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The causal role of endocrine disrupting chemicals in pubertal timing: a Mendelian randomization study

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Abstract

Objectives: Endocrine disrupting chemicals (EDCs) interfere with hormonal homeostasis, and have been observationally linked to altered pubertal timing, defined by the age of menarche (AAM) in girls and the age at voice change (AVC) in boys. However, the causality of these associations remains unclear. We used Mendelian randomization (MR) to investigate if genetically altered serum EDC levels affect pubertal timing.

Methods: We performed univariate MR to assess the effects of 22 EDCs on AAM and AVC, using the largest GWAS for EDCs as well as European and multi-ethnic GWAS on AAM and AVC. Multivariate MR (MVMR) and two-step MR were conducted to examine mediating effects of body mass index (BMI).

Results: We found causal MR associations with AAM for three polychlorinated biphenyls (PCBs): PCB 74 (β_{IVW}: −0.015, 95 % CI [−0.028, −0.003], p=0.014), PCB 194 (β_{IVW}: −0.015, 95 % CI [−0.024, −0.007], p=3.27×10^{-4}), and PCB 206 (β_{IVW}: −0.024, 95 % CI [−0.041, −0.006], p=0.0068) and for dibutyl phthalate (DBP, β_{IVW}: 0.006, 95 % CI [0.001, 0.010], p=0.013). One MR association was found with AVC for bisphenol A (BPA, $\beta_{Wald\ ratio}$= −0.032, 95 % CI [−0.044, −0.019], p=2.62×10^{-7}). Mediation analyses by MVMR or Two-Step MR suggested BMI's mediating role in the associations of EDCs with AAM and AVC.

Conclusions: Our findings indicate that exposure to specific PCBs leads to earlier AAM whereas exposure to DBP delays AAM. Exposure to BPA leads to earlier AVC, with BMI potentially acting as a mediator.

Keywords: endocrine disrupting chemicals; menarche; puberty; Mendelian randomization

Introduction

Puberty is a fundamental process of maturational changes which mark the transition from sexual infancy to reproductive maturity [1]. Normal puberty onset ranges between 8 and 13 years old for girls, and 9–14 years old for boys, and is clinically defined by the presence of breast development in girls and of testicular enlargement in boys [1]. Age at menarche (AAM) in girls and age at voice change (AVC) in boys are also landmarks that can be used to evaluate pubertal timing [2]. It has been observed that the age of onset of breast development has declined in recent decades [3]. While data on pubertal timing in boys are inconsistent [4, 5]. Early puberty leads to negative impacts on growth (such as achieved adult height) and youth's mental health and adverse health outcomes in adulthood [6–11]. Better understanding of the factors that affect pubertal timing is key in order to prevent earlier occurrences of pubertal-related changes in both sexes and their negative consequences.

It is speculated that exposure to endocrine disrupting chemicals (EDCs), among other factors, such as pediatric obesity or improvements in nutrition, contributes to the observed shifts in pubertal timing [3, 4, 12, 13]. EDCs have become increasingly prevalent in the past century as man-made products and chemicals have been made essential in our daily lives [14]. These substances have similarities in their molecular structure to that of hormones, such as estrogens, which enables their interaction with hormone receptors [15]. There are two main types of EDCs: the persistent organic compounds with decade-long half-lives such as dichloro-diphenyl-trichloroethane (DDT) and polychlorinated

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biphenyls (PCBs), and those that have a shorter half-life but can cause long-term health consequences such as phthalates, parabens and bisphenol A (BPA) [16]. DDT was used in many organochlorine pesticides and insecticides before being banned for agricultural uses in 2001 [17]. PCBs were present in lubricants, electrical equipment and plasticizers up until they were banned in multiple developed countries in the 1980s [14]. Phthalates are a large group of omnipresent compounds used as liquid plasticizers [14]. Parabens are a family of chemicals that are commonly used as preservatives in cosmetic products and appear as water contaminants [18]. BPA is used to make polycarbonate plastic and epoxy resin, and it can be found in food packaging, toys, and canned beverages' linings [14].

EDCs are ubiquitous in the environment, and more than 90 % of people have detectable levels in their urine [19, 20]. Understanding if these chemicals interfere with the onset of puberty is important, but the available evidence is derived mostly from associations in epidemiological studies [21, 22]. Such studies are often plagued by unmeasured confounding, as EDC exposure is related to sociodemographic factors and lifestyle. Randomized controlled trials (RCT) are the gold standard design to study causality, but such trials involving exposure to toxic chemicals are unethical. However, identifying causal associations between EDCs and altered pubertal timing could represent a major public health opportunity to prevent disordered puberty in youth.

Mendelian Randomization (MR) is an established method to study causality, by using genetic variants (single-nucleotide polymorphisms -SNPs) to infer levels of a modifiable biomarker, such as an EDC [23]. Due to the random allocation of genetic variants at conception (Mendel's second law), MR allows for causal inference by limiting bias caused by confounders and reverse causation. Under three main assumptions, two-sample MR uses SNPs derived from large genome-wide associations studies (GWAS) for the exposures and seeks their effects on the outcomes of interest in independent large GWAS. MR thus provides evidence of the effects of a genetically predicted exposure throughout the lifetime on an outcome.

In this study, we aimed to test whether exposure to dibutyl phthalate (DBP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), BPA, DDT, 16 PCBs and n-butyl paraben (nBuP) would cause altered AAM in girls or AVC in boys using two-sample MR. We also tested for a potential mediating effect of body mass index (BMI) using multivariable MR (MVMR).

Materials and methods

We followed the MR-STROBE checklist in designing our MR study and reporting our findings.

The design of our study appears in Figure 1.

MR exposures and outcomes

We first obtained SNPs independently associated with the exposures of interest (DBP, MECPP, MEHHP, BPA, DDT, nBuP and 16 PCBs) in available GWAS for these traits in GWAS catalog (Table S1). For DBP, we used SNPs derived from a GWAS in 146 individuals of European and Sub-Saharan African ancestry [24] which measured the adverse reactions of lymphoblastic cells to chemical compounds. For BPA, we used SNPs derived from a GWAS [25] which evaluated effects of different chemicals on red blood cell metabolism in 243 individuals of unreported ancestry. For DDT, we used a GWAS [26] which measured blood levels of a major DDT breakdown product (*p*,*p*′-DDE levels) in 1,016 individuals of European ancestry. For PCBs, we used a GWAS [27] on plasma levels of PCBs in the same cohort of 1,016 individuals of European ancestry. For MECPP, MEHHP and nBuP, SNP-instruments were obtained from a GWAS which evaluated effects of different chemicals in urine in 1,085 individuals of European ancestry.

As per the first assumption of MR which states that the genetic variant should be strongly associated with the exposure, the SNPs selected for the BPA, DDT and PCBs exposures had GWAS p-values $<5\times10^{-6}$. However, in the absence of genome-wide significant SNPs for DBP, we were less strict in our selection of SNPs using SNPs at a suggestive p-value of $<5\times10^{-3}$. We also calculated the *F*-statistic of each SNP to ensure that all our instruments were strong (*F*-statistic >10) using a published formula [28], or the get_r_from_bsen() function from the TwoSampleMR R package for MECPP, MEHHP and nBuP [29].

We queried the effects of the SNPs instrumenting our 22 exposures in the ReproGen GWAS by Kentistou et al. [30] for AAM (GWAS meta-analysis excluding 23andMe; ancestry combined: n=723,014 females and European-only: n=556,124 females) and in a subset of the ReproGen consortium used in the GWAS by Hollis et al. for AVC, including UK Biobank and excluding 23andMe (n=191,270 males) [31, 32]. Descriptives of the exposure and outcome GWAS appear in Table S1. There was no sample overlap between exposure and outcome GWAS.

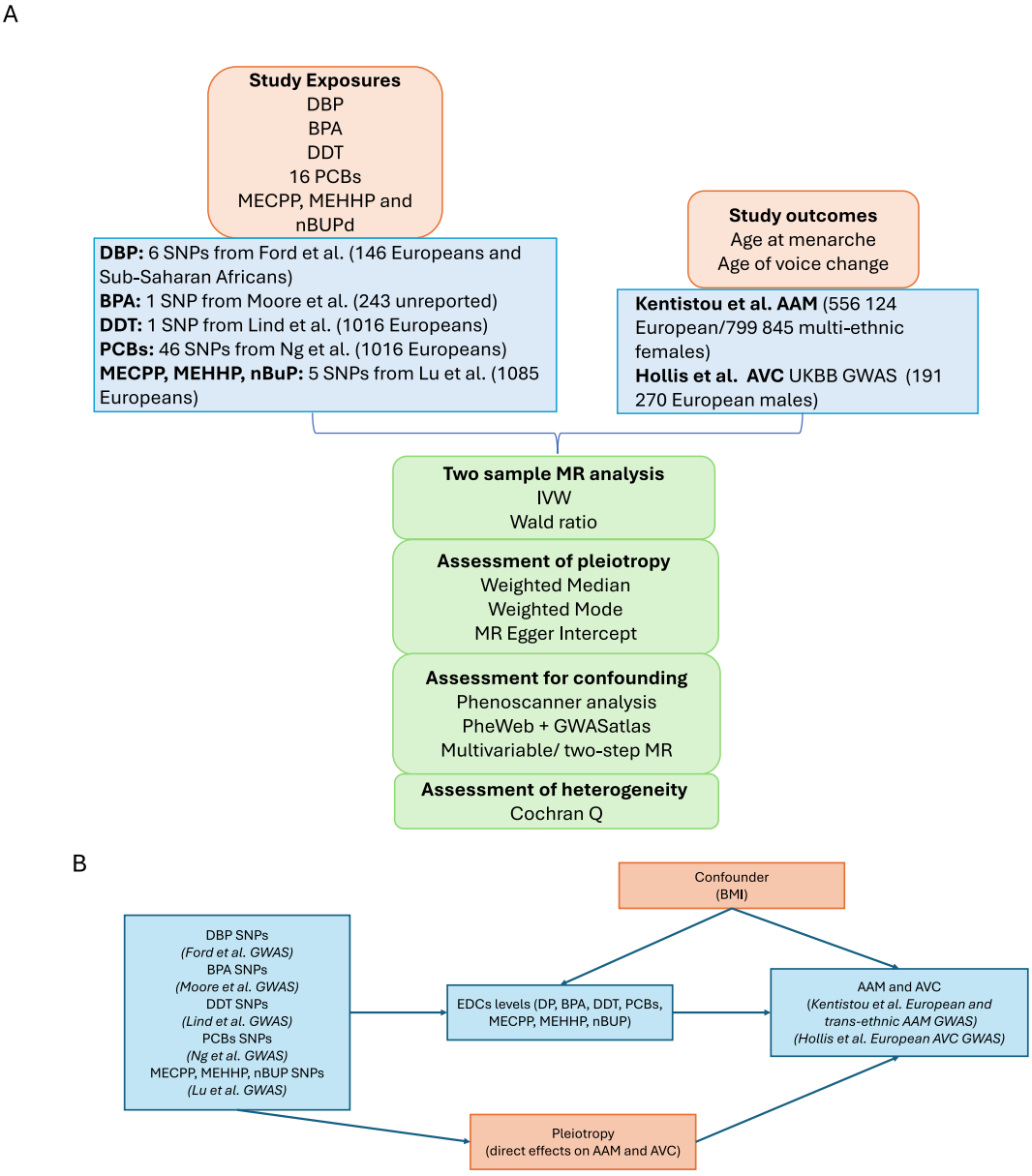

Figure 1: Summary figure of the MR study design as a flowchart (A) and a direct acyclic graph (B).

Main MR analysis

To calculate our MR estimates, we computed the Wald ratio for metabolites with a single SNP-instrument and an inverse-variance weighted (IVW) estimate for metabolites with multiple SNP-instruments using the TwoSampleMR R package [33]. The MR estimates express changes in the AAM in years per unit increase in the level of each EDC, while the exact unit of change in AVC categories per unit change in EDC cannot be provided (the AVC was treated as a categorical variable in the GWAS), although the direction of effect on AVC (i.e. increase or decrease) is informative. We adopted a Bonferroni corrected p-value threshold of 0.01 (0.05/5 groups of EDCs) for statistical significance; associations at p=0.01 to 0.05 were considered as suggestive.

Sensitivity analyses for mediation by BMI

According to the second MR assumption, the SNP-instruments in an MR study should not be associated with confounders of the exposure-outcome association. To test this assumption, we used multivariable MR (MVMR) analysis or two step network MR to test the confounding or mediating effect of BMI in the association between EDCs and pubertal timing. The effects of the SNP-instruments for the various

EDCs on BMI were extracted from the Vogelezang et al. discovery GWAS [34] on 39,620 European children. Moreover, we used three online databases (PhenoScanner [35], PheWeb [36] and GWASatlas [37]) to filter out SNPs with previously reported GWAS associations with body composition traits, BMI and socioeconomic status at a GWAS p-value $<1\times10^{-5}$.

Sensitivity analyses addressing pleiotropy

In regards to the third MR assumption (exclusion restriction assumption), we sought to discover if the SNP-instruments for the EDCs mapped in genes that were relevant to EDC metabolism, which would decrease the risk of pleiotropy. We found the associated gene for each SNP with the online tool "dbSNP" by NIH (https://www.ncbi.nlm.nih.gov/snp/), then looked up the gene function with the online tool "OMIM", an "Online Catalog of Human Genes and Genetic Disorders" (https://www.omim.org/). We also used the MR-Egger method [38] and its intercept, and the weighted median [39] and weighted mode [40] methods to test for horizontal pleiotropy. Moreover, in order to evaluate the presence of heterogeneity among the SNP-instruments of the EDCs, we computed heterogeneity estimates for the IVW and MR-Egger analyses using the Cochran Q metric.

The TwoSampleMR R package (version 0.5.6) and its default parameters (LD-clumping r2=0.001) [33] was used to harmonize the SNP-instruments for our 22 exposures between the exposure and outcome GWAS and compute the various MR estimates.

For our multivariable MR analysis, we used the MVMR R package [41]. To perform the two-step MR analysis, the first step involved conducting MR to assess the relationship between EDC levels and BMI. The second step examined the association between BMI and the outcome (AAM or AVC). The indirect effect of the EDC on the outcome, mediated through BMI, was estimated by multiplying the effect of EDC on BMI and the effect of BMI on outcomes. The standard error and the test of the indirect effect were carried out with the Sobel test. For missing SNPs in the BMI GWAS, we found proxies using LDlinkR [32].

Statistical power analysis

We computed the power in our main MR analyses for each exposure by using the online tool "mRnd" (https://shiny.cnsgenomics.com/mRnd/), using the sample size of the respective outcome GWAS, and setting the alpha level at 0.05 while using an exposure variance of 1 (one standard deviation change in each exposure) and an outcome variance of 1.69 (based on a standard deviation of AAM of 1.3 years [42]).

Results

From available GWAS data, we found 6 independent SNPs for DBP with a suggestive p-value $<5\times10^{-3}$. At a GWAS p-value $<5\times10^{-6}$, we found only one independent SNP each for BPA, DDT and nBuP, 46 independent SNPs associated with the 16 PCBs, and 1 and 4 SNPs for MEHHP and MECPP, respectively.

All 60 SNP-instruments were present in the AAM GWAS. We only found 56 of the 60 SNPs in the AVC GWAS, and no proxies for the missing SNPs. All SNP-instruments had an *F*-statistic >10 (the average *F*-statistic was 49.6). The average variance explained (R^2) of an EDC by its respective SNP-instrument(s) was 0.053 (Tables S2 and S3). We could not calculate the variance explained of MECPP, MEHHP and nBuP since information on effect allele frequency was not available in the respective GWAS.

Our gene function search showed that DDT, PCB 138 and PCB 153 are instrumented by SNPs within the gene *CYP2B6*, which is strongly associated to hepatic metabolism as it represents 2–8% of the hepatic CYP content [43]. PCB 105 is instrumented by SNPs in *CIGALT1*, which plays an important role in kidney development [44]. We identified potential confounders for rs2367809 SNP related to PCB 194 and rs1359232 SNP related to MECPP in our GWASatlas search (Table S4). Consequently, we excluded those two SNPs from subsequent analyses. Our main results for AAM and AVC can be found in Tables S5 and S6 respectively.

Among the 22 tested exposures for AAM, we found significant associations for PCB 206 (IVW beta: −0.024, 95 % CI [−0.041, −0.006], p=0.0068) in girls of European ancestry, and PCB 194 (IVW beta: −0.015, 95 % CI [−0.024, −0.007], $p=3.27\times10^{-4}$) in both European and multi-ethnic analyses. We also found two suggestive MR associations with AAM, for PCB 74 (IVW beta: −0.015, 95 % CI [−0.028, −0.003], p=0.014) in both European and multi-ethnic analyses, and for DBP (IVW beta: 0.006, 95 % CI [0.001, 0.010], p=0.013) only in Europeans. PCB 74 showed a suggestive association in the weighted median analysis as well (beta: −0.014, 95 % CI [−0.026, −0.001], p=0.029), and had the same direction of effect in all four MR analyses (IVW, weighted median, weighted mode and MR-Egger). DBP had the same direction of effect in three out of the four MR analyses. PCB 194 also showed a suggestive result in the weighted median analysis (beta: −0.014, 95 % CI [−0.026, −0.002], p=0.017), and had the same direction of effect with all four MR methods.

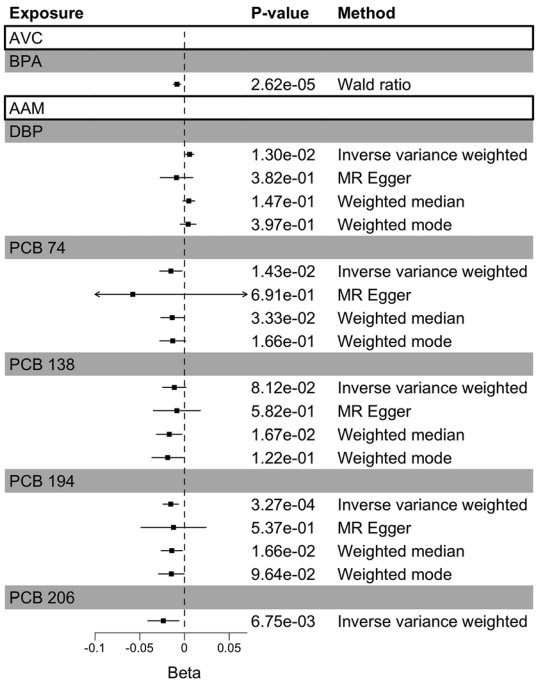

Figure 2: Forest plot of MR study investigating effects of significant EDC on AAM and AVC in Europeans. AVC=Age at Voice Change; BPA=Bisphenol A; DBP=Dibutyl Phthalate; PCB=Polychlorinated Biphenyls; AAM=Age at Menarche.

For AVC, we found a single significant MR association for BPA (MR Wald ratio= −0.008, 95 % CI [−0.012, −0.004], $p=2.62\times10^{-5}$). Since this was a single-instrument MR, sensitivity analyses for pleiotropy were not performed. We did not obtain MR results for PCB 157, since its single SNP-instrument was palindromic and ambiguous in frequency. Forest plots with the results of our significant MR results appear in Figure 2.

We then undertook MVMR to evaluate the potential mediating effect of BMI in the associations between DBP and PCB 74, which had 3 or more SNP-instruments allowing such analysis, with AAM (Table S7A). The IVW p-value for the two EDCs in these MVMRs became non-significant, suggesting that BMI may mediate the associations between DBP, PCB 74, and AAM in girls of European ancestry.

Since the small number of SNP-IVs limited our ability to perform MVMR, we undertook Two-Step MR for all MR-prioritized EDCs to assess the potential mediating effect of BMI. The results indicated a mediating effect in the association between PCB 74 and AAM (Table S7B) and in the association between BPA and AVC (Table S7C).

The power calculations for the AAM European MR analyses showed a power of 83 % for DBP, of 97 % for PCB 74, of 99 % for PCB 206 and of 90 % for PCB 194. The power analysis MR for the role of BPA on AVC showed a power of 99 % (Tables S8A and S8B). PCB 156 was the only EDC with a non-significant MR result and power >80 % in its MR, while the analyses for the remaining EDCs were underpowered.

Discussion

In this study, we used an unbiased approach based on MR to investigate whether genetically determined levels of endocrine-disrupting chemicals (EDCs) are causally associated with outcomes related to pubertal timing in boys and girls. By examining MR effects of 22 EDCs on age at menarche (AAM) in both European and multi-ethnic populations and age at voice change (AVC) in Europeans, we observed evidence suggesting causal links between DBP, PCB 74, PCB 194, and PCB 206 with AAM. Higher levels of DBP were associated with later AAM, whereas higher levels of PCB 74, PCB 194, and PCB 206 were associated with earlier AAM. Additionally, higher levels of BPA appeared to promote earlier AVC. Our MVMR analyses suggested a possible mediating effect of BMI in the association between PCB 74 and AAM, and BPA and AVC. The effect sizes on AAM of these EDCs were of a few days to a week per unit change in the EDC level.

Our results suggest a potential link between exposure to BPA and earlier AVC in boys. This aligns with a Vietnamese case-control study on 124 boys with precocious puberty, which detected urinary levels of BPA in boys with precocious puberty but not in controls [45]. However, our BPA result contradicts previous research which reported a significant correlation between higher urinary BPA levels and delayed genital maturation in boys [46]. Interestingly, the impact of BPA on pubertal timing may vary with the age of exposure. A recent study conducted in three European cohorts showed that prenatal exposure, especially in the third trimester of pregnancy for boys and the first trimester for girls, to BPA and parabens was associated with delayed puberty in both sexes [47]. Ferguson et al. compared prenatal vs. infantile exposure to phthalates and BPA in 118 boys aged 8–14 years [48]. Prenatal exposure was found to delay adrenarche, whereas childhood exposure (the ages of exposure were not defined) did not affect pubertal timing, with the duration and dose of exposure also playing a role in the association [48]. An interesting study done in rodents also showed the transgenerational effect of BPA exposure, as the offspring of prenatally exposed female rats and unexposed male rats displayed delayed pubertal onset [49].

Our MR study provides evidence linking exposure to DBP to a later puberty onset in girls. Literature about the impact of DBP exposure on female puberty is limited, and contradictory to our findings, whereas the literature about DBP exposure on male puberty is mostly in line with our findings. A Swedish study on 196 young boys found an

association between prenatal exposure to diisononyl phthalate (DiNP) metabolites (DiNP being an isomer with the same core structure as DBP) and a virilization defects in newborn boys [50]. A recent study also showed that males with higher urinary concentrations of ∑DiNP metabolites in late puberty had poorer semen quality than those with lower urinary ∑DiNP metabolite concentrations [51]. A study in 106 Chinese girls found that exposure to DBP and other types of phthalates increased kisspeptin protein's activity, a strong gonadotropin-releasing hormone (GnRH) simulator [52]. GnRH in turn stimulates the release of luteinizing hormone and follicle-stimulating hormone, which are responsible for sexual maturation and advanced puberty [53].

Our findings indicate that PCB 74, PCB 206 and PCB 194 are linked to an earlier puberty onset. Similarly to DBP, previous evidence on the impact of PCBs exposure on female puberty is sparse, as observational studies have mostly focused on male puberty. A study found a correlation between dioxin-like compounds (such as PCB 156) and organochlorine pesticides and delayed pubertal onset in boys [54], which is in the same direction with our findings in regards to the role of PCB 74 on AAM in girls. Contrarily, non-dioxin-like PCBs (such as PCB 74, 206 and 194) tend to advance pubertal start in boys [54], which is in the same direction as our findings for these metabolites in puberty in girls. Timing of exposure to these chemicals appears to play an important role. Leijs et al. found that 18 girls exposed to dioxin-like PCBs-contaminated breast milk experienced delayed breast development during puberty, but they found no association with AAM [55]. However, an earlier study that measured the PCBs serum levels in women found that their daughters, exposed to these chemicals *in-utero*, experienced earlier AAM [56].

An interesting finding of our study was a possible mediating effect of BMI in the association of PCBs and DBP with AAM. In keeping with our results, Vasiliu et al. showed that when adjusting for BMI, the association between dichlorodiphenyldichloroethylene (DDE), a toxic by-product of the DDT breakdown, and AAM was no longer significant [56], but evidence on effect modification by the BMI specifically for PCBs is lacking. In terms of the BPA, Wang et al. did not observe a mediating effect of BMI on the association between BPA and puberty, but Freire et al. noted a stronger effect on boys with a normal/underweight BMI [46, 47], which is in keeping with our results.

Taken together, the evidence from observational studies has generated conflicting findings on the association of EDCs with pubertal timing, possibly due to the layered, multifactorial nature of these associations. While our findings support a causal role of these chemicals on pubertal timing, the exact underlying mechanisms of hormonal EDC disruptions are still largely unknown.

Our study has a few limitations. Due to the small sample size of the EDCs GWAS, and the limited number of SNPs for certain exposures, explaining a small portion of the variance in the levels of these chemicals, our MR analyses for several EDCs were underpowered. This may explain the null results for most of the studied associations. The unit of change in the EDC levels in the GWAS was not uniform across the various EDCs, which complicates the interpretation of the effect sizes of our MR analysis. Due to the scarcity of SNP-instruments for many EDCs, including BPA, it was not possible to perform sensitivity analyses for pleiotropy. The SNP-instruments for DBP can be considered weak, as their GWAS p-value was of 5×10^{-3}, which could lead to false negative results for this exposure. Nevertheless, the F-statistic for these SNPs was higher than 10. Another limitation is the fact that the MR exposures (EDC levels) were measured at a more advanced age than the outcomes. All EDC GWAS were conducted in adults, but both our outcomes (AAM and AVC) occur in childhood. However, genetic variants affecting levels of modifiable exposures may be assumed to overlap between children and adults. Some EDCs are reported to lead to different pubertal consequences depending on if the exposure was *in-utero* or post-natal. Future GWAS on EDC levels in mother-child cohorts could help elucidate the role of *in-utero* exposure to these chemicals on pubertal outcomes in the offspring using the MR framework. Finally, the majority of the GWAS for the exposures and outcomes were in populations of European ancestry, which limits their generalizability in other ancestries.

Conclusions

In conclusion, our study points to a causal effect of DBP in delaying AAM, of PCB 74, PCB194 and of PCB 206 in advancing AAM, and of BPA in advancing AVC, with BMI acting as a mediator in some of the above associations. While the existing literature on the effects of some of these EDCs on female or male puberty is limited, our findings are generally concordant with the results of these studies. While EDCs appear to be causally associated with children's pubertal timing, more research is needed to deepen our knowledge of their health consequences and molecular interactions, the role of the timing of the exposure, and the mediating effect of BMI. This knowledge could support public health decisions geared toward the ban of EDCs, and ultimately contribute to a healthier pediatric population.

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Research ethics: Not applicable.

Informed consent: Not applicable.

Author contributions: DM conceived the study and supervised the analyses. MZ, IG and KF drafted the manuscript and performed the analyses. All authors contributed in study design, reviewing and writing the manuscript. All authors critically reviewed and approved the final version of the manuscript.

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Data availability: Data from the EDC GWAS were obtained from the following sources: DBP GWAS: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9413237/. BPA GWAS: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9763692/. DDT GWAS: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5152752/. PCBs GWAS: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4509719/. MECPP, MEHHP and nBUP GWAS: https://www.sciencedirect.com/science/article/pii/S0160412023006694?via%3Dihub. BMI GWAS: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7581004/. Data on age at menarche and age at voice change were obtained from the REPROGEN Consortium GWAS: https://www.reprogen.org/data_download.html. Details for each dataset can be obtained from Table S1. All R codes used to generate the results of the MR and colocalization analyses are available upon request to the corresponding author.

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