohort. *Epigenetics* 14, 977–988. <https://doi.org/10.1080/15592294.2019.1629230>

Geyer, H.J., Schramm, K.-W., Darnerud, P.O., Aune, M., Feicht, A., Fried, K.W., Henkelmann, B., Lenoir, D., Schmid, P., McDonald, T.A., 2004. Terminal elimination half-lives of the brominated flame

retardants TBBPA, HBCD, and lower brominated PBDEs in humans. *ORGANOHALOGEN Compd.* 66, 7.

Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., Toppari, J., Zoeller, R.T., 2015. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. *Endocr. Rev.* 36, E1–E150.
<https://doi.org/10.1210/er.2015-1010>

Greeson, K.W., Fowler, K.L., Estave, P.M., Kate Thompson, S., Wagner, C., Clayton Edenfield, R., Symosko, K.M., Steves, A.N., Marder, E.M., Terrell, M.L., Barton, H., Koval, M., Marcus, M., Easley, C.A., 2020. Detrimental effects of flame retardant, PBB153, exposure on sperm and future generations. *Sci. Rep.* 10, 8567.
<https://doi.org/10.1038/s41598-020-65593-x>

Handy, D.E., Castro, R., Loscalzo, J., 2011. Epigenetic Modifications: Basic Mechanisms and Role in Cardiovascular Disease. *Circulation* 123, 2145–2156.
<https://doi.org/10.1161/CIRCULATIONAHA.110.956839>

He, J., Yang, D., Wang, C., Liu, W., Liao, J., Xu, T., Bai, C., Chen, J., Lin, K., Huang, C., Dong, Q., 2011. Chronic zebrafish low dose decabrominated diphenyl ether (BDE-209) exposure affected parental gonad development and locomotion in F1 offspring. *Ecotoxicology* 20, 1813–1822. <https://doi.org/10.1007/s10646-011-0720-3>

Hendriks, H.S., Westerink, R.H.S., 2015. Neurotoxicity and risk assessment of brominated and alternative flame retardants. *Neurotoxicol. Teratol.* 52, 248–269.
<https://doi.org/10.1016/j.ntt.2015.09.002>

Houseman, E.A., Accomando, W.P., Koestler, D.C., Christensen, B.C., Marsit, C.J., Nelson, H.H., Wiencke, J.K., Kelsey, K.T., 2012. DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics* 13, 86.
<https://doi.org/10.1186/1471-2105-13-86>

Huen, K., Yousefi, P., Bradman, A., Yan, L., Harley, K.G., Kogut, K., Eskenazi, B., Holland, N., 2014. Effects of age, sex, and persistent

organic pollutants on DNA methylation in children: DNA Methylation in Children. *Environ. Mol. Mutagen.* 55, 209–222. <https://doi.org/10.1002/em.21845>

Kappil, M.A., Li, Q., Li, A., Dassanayake, P.S., Xia, Y., Nanes, J.A., Landrigan, P.J., Stodgell, C.J., Aagaard, K.M., Schadt, E.E., Dole, N., Varner, M., Moye, J., Kasten, C., Miller, R.K., Ma, Y., Chen, J., Lambertini, L., 2016. *In utero* exposures to environmental organic pollutants disrupt epigenetic marks linked to fetoplacental development. *Environ. Epigenetics* 2, dvv013. <https://doi.org/10.1093/eep/dvv013>

Karimi, M., Luttrupp, K., Ekström, T.J., 2011. Global DNA Methylation Analysis Using the Luminometric Methylation Assay, in: Tollefsbol, T.O. (Ed.), *Epigenetics Protocols*. Humana Press, Totowa, NJ, pp. 135–144. https://doi.org/10.1007/978-1-61779-316-5_11

Kassotis, C.D., Vandenberg, L.N., Demeneix, B.A., Porta, M., Slama, R., Trasande, L., 2020. Endocrine-disrupting chemicals: economic, regulatory, and policy implications. *Lancet Diabetes Endocrinol.* 8, 719–730. [https://doi.org/10.1016/S2213-8587\(20\)30128-5](https://doi.org/10.1016/S2213-8587(20)30128-5)

Khalil, A., Parker, M., Brown, S.E., Cevik, S.E., Guo, L.W., Jensen, J., Olmsted, A., Portman, D., Wu, H., Suvorov, A., 2017. Perinatal exposure to 2,2',4'4' –Tetrabromodiphenyl ether induces testicular toxicity in adult rats. *Toxicology* 389, 21–30. <https://doi.org/10.1016/j.tox.2017.07.006>

Kim, K.-Y., Kim, D.-S., Lee, S.-K., Lee, I.-K., Kang, J.-H., Chang, Y.-S., Jacobs, D.R., Steffes, M., Lee, D.-H., 2010. Association of Low-Dose Exposure to Persistent Organic Pollutants with Global DNA Hypomethylation in Healthy Koreans. *Environ. Health Perspect.* 118, 370–374. <https://doi.org/10.1289/ehp.0901131>

La Merrill, M.A., Vandenberg, L.N., Smith, M.T., Goodson, W., Browne, P., Patisaul, H.B., Guyton, K.Z., Kortenkamp, A., Cogliano, V.J., Woodruff, T.J., Rieswijk, L., Sone, H., Korach, K.S., Gore, A.C., Zeise, L., Zoeller, R.T., 2020. Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification. *Nat. Rev. Endocrinol.* 16, 45–57.

<https://doi.org/10.1038/s41574-019-0273-8>

Laing, L.V., Viana, J., Dempster, E.L., Trznadel, M., Trunkfield, L.A., Uren Webster, T.M., van Aerle, R., Paull, G.C., Wilson, R.J., Mill, J., Santos, E.M., 2016. Bisphenol A causes reproductive toxicity, decreases *dnmt1* transcription, and reduces global DNA methylation in breeding zebrafish (*Danio rerio*). *Epigenetics* 11, 526–538. <https://doi.org/10.1080/15592294.2016.1182272>

Liberzon, A., Birger, C., Thorvaldsdóttir, H., Ghandi, M., Mesirov, J.P., Tamayo, P., 2015. The Molecular Signatures Database Hallmark Gene Set Collection. *Cell Syst.* 1, 417–425. <https://doi.org/10.1016/j.cels.2015.12.004>

Liu, S., Zhao, G., Li, J., Zhao, Hongxia, Wang, Y., Chen, J., Zhao, Haidong, 2017. Association of polybrominated diphenylethers (PBDEs) and hydroxylated metabolites (OH-PBDEs) serum levels with thyroid function in thyroid cancer patients. *Environ. Res.* 159, 1–8. <https://doi.org/10.1016/j.envres.2017.07.042>

Liu, Y., Zhang, Y., Tao, S., Guan, Y., Zhang, T., Wang, Z., 2016. Global DNA methylation in gonads of adult zebrafish *Danio rerio* under bisphenol A exposure. *Ecotoxicol. Environ. Saf.* 130, 124–132. <https://doi.org/10.1016/j.ecoenv.2016.04.012>

Mancini, F.R., Cano-Sancho, G., Mohamed, O., Cervenka, I., Omichessan, H., Marchand, P., Boutron-Ruault, M.-C., Arveux, P., Severi, G., Antignac, J.-P., Kvaskoff, M., 2020. Plasma concentration of brominated flame retardants and postmenopausal breast cancer risk: a nested case-control study in the French E3N cohort. *Environ. Health* 19, 54. <https://doi.org/10.1186/s12940-020-00607-9>

Ongono, J.S., Dow, C., Gambaretti, J., Severi, G., Boutron-Ruault, M.-C., Bonnet, F., Fagherazzi, G., Mancini, F.R., 2019. Dietary exposure to brominated flame retardants and risk of type 2 diabetes in the French E3N cohort. *Environ. Int.* 123, 54–60. <https://doi.org/10.1016/j.envint.2018.11.040>

Robinson, J.F., Kapidzic, M., Hamilton, E.G., Chen, H., Puckett, K.W., Zhou, Y., Ona, K., Parry, E., Wang, Y., Park, J.-S., Costello,

J.F., Fisher, S.J., 2019. Genomic Profiling of BDE-47 Effects on Human Placental Cytotrophoblasts. *Toxicol. Sci.* 167, 211–226. <https://doi.org/10.1093/toxsci/kfy230>

Shrestha, S., Bloom, M.S., Yucel, R., Seegal, R.F., Wu, Q., Kannan, K., Rej, R., Fitzgerald, E.F., 2015. Perfluoroalkyl substances and thyroid function in older adults. *Environ. Int.* 75, 206–214. <https://doi.org/10.1016/j.envint.2014.11.018>

Singmann, P., Shem-Tov, D., Wahl, S., Grallert, H., Fiorito, G., Shin, S.-Y., Schramm, K., Wolf, P., Kunze, S., Baran, Y., Guarrera, S., Vineis, P., Krogh, V., Panico, S., Tumino, R., Kretschmer, A., Gieger, C., Peters, A., Prokisch, H., Relton, C.L., Matullo, G., Illig, T., Waldenberger, M., Halperin, E., 2015. Characterization of whole-genome autosomal differences of DNA methylation between men and women. *Epigenetics Chromatin* 8, 43. <https://doi.org/10.1186/s13072-015-0035-3>

Storey, J.D., 2011. False Discovery Rate, in: Lovric, M. (Ed.), *International Encyclopedia of Statistical Science*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 504–508. https://doi.org/10.1007/978-3-642-04898-2_248

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P., 2005. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci.* 102, 15545–15550. <https://doi.org/10.1073/pnas.0506580102>

Udesky, J.O., Dodson, R.E., Perovich, L.J., Rudel, R.A., 2019. Wrangling environmental exposure data: guidance for getting the best information from your laboratory measurements. *Environ. Health* 18, 99, s12940-019-0537–8. <https://doi.org/10.1186/s12940-019-0537-8>

van der Laan, M.J., 2008. Estimation Based on Case-Control Designs with Known Prevalence Probability. *Int. J. Biostat.* 4. <https://doi.org/10.2202/1557-4679.1114>

VanderWeele, T.J., Vansteelandt, S., 2010. Odds Ratios for

Mediation Analysis for a Dichotomous Outcome. *Am. J. Epidemiol.* 172, 1339–1348. <https://doi.org/10.1093/aje/kwq332>

Woods, R., Vallero, R.O., Golub, M.S., Suarez, J.K., Ta, T.A., Yasui, D.H., Chi, L.-H., Kostyniak, P.J., Pessah, I.N., Berman, R.F., LaSalle, J.M., 2012. Long-lived epigenetic interactions between perinatal PBDE exposure and Mecp2308 mutation. *Hum. Mol. Genet.* 21, 2399–2411. <https://doi.org/10.1093/hmg/dds046>