RESEARCH

Levels of endocrine-disrupting chemicals are associated with changes in the peri-pubertal epigenome

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Abstract

Puberty marks a transition period, which leads to the attainment of adult sexual maturity. Timing of puberty is a strongly heritable trait. However, large genetic association studies can only explain a fraction of the observed variability and striking secular trends suggest that lifestyle and/or environmental factors are important. Using liquid-chromatography tandem-mass-spectrometry, we measured endocrine-disrupting chemicals (EDCs; triclosan, bisphenol A, benzophenone-3, 2,4-dichlorophenol, 11 metabolites from 5 phthalates) in longitudinal urine samples obtained biannually from peri-pubertal children included in the COPENHAGEN puberty cohort. EDC levels were associated with blood DNA methylation profiles from 31 boys and 20 girls measured both pre- and post-pubertally. We found little evidence of single methylation sites that on their own showed association with urinary excretion levels of EDCs obtained either the same-day or measured as the yearly mean of dichotomized EDC levels. In contrast, methylation of several promoter regions was found to be associated with two or more EDCs, overlap with known gene–chemical interactions, and form a core network with genes known to be important for puberty. Furthermore, children with the highest yearly mean of dichotomized urinary phthalate metabolite levels were associated with higher promoter methylation of the thyroid hormone receptor interactor 6 gene (*TRIP6*), which again was mirrored by lower circulating TRIP6 protein levels. In general, the mean *TRIP6* promoter methylation was mirrored by circulating TRIP6 protein levels. Our results provide a potential molecular mode of action of how exposure to environmental chemicals may modify pubertal development.

Key Words

- DNA methylation
- endocrine disruption
- puberty
- phthalates
- epigenetics

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Introduction

Central puberty is a major reproductive hallmark where sexual maturation is achieved. It is initiated in the brain by reactivation of the hypothalamic–pituitary–gonadal (HPG) axis. The HPG axis is initially and transiently activated during mini-puberty (1) right after birth and until approximately 3–6 months of age. However, when puberty is about to start, the HPG-axis is reactivated by activation of the so-called KNDy neurons (Kiss, NKB and Dyn positive neurons) (2). Activated KNDy neurons start the hypothalamic GnRH pulse generator and cause pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). When the HPG-axis is activated the subsequent cascade of physiological events linked to sexual maturity can take place. The age at pubertal onset varies markedly both among healthy boys (9–14 years) and girls (8–13 years), and both early and late pubertal onset is related to a higher risk of disease development later in life (3). In girls, early age

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at menarche is associated with substantially higher risks for subsequent obesity, type 2 diabetes, cardiovascular disease as well as higher risks for breast cancer and all-cause mortality and late age at menarche is associated with asthma (3). Because the signs of pubertal onset in boys are more difficult to measure on a large scale, correlations to increased lifetime risks in males are harder to establish. However, similar associations between early pubertal onset and cardiovascular and metabolic diseases, as well as late voice break and asthma have been reported (3). The age at pubertal onset has decreased substantially among Danish girls the last two decades (4), and it is suspected that this may be caused by changes in lifestyle as well as in exposure to endocrine-disrupting chemicals (EDCs). Several studies have been able to associate urinary levels of several EDCs, with the age at pubertal onset (5, 6). Especially non-persistent phthalates and phenols have been investigated and are found at considerable levels in many individuals in the Danish population (7).

Despite the inclusion of several hundred thousand subjects in the analysis, recent genome-wide association studies (GWAS) are only able to explain a fraction of the observed variation in pubertal onset (8, 9). We have recently described single genetic variants in the promoters of *FSHR* and *FSHB*, mediating the largest known effect on age at pubertal onset in girls, explaining nearly a year of pubertal timing (10). Twin studies have, however, indicated that approximately 60% of the timing is heritable (11). Consequently, there is a gap of explanation between the observed heritability and what GWAS can account for, and this has sparked an interest in epigenetic studies. We have previously published one of the first studies demonstrating changes in DNA methylation patterns with the onset of puberty in healthy children (12) and identified the promotor of the thyroid hormone receptor interactor 6 gene (*TRIP6*) to be differentially methylated according to pubertal progression. In accordance, we found the TRIP6 protein induced in testicular Leydig cells as well as circulating levels of TRIP6 to be significantly induced during pubertal onset. The promoter of *TRIP6* was later also identified to be differentially methylated in at least three other puberty cohorts with different ethnicity (13, 14, 15).

It is well-established that EDCs can modify the epigenome and also can cause adverse reproductive outcomes (16), but a link between EDC exposure, changes in the epigenome, and pubertal onset remains to be established. Here we use the COPENHAGEN puberty cohort with comprehensive and longitudinal data on pubertal development to combine measurements of genome-wide DNA methylation and urinary levels of several well-known EDCs in order to investigate whether exposure to EDCs may be associated with changes the peri-pubertal epigenome.

Methods

Several parts of the data included in this study have been published individually before. This includes the DNA methylation data and TRIP6 protein measurements (12) as well as the phthalate measurements (17). The remaining parts of the EDC measurements have not been published before, and the DNA methylation data has never been analyzed in the context of EDC levels before.

Study population

The COPENHAGEN Puberty Study is a cross-sectional study of healthy Danish children with a nested longitudinal sub-cohort of 108 girls and 101 boys, who were examined every 6 months during puberty, starting from 6–8 years of age and up to 7 years after inclusion. The study population has been described in detail previously (4, 18, 19). Trained physicians performed all clinical evaluations including pubertal staging according to Tanner's classification evaluated by breast palpation in girls and testicular volume in boys using orchidometry as described by Marshall and Tanner (20). A breast Tanner stage of two or above and a testicular volume of 4 mL or more were indicative of pubertal onset in girls and boys, respectively.

In the present study, longitudinal biannual measurements of morning urinary levels of a range of EDCs were available. Based on the number of samples with levels above the detection limit and clinical relevance, a subset consisting of four phenols as well as 11 metabolites of five phthalates (see subsequently) were chosen for analysis. In addition, DNA methylation profiles from 31 boys and 20 girls that had paired pre- and post-puberty DNA methylation profiles performed earlier were included (see subsequently). The study setup is illustrated in Fig. 1A and in Table 1. In order to obtain as much analytical power as possible, all samples were included and analyzed as one cohort, but with age and sex included as confounders.

DNA methylation profiling

From our earlier studies on DNA methylation patterns and pubertal development (12) 102 genome-wide DNA methylation profiles originating from matched pre- and

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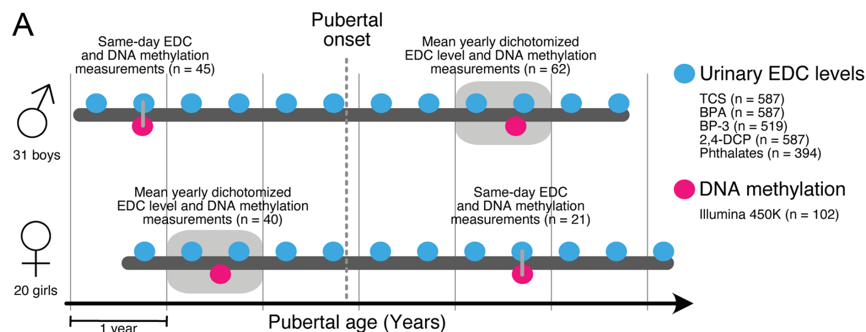

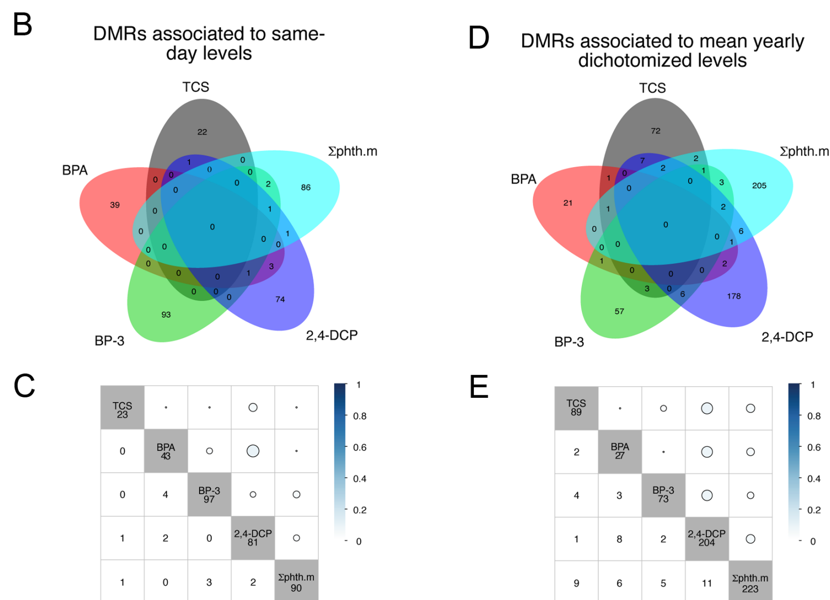

Figure 1
Study setup overview and overlap between differentially methylated regions (DMRs) associated with urinary levels of EDCs. (A) Illustration of the study setup indicating biannual measurements of EDCs (TCS, BPA, BP-3, 2,4-DCP, and phthalate metabolites) in longitudinal urine samples obtained from peri-pubertal children included in the COPENHAGEN puberty cohort together with pre- and post-pubertal measurements of blood DNA methylation profiles (Illumina 450K). The dataset consisted of 31 boys and 20 girls each with both pre- and post-pubertally DNA methylation profiles (equaling 102 DNA methylation profiles) and longitudinal EDC measurements (equaling on average 10.5 measurements of each EDC). The *n* indicates the number of measurements performed. (B) Venn diagram showing the overlap between all DMRs identified to be associated with the same-day level of the indicated EDCs at an FDR < 0.05. (C) Similarity matrix showing the number of shared DMRs between two different EDCs when same-day measurements of urinary EDC levels and DNA methylation were analyzed. The lower left triangle of the matrix shows the actual numbers of overlapping DMRs, which has been translated into relative (to the total number identified DMRs) color and size coded circles in the upper right triangle of the matrix. (D) Venn diagram and (E) similarity matrix when the mean of yearly dichotomized levels of EDCs were used to identify associated DMRs at an FDR < 0.05.

post-pubertal blood samples of 20 girls and 31 boys were available. Detailed description of the experimental procedures can be found in Almstrup et al. (12). In brief, DNA methylation profiles were obtained after bisulfite treatment of DNA and hybridization to the Infinium HumanMethylation450 BeadChips (Illumina, San Diego, CA, USA) using standard protocols.

The dataset is available in the ArrayExpress repository (www.ebi.ac.uk/arrayexpress), under accession number E-MTAB-4187.

Table 1 Descriptive table of the study population.

Median (range)	**All** (*n* = 102)	**Pre-pubertal boys** (*n* = 31)	**Post-pubertal boys** (*n* = 31)	**Pre-pubertal girls** (*n* = 20)	**Post-pubertal girls** (*n* = 20)
Age (years)	9.7 (5.6 to 16.4)	9.28 (6.2 to 10.5)	15.8 (12.6 to 16.4)	9.3 (5.6 to 11.3)	12.59 (12.2 to 16.3)
Years relative to pubertal onset (years)	−0.21 (−6.0 to 6.0)	−2.7 (−5.1 to −0.4)	3.5 (0 to 6)	−1.0 (−6.0 to −0.4)	3.9 (0.5 to 6)
Children with same-day measurements of urinary EDC levels and DNA methylation	66	28	17	16	5
TCS levels (ng/mL)	1.3 (0 to 2401)	2.0 (0 to 376)	2.0 (0.2 to 69.4)	1.12 (0 to 2401)	0.48 (0.1 to 4.1)
BPA levels (ng/mL)	2.0 (0 to 75.7)	1.4 (0 to 75.7)	2.9 (0.4 to 7.7)	1.8 (0 to 10.3)	0.9 (0.2 to 14.8)
BP-3 levels (ng/mL)	2.2 (0 to 55.6)	1.9 (0 to 26.2)	1.9 (0.2 to 55.6)	3.8 (0.2 to 24.8)	2.0 (1.3 to 32.1)
2,4-DCP levels (ng/mL)	0.35 (0 to 11.3)	0.3 (0 to 4.2)	0.5 (0 to 1.7)	0.3 (0 to 7.0)	0.18 (0 to 0.4)
Σphth.m levels (ng/mL)	325 (51.1 to 1825)	346 (87.1 to 1227)	165 (46.2 to 1415)	401 (51.4 to 811)	123 (45.9 to 265)

Σphth.m is the sum of the molar concentrations of MiBP, MnBP, MBzP, MEHP, MEHHP, MEOHP, MECPP, MiNP, MHiNP, MOiNP and, MCiOP multiplied with the molar weight of MEHP.

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Measurements of EDCs in urine

In this study, we included data from liquid-chromatography tandem-mass-spectrometry (LC-MS/MS) measurements of triclosan (TCS), bisphenol A (BPA), benzophenone-3 (BP-3), 2,4-dichlorophenol (2,4-DCP), and the 11 metabolites: mono-iso-butyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), mono-benzyl phthalate (MBzP), mono-(2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-iso-nonyl phthalate (MiNP), mono-hydroxy-iso-nonyl phthalate (MHiNP), mono-oxo-iso-nonyl phthalate (MOiNP), and mono-carboxy-iso-octyl phthalate (MCiOP) of the five phthalate diesters: di-iso-butyl phthalate (DiBP), di-*n*-butyl phthalate (DnBP), butylbenzyl phthalate (BBzP), di-(2-ethylhexyl) phthalate (DEHP), and di-iso-nonyl phthalate (DiNP). The phenols and phthalate metabolites were analyzed according to previously described methods (21, 22) and the measured urinary concentrations of phthalate metabolites have been published before (17). The EDCs were measured in samples collected twice each year during pubertal transition (Fig. 1A and Table 1) and the number of longitudinal EDC measurement available were in total 587 (23 < LOD), 587 (43 < LOD), 519 (0 < LOD), 587 (75 < LOD) and 394 for TCS, BPA, BP-3, 2,4-DCP, and phthalate metabolites, respectively. At 66 visits, the urine samples were obtained the same-day as blood was drawn for DNA methylation analysis. For the remaining 36 visits, the closest sample in time was used. On average, these were obtained 220 days prior to when the sample for DNA methylation analysis was obtained. When the mean of the yearly dichotomized levels of EDCs were analyzed (see 'Results' section), the yearly bin started on average 237 days earlier than the measure of DNA methylation.

Measurement of circulating TRIP6 protein levels

Data were obtained from Almstrup *et al.* (12). In brief, quantitative sandwich ELISA kit (Cat. No MBS9328439, MyBioSource, Inc., San Diego, CA) was used to measure the concentration of TRIP6 protein in serum samples from boys ($n=20$) and girls ($n=18$) at three different time points during puberty representing pre-, peri-, and post-pubertal stages (12). In 73 serum samples, the measurement of circulating TRIP6 protein levels was performed on serum obtained the same-day as where DNA methylation measurements were available and therefore further analyzed here.

Data analysis

For the analysis of associations between chemical excretion levels of the phthalate metabolites and DNA methylation, the phthalate metabolites were summed (denoted Σphth.m) by adding the molar metabolite concentrations in each sample and expressed in ng/mL by multiplying with the molar weight of MEHP.

The data were analyzed in R version 3.3.3 integrated into RStudio Version 1.0.136. Using the R package *minfi* (23) data was normalized by subset quantile within-array normalization (24) and probes containing SNPs in the CpG or extension sites were removed. The R package *SmartSVA*, which applies a surrogate variable analysis (SVA) (25, 26), was used to efficiently control the genomic inflation originating from, for example, differences in blood cell type composition. Age and sex were included as specific covariates. The R package *CpGAssoc* (27) was used to analyze differential methylation at single CpG sites where the surrogate variables from *SmartSVA* were included as covariates.

Differentially methylated regions were investigated using the *DMRcate* package (28). *DMRcate* identifies and ranks the most differentially methylated regions across the genome based on the tunable kernel smoothing method. A bandwidth of 1000 nucleotides (lambda = 1000) and a scaling factor of 2 (C = 2) were used as recommended by the authors of the *DMRcate* package (28) and results were corrected for multiple testing by using the Benjamini–Hochberg method (29).

Genomic ranges identified in the analysis were plotted together with publicly available genome tracks using the R package *GViz* (30). Annotation was done according to hg19. The R package *ChIPpeakAnno* (31) was used to draw Venn plots of genomic regions.

Curated chemical–gene interactions data were retrieved from the Comparative Toxicogenomics Database (CTD), MDI Biological Laboratory, Salisbury Cove, Maine, and NC State University, Raleigh, North Carolina (http://ctdbase.org/) as of November 2019 (32). Protein–protein interactions were acquired from the STRING database (33) version 10 using only high confidence (0.7) interactions.

Depending on whether or not correction for multiple testing was applied, a *P*-value or a false discovery rate (FDR) of 0.05 was considered significant.

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Ethics and data protection

The COPENHAGEN Puberty Study (ClinicalTrials.gov ID: NCT01411527) has been approved by the local Danish ethical committee (KF 01 282214; V200.1996/90) and the Danish Data Protection Agency (2010-41-5042). The study was carried out in accordance with the approved guidelines and written informed consent was obtained from all children and parents.

Results

Association between single methylation-sites and EDC levels

For a subset of the samples ($n=66$), same-day measurements of urinary EDC levels and genome-wide methylation data on peripheral blood DNA was available (Fig. 1A) and were used to search for associations between EDC levels and levels of methylation at single methylation-sites (CpG-sites) in the genome. Both the EDC levels and DNA methylation data contained a lot of biological variability and we, therefore, tested five different approaches to adjust for the variance in the data. Associations of EDC levels were analyzed both as raw values, log-transformed values and as quartiles. Moreover, the DNA methylation data was analyzed with or without correction of the potential bias originating from, for example, different blood cell counts using surrogate variables and specifically corrected for age and sex. The number of identified single CpGs associated with same-day measurements of the different EDCs are listed in Table 2. The genomic inflation factor, which quantifies the extent of the bulk inflation and the excess false positive rate, was in all cases close to 1 (Table 2) indicating a low amount of bias in the data (34). We, nevertheless, observed a pattern of either no/few associations (BP-3 and Σphth.m; Table 2) or many associations (TCS, BPA, and 2,4-DCP; Table 2) when a standard FDR threshold of 0.05 was applied. However, for all EDCs except Σphth.m, the associations appeared to be driven by few extreme EDC values (Supplementary File 1, see section on supplementary materials given at the end of this article), probably reflecting the biological variability in the data. This was further substantiated when quartiles of EDC levels were used as input values, which resulted in loss of all associations to single CpGs (Table 2).

Only for Σphth.m we found three single CpGs (cg23675323, cg10328831, cg25462190) that appeared to be driven by a true correlation with methylation levels at an

Table 2 Association between EDCs and single CpGs under different analytical conditions.

Input values/correction/transformation	Raw/No/No	Raw/No/Log	Raw/SVA/No	Raw/SVA/Log	Quartiles/SVA/No
TCS (triclosan)					
Genomic inflation factor	1.40	1.56	1.24	1.31	1.05
CpGs at FDR < 0.05	327[a]	1156[a]	521[a]	898[a]	0
BPA (bisphenol A)					
Genomic inflation factor	1.77	1.38	1.35	1.09	1.01
CpGs at FDR < 0.05	3383[a]	227[a]	757[a]	210[a]	0
BP-3 (benzophenone-3)					
Genomic inflation factor	0.77	0.77	1.15	1.13	1.09
CpGs at FDR < 0.05	19[a]	13[a]	147[a]	95[a]	0
2,4-DCP (2,4-dichlorophenol)					
Genomic inflation factor	1.06	1.21	1.14	1.18	1.06
CpGs at FDR < 0.05	101[a]	305[a]	198[a]	263[a]	0
Σphth.m (11 phthalate metabolites)[b]					
Genomic inflation factor	0.89	0.91	1.12	1.11	1.10
CpGs at FDR<0.05	0	0	2	3	0

[a]Driven by single point measurements (Supplementary File 1); [b]See 'Methods' section for explanation on which phthalate measurements that are included.
CpG, DNA methylation loci; FDR, false discovery rate; SVA, surrogate variable analysis .

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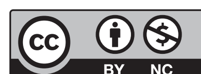

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FDR<0.05 and were located in introns of *RASA3*, *MIR1322*, and *N4BP3*, respectively (Supplementary File 1).

In general, we did not find evidence for a strong association between methylation levels of single CpGs sites and the level of urinary EDCs measured in same-day samples of peri-pubertal children. We, therefore, investigated whether methylation levels of whole regions of CpGs in a coordinated manner could be associated with EDC levels.

Differentially methylated regions associated with same-day EDC excretion levels

Using data from the same 66 children with same-day measurements of EDCs and DNA methylation levels (Fig. 1A), we identified 24, 42, 97, 83, 92 differentially regulated regions (DMRs) that were significantly (FDR < 0.05) associated with the same-day urinary level of TCS, BPA, BP-3, 2,4-DCP, and Σphth.m, respectively (Supplementary File 2). The identified regions were scattered throughout the genome (Supplementary File 2) and to narrow down the list we searched for genomic regions that were associated with levels of more than one EDC. Only 11 (3%) DMRs were associated with the same-day level of more than one of the EDCs (Fig. 1B, C and Supplementary File 2). Of particular notice were promoters of *VTRNA2*, which was found associated with BPA, BP-3, and 2,4-DCP and the promoter of *NPFFR2*, which was associated with BP-3, 2,4-DCP, and Σphth.m (Supplementary File 2). The overall low overlap between identified promoters, nevertheless, indicates that there is no single genomic region that changes methylation level according to exposure of EDCs, in general, at least when same-day measurements were analyzed. We, therefore, assessed whether longitudinal levels of EDCs could be associated with changes in methylation levels.

Differentially methylated regions associated with the mean of yearly dichotomized levels

To minimize the influence of biological day-to-day variation of the EDC measurements, to include more EDC and DNA methylation measurements of the same individuals and to have a better picture of overall individual exposure levels we took advantage of the longitudinal measurements of EDCs in our cohort (Fig. 1A). We dichotomized each urinary measurement into high (assigned a value of 2) and low (assigned a value of 1) and subsequently averaged measurements into yearly bins. This left subjects with a yearly mean value of 2, 1.5, or 1, representing a high, intermediate, or low yearly exposure level. In other words, a highly exposed child (with a mean yearly dichotomized level of 2) will have two successive EDC measurements within a year at a level corresponding to the higher half of all measurements. The mean yearly dichotomized levels were then associated with regional changes in DNA methylation obtained within the same yearly bin.

Using an FDR cut-off of 0.05, 91, 27, 73, 210, 248 DMRs were found to be associated with the mean of the yearly dichotomized level of TCS, BPA, BP-3, 2,4-DCP, and Σphth.m, respectively (Supplementary File 3). When compared to the number of DMRs associated with same-day levels (Fig. 1B and C), a reduced number of associated DMRs was observed for BPA and BP-3, whereas more DMRs were observed for TCS, 2,4-DCP and Σphth.m (Fig. 1D and E). We identified 54 (8%) DMRs that were associated with the level of more than one EDC at a time (Fig. 1D and Supplementary File 3), which represented a significant (*P*-value: 0.003) increase compared to same-day exposure associations. Of particular notice were the promoters of *CLEC4GP1* and *FAM71F1*, which were found associate with both TCS, 2,4-DCP, and Σphth.m, the promoter of *VTRNA2-1*, which was associated with TCS, BP3, and Σphth.m, and the promoter of *LYPD3*, which was associated with 2,4-DCP, BP-3, and Σphth.m. Also, of interest was the promoter of *TRIP6*, which was associated with both BP-3 and Σphth.m (Supplementary File 3).

Overlap between same-day levels and mean of yearly dichotomized levels

Since DMRs associated to the same-day EDC level and the mean of yearly dichotomized values may be a measure of different exposure patterns, we looked at the overlap between the two. This revealed a quite modest overlap with only 10, 6, 4, 28, and 29 DMRs overlapping for TCS, BPA, BP-3, 2,4-DCP, and Σphth.m, respectively (Fig. 2). Of particular interest were promoters also identified above to be associated to more than one EDC at a time, which included promoters of *BAALC*, *CCDC79*, *CERS4*, *CLEC4GP1*, *CNKSR1*, *Corf26*, *FAM71F1*, *KLHDC4*, *LYPD3*, *OR2L13*, *PRKCZ*, *SYCP1*, *TAPBP*, *TRIP6*, and *VTRNA2-1* (Supplementary Files 2 and 3). Interestingly, the promoter of *LYPD3* was detected to overlap both for 2,4-DCP and Σphth.m.

When boys and girls were analyzed individually using the mean of yearly dichotomized values, we observed that most signals originated from boys, except for TCS and Σphth.m (Supplementary File 1), which probably reflect that less girls were included in the study (31 boys vs 20 girls).

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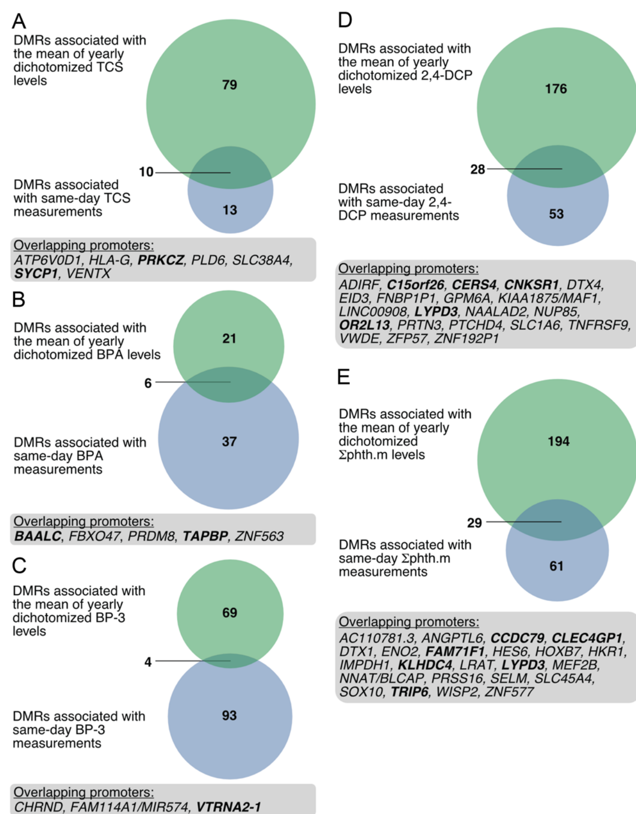

Figure 2
Overlap between DMRs identified as significantly associated with same-day EDC levels and with the mean of yearly dichotomized levels of EDCs. (A) TCS, (B) BPA, (C) BP-3, (D) 2,4-DCP, (E) Σphth.m. The size of the circles is proportional to the number of DMRs. Below each Venn plot DMRs overlapping known promoters are listed and promoters also associated with more than one EDC at a time (Fig. 1D) are highlighted in bold font. The full list of DMRs are listed in Supplementary Files 2 and 3.

Gene–chemical and protein–protein interactions

In order to deduce whether the observed association between EDCs and DMRs potentially also could cause downstream effects, we took advantage of the Comparative Toxicogenomics Database (CTD), which holds curated data on the interaction between chemicals and genes (32). We searched for overlap between genes listed in CTD to be associated with a particular EDC and the list of genes with promoter DMRs associated with the mean of yearly dichotomized EDC levels (Supplementary File 3). This revealed a small overlap for TCS (9 genes), BPA (11 genes) and no overlap for BP-3 and 2,4-DCP. For the Σphth.m a more substantial overlap was observed when di-ethylhexyl phthalate (DEHP; 34 genes) and di-butyl phthalate (DBP; 32 genes) were used as input (Fig. 3A, B, C, D and E).

Promoters of genes that also were identified as overlapping in association to more than one EDC at a time (Supplementary File 3) were *BAALC*, *CLDN9*, *FAM71F1*, *FAM83A*, *PNOC*, and *TRIP6*, and promoters of genes also identified to overlap between same-day levels and the mean of yearly dichotomized EDC levels (Fig. 2) were *BAALC*, *DTX1*, *FAM71F1*, *FBXO47*, *HES6*, *SLC38A4*, *SLC45A4*, *SOX10*, *TAPBP*, and *TRIP6* (Table 3).

Furthermore, all genes identified with promoter methylation levels associated with EDC levels were used to search the STRING database (33) for protein–protein interactions. This identified a high confident core network of proteins (small networks of 6 or less proteins were excluded; Fig. 3F) that included 27 genes previously identified in the analysis of overlapping DMRs (Figs 1, 2, 3A, B, C, D and E and Table 3). Furthermore, the network included at least nine proteins with well-known functions in pubertal development and the HPG-axis (e.g. BMP4, NFKB1, SOX10, TGFB2, and TRIP6; Fig. 3F). Of particular interest was TRIP6, which was identified in several of the analyses (Figs 2, 3, Table 3 and Supplementary File 3) and we, therefore, investigated this association further.

Effects on the TRIP6 promoter and circulating levels of *TRIP6*

Methylation of the *TRIP6* promoter was identified to be associated with both BP-3 and Σphth.m levels. The association with Σphth.m was evident for both same-day levels and the mean of yearly dichotomized values and an interaction between DBP and *TRIP6* was also listed in CTD.

The group of children with intermediate levels of BP-3 (mean of yearly dichotomized values equal to 1.5) had a higher methylation level of the *TRIP6* promoter compared to the groups with low and high levels (Fig. 4A and B). From a subset of the study population ($n=73$) circulating levels of the TRIP6 protein was measured previously (12) and higher *TRIP6* promoter methylation level in the intermediate group resulted in lower circulating TRIP6 levels (Fig. 4C).

A higher level of *TRIP6* promoter methylation was observed in the group of children with high Σphth.m levels (Fig. 4D and E) and this was mirrored by lower circulating TRIP6 levels (Fig. 4F).

In general, we found significant negative correlation (Pearsons *R*: −0.47, *P*-value: 3.5e−05) between *TRIP6* promoter methylation and circulating TRIP6 levels (Supplementary File 1), which indicates that the observed association between urinary EDC levels and methylation levels of the *TRIP6* promoter could have downstream functional consequences. It was not possible

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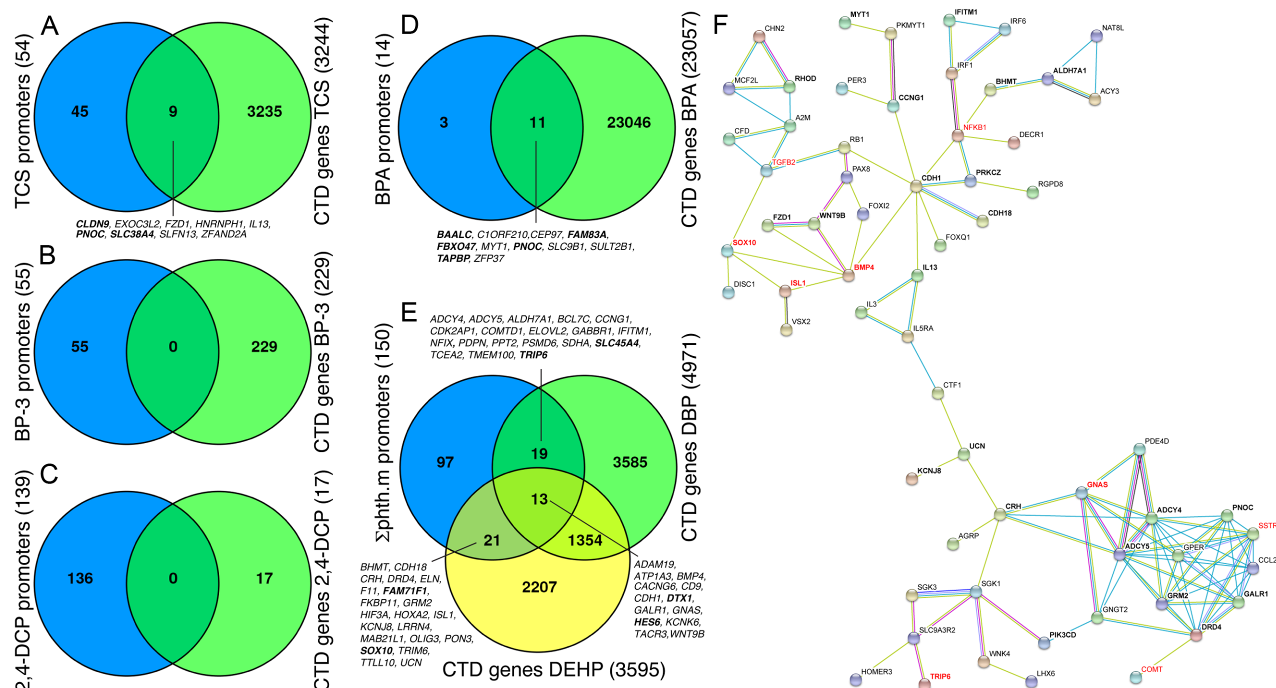

Figure 3

Overlap with the Comparative Toxicogenomics Database (CTD) and core protein–protein interacting network. Venn plots show the overlap between genes with promoter methylation associated with urinary levels of EDCs and genes known to be interacting with the EDCs as listed in the CTD. (A) TCS, (B) BP-3, (C) 2,4-DCP, (D) BPA, and (E) Σphth.m. Intersecting promoters are listed below each Venn plot and promoters also identified as overlapping between several EDCs (*BAALC*, *CLDN9*, *FAM71F1*, *FAM83A*, *PNOC*, and *TRIP6*) and promoters also identified between same-day EDC levels and the mean of yearly dichotomized levels of EDCs (*BAALC*, *DTX1*, *FAM71F1*, *FBXO47*, *HES6*, *SLC38A4*, *SLC45A4*, *SOX10*, *TAPBP*, and *TRIP6*) are highlighted in bold font. (F) All genes identified as having promoter methylation associated with EDC levels were used to search the STRING database for high confident protein–protein interactions. This identified a single core network of 63 proteins (small networks of six or less proteins were excluded), which included 27 genes previously identified to be shared in the different analyses (Figs 2 and 3A, B, C, D, E) and are marked with bold font. The core network also included nine proteins with well-known functions in pubertal development and the HPG-axis (e.g. BMP4, NFKB1, SOX10, TGFB2, and TRIP6) and these are marked with red font. Note that five proteins are both in red and bold font (BMP4, ISL1, SOX10, GNAS, and TRIP6) indicating that they both have promoter methylation associated with EDCs and have been described in relation to puberty.

to measure the circulating *TRIP6* transcript level in stored serum samples.

Discussion

We here show a potential relationship between chemical exposure, specific changes in the epigenome, and association to downstream changes in protein levels indicating a potential direct effect of chemical exposure on the human epigenome. Higher urinary phthalate levels were associated with higher *TRIP6* promoter methylation and in concordance lower circulating TRIP6 protein levels (Fig. 5). For BP3, we observed nearly the opposite pattern, albeit the intermediate group showed the highest *TRIP6* promoter methylation level. We have previously found that *TRIP6* promoter methylation correlates with pubertal transition (12); a correlation, which has been confirmed in several other pubertal cohorts (13, 14, 15). The *TRIP6* promoter methylation levels gradually declined during pubertal development and a concordant rise in circulating TRIP6 levels was observed (12). Together, these results suggest that higher phthalate levels lead to higher *TRIP6* promoter methylation, concordant lower circulating levels of TRIP6 and subsequently later pubertal onset (Fig. 5). For BP3, the opposite would be true. Indeed, direct relations between exposure levels and pubertal onset has been reported before – also in the same cohort of children (35). In general, higher phthalate levels were associated to a later pubarche and menarche (5, 6, 35, 36). Also, higher BP-3 levels have been associated to earlier menarche (36). Phthalate and BP-3 exposure hence seem to show opposite associations to pubertal development which fits to the observed association with TRIP6 promoter methylation.

TRIP6 interacts with the TR-beta only in the presence of thyroid hormone (37) and is induced in steroidogenic Leydig cells at puberty (12). As thyroid hormone is crucial for testicular development, it is highly likely that TRIP6

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Table 3 Selected genes showing substantial overlap in our study.

Promoter	Associated with the same-day levels of [a]	Associated with the mean of yearly dichotomized levels of [a]	Overlap with gene-chemical interactions listed by CTD[b]	In core network[b]	Comments
BAALC	BPA	BPA, BP-3,	BPA	Yes	Mainly expressed in brain. Mediates PTH action on bone (45)
CLDN9		TCS, 2,4-DCP	TCS	No	Mainly expressed in brain. Enriched in GnRH neurons (46)
DTX1	Σphth.m	Σphth.m	Σphth.m	No	Ubiquitin ligase involved in Notch signalling
FAM71F1	Σphth.m	TCS, 2,4-DCP, Σphth.m	Σphth.m	No	Uniquely expressed in testis. Related to azoospermia (47)
FAM83A		BPA, 2,4-DCP	BPA	No	Highly expressed in vagina and oesophagus
FBXO47	BPA, BP-3	BPA	BPA	No	Uniquely expressed in testis. Involved in meiosis (48)
HES6	Σphth.m	Σphth.m	Σphth.m	No	Highly expressed in brain and testis. Involved in neurogenesis (49)
PNOC		TCS, BPA	TCS	Yes	Induced in theca cells by hCG administration (50)
SLC38A4	TCS	TCS	TCS	No	System A amino acid transporter, highly expressed in liver
SLC45A4	Σphth.m	Σphth.m	Σphth.m	No	Involved in cognitive functions (51)
SOX10	Σphth.m	Σphth.m	Σphth.m	Yes	Involved in Kallmann syndrome (52) and hypogonadotropic hypogonadism (53)
TAPBP	BPA, Σphth.m	BPA, BP-3	BPA	No	MHC class I antigen-processing
TRIP6	Σphth.m	BP-3, Σphth.m	Σphth.m	Yes	Induced in testicular Leydig cells during puberty (12)

[a]See Fig. 2 for further details; [b]See Fig. 3 for further details.

also is important for testicular development at puberty. TRIP6 is however also involved dendritic morphogenesis of hippocampal neurons (38) and can be found differential expressed during rodent development of the mammary gland (GEO:GDS2721 and GEO:GDS2360) (39, 40), gonads (GEO:GDS4503) (12, 41) as well as in the hypothalamic hamartomas of patients with central precocious puberty (GEO:GDS3110) (42). It is, therefore, highly likely that TRIP6 plays an active role in multiple relevant endocrine tissues during puberty of both boys and girls. More research is, however, needed to establish whether there is a direct causal relationship between exposure and changes in the epigenome, for example, in the *TRIP6* promoter in endocrine tissues. It is possible that some circular associations exist for the *TRIP6* promoter where pubertal transition is associated with changes in promoter demethylation but also with changes in behavioral patterns, which may lead to changes in EDC exposure levels. However, promoters that previously was not found associated with pubertal transition (12) were also found associated with EDC levels. Besides the promoters and their putative functions listed in Table 3, the *CLEC4GP1* promoter, was found associated to both TCS, 2,4-DCP, and Σphth.m. According to the GTeX database *CLEC4GP1* is primarily expressed the brain, adrenals and gonads and it would be interesting to see whether methylation of the *CLEC4GP1* promoter in these endocrine active tissues also can be affected by exposure to EDCs. Functional studies are, nevertheless, needed to support that the effects observed in blood also have effects in target tissues. We have earlier shown that the peri-pubertal demethylation of the *TRIP6* promoter in blood is mirrored by increased TRIP6 protein expression in steroidogenic Leydic cells (12), indicating that blood indeed can act as a surrogate tissue for endocrine active target tissues. Interestingly, in this study, we also identified promoters of genes like *BMP4*, *TACR3* and *GNAS* to be associated with urinary EDC levels. These genes are all well-known to be involved in the activation of the HPG-axis at pubertal onset (43) and demethylation of the *GNAS* promoter has been shown to control its expression in the rat brain (44). It is therefore likely that the observed associations between exposure levels and DNA methylation can be mirrored in target tissues like the gonads and brain, albeit it remains to be directly proven. We speculate that the epigenome may serve as an intermediate molecular mode of action of how exposure to environmental chemicals can modify pubertal development.

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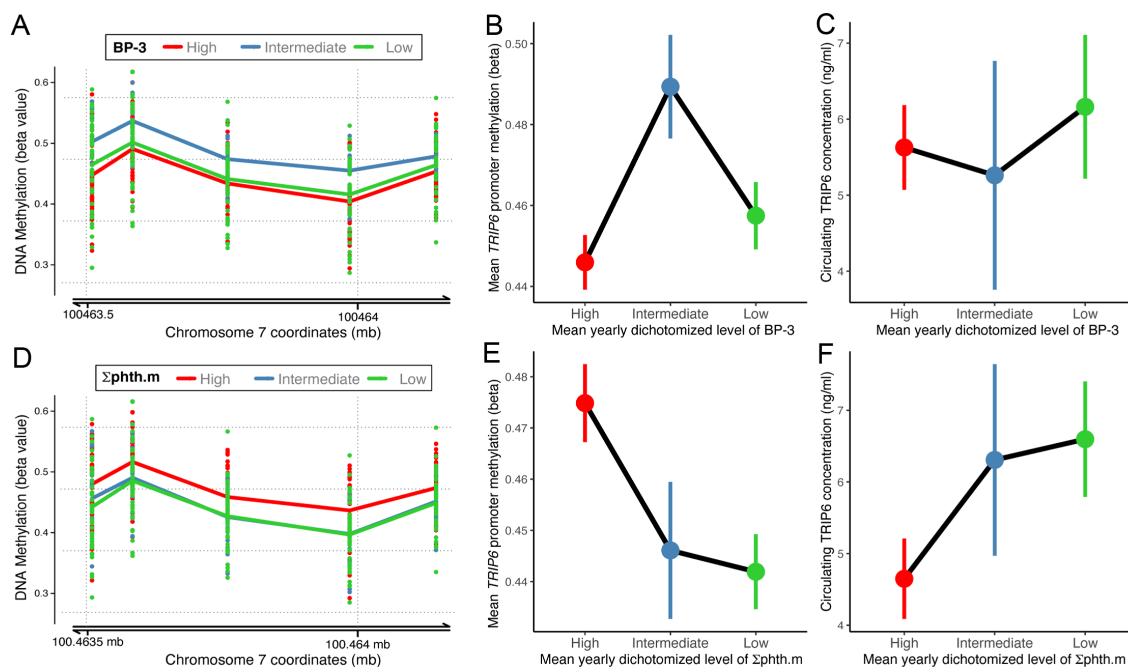

Figure 4

Association between *TRIP6* promoter methylation, EDCs, and circulating levels of TRIP6. (A) Genome tracks showing the *TRIP6* promoter and methylation levels in groups of children with a mean of yearly dichotomized levels of BP-3 equal to 2 (high; red), 1.5 (intermediate; blue), and 1 (low; green). (B) The mean methylation level of the *TRIP6* promoter (mean of the 5 CpG) plotted according to the same groups as in A. (C) Circulating levels of TRIP6 protein in children divided into the same groups as in A. (D) Genome tracks of the *TRIP6* promoter and methylation levels divided into groups based on the yearly dichotomized levels of Σphth.m. (E) The mean methylation level of the *TRIP6* promoter plotted according to the same groups as in D. (F) Circulating levels of TRIP6 in children divided into the same groups as in D. A significant association (*P*-value: 3.5e−05) was observed between the mean *TRIP6* promoter methylation level and the circulating TRIP6 levels (Supplementary File 1).

Our study nevertheless also has some limitations. It is based on a rather small study population and larger cohorts are need to more firmly establish the relationship between exposure levels and the epigenome.

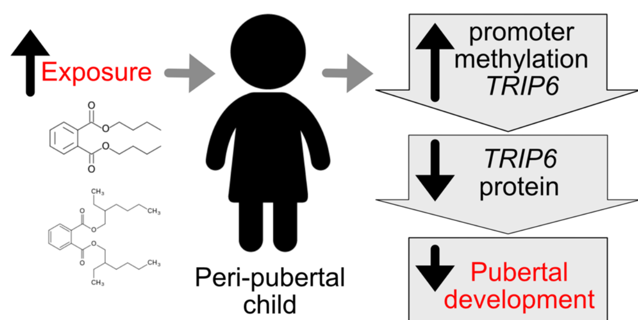

Figure 5

Conceptual figure illustrating the proposed impact of EDC exposure on the peri-pubertal epigenome and subsequent effects leading to changes in pubertal timing. High urinary levels of phthalates, presumably caused by higher exposure, were shown to be associated with higher promoter methylation of the *TRIP6* promoter and lower circulating levels of TRIP6 protein. We have earlier shown that lower circulating levels of TRIP6 protein were associated with later pubertal onset. Also, higher urinary phthalate levels have earlier been shown to be directly associated with later pubarche and menarche (5, 6, 35, 36).

Especially since both of these measures show a high degree of biological variability. This was particularly evident when we analyzed single-CpGs and found associations driven by single data points. Albeit, the few associations to single-CpGs that appeared real might be of biological importance, we believe that regional changes in promoter regions are more important since they are more likely to cause downstream transcriptional changes. Furthermore, using the mean of yearly dichotomized values seemed to give more coherent associations (with smaller *P*-values and more overlapping DMRs) than using same-day levels. This may, however, simply be due to inclusion of more samples when using the mean of yearly dichotomized values, but it could also reflect a more general exposure and hence a more stable effect on the epigenome in contrast to same-day levels.

Our study population was peri-pubertal children, which represent a sensitive window in terms of endocrinology, and it needs to be established whether the same relationship can be observed among, for example, adults or younger children. Also, it would be interesting to investigate cohorts of children with early or late pubertal onset. Finally, our study only provides associations and

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functional validation is needed to investigate if there is a direct relationship between exposure and methylation changes, for example, of the *TRIP6* promoter. At present, it is unknown whether the observed methylation changes are caused by physiological processes derived from exposures or whether, for example, phthalates can directly bind to proteins that modify DNA methylation at specific sites. It is however difficult to investigate as the experimental system also needs to be physiological relevant to peri-pubertal children.

Conclusions

We identified associations between urinary excretion levels of several endocrine-disrupting chemicals and changes in regional DNA methylation levels in peri-pubertal children. Changes in methylation of several promoters were found to be associated with more than one chemical at the time and overlapped with known gene–chemical interactions. EDC-associated changes in methylation of the *TRIP6* promoter were mirrored by changes in circulating levels of the TRIP6 protein. Our results provide a potential molecular mode of action of how exposure to environmental chemicals potentially can modify pubertal development.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EC-20-0286.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

K A and A J designed and conceptualized the study. K A and H F conducted the experiments and gathered the data. K A and H F analyzed the data. K A wrote the paper and all participated in the final writing of the paper. All authors endorsed the results and agreed to publish the manuscript.

Accession codes

ArrayExpress (www.ebi.ac.uk/arrayexpress) accession number E-MTAB-4187.

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