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Associations between Prenatal Phthalate Exposure and Childhood Epigenetic Age Acceleration

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Abstract

Background: Phthalates, a group of pervasive endocrine-disrupting chemicals found in plastics and personal care products, have been associated with a wide range of developmental and health outcomes. However, their impact on biomarkers of aging has not been characterized. We tested associations between prenatal exposure to 11 phthalate metabolites on epigenetic aging in children at birth, 7, 9, and 14 years of age. We hypothesized that prenatal phthalate exposure will be

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Conflict of Interest
The authors declare they have nothing to disclose.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Dennis Khodasevich: Conceptualization, Methodology, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Visualization
Nina Holland: Conceptualization, Methodology, Writing - Review & Editing, Supervision
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associated with epigenetic age acceleration measures at birth and in early childhood, with patterns dependent on sex and timing of DNAm measurement.

Methods: Among 385 mother-child pairs from the CHAMACOS cohort, we measured DNAm at birth, 7, 9, and 14 years of age, and utilized adjusted linear regression to assess the association between prenatal phthalate exposure and Bohlin's Gestational Age Acceleration (GAA) at birth and Intrinsic Epigenetic Age Acceleration (IEAA) throughout childhood. Additionally, quantile g-computation was utilized to assess the effect of the phthalate mixture on GAA at birth and IEAA throughout childhood.

Results: We found a negative association between prenatal di(2-ethylhexyl) phthalate (DEHP) exposure and IEAA among males at age 7 (−0.58 years; 95% CI: −1.02 to −0.13), and a marginal negative association between the whole phthalate mixture and GAA among males at birth (−1.54 days, 95% CI: −2.79 to −0.28), while most other associations were nonsignificant.

Conclusions: Our results suggest that prenatal exposure to certain phthalates is associated with epigenetic aging in children. Additionally, our findings suggest that the influence of prenatal exposures on epigenetic age may only manifest during specific periods of child development, and studies relying on DNAm measurements solely from cord blood or single time points may overlook potential relationships.

Keywords

phthalates; epigenetic aging; childhood; prenatal

1. Background

Endocrine-disrupting chemicals (EDCs) are a class of chemicals that can interact with the endogenous endocrine system and have been associated with a wide range of outcomes including reproductive anomalies, certain cancers, obesity, and birth weight.(Diamanti-Kandarakis et al., 2009) The Developmental Origins of Health and Disease (DOHaD) hypothesis and life course epidemiology framework emphasize the role of the early life environment in establishing adult trajectories of health and disease,(Eriksson, 2016) which highlights the importance of studying EDC exposure during fetal development.(Perera and Herbstman, 2011) Phthalates, a group of pervasive EDCs commonly found in plastics and personal care products, have received attention due to their suggested effects on human health and development, as well as ubiquitous human exposure and detection. (Dutta et al., 2020) Previous studies have reported heterogeneous associations between phthalate exposure and several developmental outcomes in childhood dependent on timing of exposure, child sex, and specific phthalate metabolites. Additionally, there is some evidence that prenatal phthalate exposure is associated with other biomarkers of aging. For example, one study involving 181 newborns reported that prenatal DEHP and MiBP exposure was associated with longer telomere length at birth among boys,(Michels et al., 2020) while another study involving 762 newborns reported that prenatal MEP, MBP, and DEHP exposure was associated with shorter telomere length at birth in both boys and girls. (Song et al., 2019)

Biological aging is a complex process tied to age-associated increased susceptibility to disease and mortality.(Harman, 1981) The development of epigenetic aging biomarkers has allowed for an improved characterization of the biological aging process using DNA methylation measurements across the genome.(Horvath, 2013) While many commonly used epigenetic biomarkers were developed using adult populations, several epigenetic clocks perform well predicting chronological age in pediatric populations. Notably, Horvath's pan-tissue clock(Horvath, 2013) accurately predicts chronological age across the lifespan and provides valuable information on the underlying process of biological aging across most human tissues. The processes of growth early in life and aging later in life are thought to share some common foundations, with the TOR signaling pathway playing a key role in both.(Blagosklonny and Hall, 2009)(Katewa and Kapahi, 2011) Some researchers have expanded the use of epigenetic clocks accordingly, finding associations between epigenetic aging in children and several measures of early life growth including height, weight, and pubertal timing.(Simpkin et al., 2017)(Binder et al., 2018)(Bright et al., 2019) Prenatal exposure to phthalates has been associated with altered DNA methylation in cord blood, (Solomon et al., 2017)(Chen et al., 2018; Miura et al., 2021) male placenta,(Jedynak et al., 2022) and peripheral blood and buccal cells, (England-Mason et al., 2022) but it is unknown how prenatal exposure to phthalates might influence epigenetic aging rates.

Several environmental exposures have been found to be associated with greater epigenetic age acceleration, including organochlorine pesticides, tobacco smoke, ambient air pollution, as well as benzene and trichloroethylene.(Lind et al., 2018)(de Prado-Bert et al., 2021) (Nwanaji-Enwerem et al., 2016)(van der Laan et al., 2022) However, to date, no studies have aimed to perform a targeted characterization of the influence of prenatal phthalate exposure on epigenetic aging during child development, despite reported associations between phthalates and early life growth. To address this gap in existing literature, we set out to utilize 15 years of longitudinal data from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort to test associations between prenatal urinary concentrations of 11 common phthalate metabolites and epigenetic age acceleration early in life. We hypothesized that prenatal phthalate exposure will be associated with epigenetic age acceleration measures in early childhood, with patterns dependent on child sex and timing of DNAm measurement.

2. Methods

2.1: Study Population

Between October 1999 and October 2000, the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) study recruited 601 pregnant women from farmworker communities of the Salinas Valley in California. Women were recruited during prenatal care visits at one of several partner clinics in the area. Eligible participants were ≤20 weeks gestation at enrollment, English- or Spanish-speaking, Medicare eligible, planning to deliver at the county hospital, and attending prenatal care visits at one of six local community clinics or hospitals. Of 601 initial enrollees of the cohort, we followed 526 to delivery of live, singleton newborns in 2000–2001. The study continues to follow up participants and their children, with data collected every few years since enrollment.

Trained bilingual staff members interviewed women twice during pregnancy, at around 13 weeks (median: 13.21, IQR: 10.29 – 18.14) and 26 weeks (median: 26.42, IQR: 25.57 – 27.57) of gestation, to collect information. At delivery hospital staff collected cord blood specimens, and at child ages 7, 9, and 14 years a phlebotomist collected child blood samples via venipuncture. The University of California, Berkeley Committee for the Protection of Human Subjects approved all study activities. Written, informed consent was obtained for all participating women, child verbal assent was obtained starting at age 7 years, child written assent was obtained starting at age 12 years, and child written consent was obtained at age 18 years.

2.2: Phthalate Measurements

Two urine samples were collected from mothers at the time of pregnancy interviews. Samples were aliquoted, barcoded, and stored at −80°C at the University of California, Berkeley until shipment on dry ice to the Centers for Disease Control and Prevention (CDC) for measurement of phthalate metabolites. Prenatal phthalate metabolites concentrations were measured in both maternal urinary samples at 13- and 26-weeks of gestation for 385 mothers in this study sample, using solid phase extraction coupled with isotope dilution high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry. (Silva et al., 2007) Quality control procedures included laboratory and field blanks, calibration standards, and controls with high and low concentrations.

Eleven phthalate metabolites from 8 parent compounds were measured in maternal pregnancy urine samples. The phthalate metabolites can be broadly divided by their molecular weight into low-molecular weight (LMW) and high-molecular weight (HMW) categories. LMW phthalates include monoethyl phthalate (MEP), a metabolite of diethyl phthalate (DEP); mono-n-butyl phthalate (MBP), a metabolite of di-n-butyl phthalate (DBP); and mono-isobutyl phthalate (MiBP), a metabolite of diisobutyl phthalate (DiBP). HMW phthalates include monobenzyl phthalate (MBzP), a metabolite of benzyl butyl phthalate (BBzP); four metabolites of di(2-ethylhexyl) phthalate (DEHP) including mono-2-ethylhexyl phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP)); monocarboxyoctyl phthalate (MCOP), a metabolite of di-isononyl phthalate (DiNP); monocarboxy-isononly phthalate (MCNP), a metabolite of di-isodecyl phthalate (DiDP); and mono(3-carboxypropyl) phthalate (MCPP), a metabolite of several HMW phthalates and a minor metabolite of DBP.

Concentrations below the limit of detection with no corresponding instrumental signal were imputed from a log-normal distribution with the fill-in method described by Lubin et al. (Lubin et al., 2004) Urinary dilution was accounted for by using specific gravity (SG), which was measured with a refractometer (National Instrument Company Inc., Baltimore, MD). Specific gravity adjustment was performed because creatinine can vary significantly over the course of pregnancy and creatinine adjustment can introduce bias in measurements of phthalates during pregnancy.(Adibi et al., 2008) SG-adjusted concentrations (μg/L) were produced using the formula: $P_C = P[(1.024 - 1)]/(SG - 1)$, where P_c is the specific gravity-adjusted concentration, P is the phthalate concentration (in μg/L), 1.024 is the median SG

of all samples, and SG is the specific gravity of the individual sample. A summary DEHP metric was used in place of individual DEHP metabolites due to high correlations between individual metabolites, and was calculated by dividing concentrations of each metabolite by its molecular weight to generate molar sums, adding the molar sums for each metabolite, and multiplying by the average molecular weight of the metabolites in the group.(Koch et al., 2005) Due to high correlation with several other metabolites, MCOP was excluded from analysis. Variation in maternal phthalate metabolite measures throughout pregnancy in the CHAMACOS cohort have been previously characterized,(Harley et al., 2017),(Holland et al., 2016) so we only briefly describe the distribution of pregnancy averaged SG-adjusted phthalate concentrations among the current study population in the supplement. (Table S1 and Figure S1)

2.3: Maternal Characteristics

Maternal characteristics data primarily came from medical records and maternal interviews conducted during pregnancy at around 13- and 26-weeks of gestation, and shortly after delivery. Maternal age at delivery and pre-pregnancy body mass index (BMI) were identified as potential confounders. Pre-pregnancy weight was obtained from prenatal medical records or from self-report at the 13-week visit if it was missing from medical records. If pre-pregnancy weight was still missing, weight at the first prenatal visit was used if the visit took place before 13 weeks gestation or, if the visit took place after 13 weeks gestation (n=8), regression models were used to impute pre-pregnancy weight based on weight at all prenatal visits. Other covariates obtained from prenatal study interviews included maternal smoking during pregnancy (no, yes), poverty status during pregnancy as determined by U.S. Census Bureau Poverty Thresholds (Bureau, n.d.) (at or below poverty, poverty - 200%, >200% poverty), and maternal parity (0, 1, 2+). Infant gestational age was obtained by asking mothers about the date of their last menstrual period (LMP) and, if unknown (n=47), was based on ultrasound methods abstracted from medical records. Medical records were systematically biased downward due to being rounded to the closest completed week. To account for this systematic downward bias, half a week was added to all gestational age estimates obtained from medical records.

2.4: DNA Methylation and Epigenetic Aging Measures

A convenience sample of children were selected for DNA methylation analysis at each timepoint, totaling up to 373 participants at birth, 189 at age 7, 241 at age 9, and 187 at age 14. After excluding participants with missing phthalate and covariate data, DNA methylation data was available from 311 participants at birth, 175 at age 7, 219 at age 9, and 179 at age 14. Detailed sample sizes for all time points as well as overlapping participants between timepoints are presented in the supplement. (Table S2).

Blood samples were refrigerated and transported to the University of California, Berkeley biorepository where samples without anticoagulant were separated into serum and clot and stored at −80° C until analysis. DNA was isolated from the banked cord blood samples from birth and blood clot samples from childhood using QIAamp DNA Blood Maxi Kits (Qiagen, Valencia, CA) according to the manufacturer's protocol with minor modifications, as previously described.(Holland et al., 2006) DNA aliquots of 1 μg were bisulfite

converted using Zymo Bisulfite Conversion Kits (Zymo Research, Orange, CA). DNA was amplified, enzymatically fragmented, purified, and applied to the Illumina Infinium HumanMethylation450 (450K) for cord blood and age 9 samples and EPIC BeadChips (Illumina, San Diego, CA) for ages 7 and 14 samples, according to the Illumina methylation protocol to measure DNA methylation. Both EPIC and 450K chips were analyzed using the Illumina Hi-Scan system. Quality control steps included the use of repeats and randomization of samples across chips and plates.(Yousefi et al., 2015) Methylation data were imported into R statistical software for preprocessing using *minfi*.(Aryee et al., 2014) We first performed quality control at the sample level, excluding samples with overall low intensities (< 10.5) and technical duplicates. We computed detection *P* values relative to control probes and excluded probes with non-significant detection ($P > 0.01$) for 5% or more of the samples. We preprocessed our data using functional normalization,(Fortin et al., 2014) adjusted for probe-type bias using the regression on correlated probes method,(Niu et al., 2016) and used ComBat from the *sva* package to adjust for sample plate as a technical batch. (Johnson et al., 2007) We visualized the data using density distributions at all processing steps and performed PC analyses to examine the associations of methylation differences with technical, biological, and measured traits with global DNAm variation using PCA plots. Cell proportions of CD8 T cells, CD4 T cells, NK cells, B cells, Monocytes, Granulocytes, and nucleated red blood cells were generated from the methylation data using the Gervin et al reference.(Gervin et al., 2019)

Epigenetic age measures, including Horvath's DNAm Age and Intrinsic Epigenetic Age Acceleration (IEAA), were generated from the processed DNA methylation data using the publicly available online Horvath's New Methylation Age Calculator.(Horvath, 2013) We did not examine other epigenetic clocks due to poor performance in children. IEAA is uncorrelated with chronological age, independent of estimated cell type composition, and provides a relative measure of one's epigenetic aging, where a positive IEAA indicates one's DNAm age is higher than their chronological age. Horvath's clock and its corresponding intrinsic epigenetic age acceleration (IEAA) measure were used for analysis of childhood data due to overall goodness-of-fit with chronological age, its multi-tissue nature, applicability throughout the human lifespan, and higher biological relevance for compounds that target the endocrine system. Bohlin's gestational age was calculated for cord blood samples using the *GAprediction* R package.(Bohlin et al., 2016)(Bohlin, 2022) A cell-adjusted gestational age acceleration (GAA) measure was generated by extracting the residuals from a linear regression of Bohlin's gestational age on chronological gestational age, adjusted for cell proportions of CD8 T cells, CD4 T cells, NK cells, B cells, Monocytes, Granulocytes, and nucleated red blood cells. Previous research has found robust correlation in DNAm age measurements obtained with 450K and EPIC,(McEwen et al., 2018)(Daredia et al., 2022) as well as high overall correlation between the two platforms,(Solomon et al., 2018) despite differences in the number of available clock sites between the two platforms. Variation in GAA at birth and IEAA throughout childhood was characterized by calculating median absolute error (MAE) from 0.

2.5: Statistical Analysis

Means and standard deviations (SD) for continuous measurements and frequencies and proportions for categorical covariates were used to describe our study sample. Previous studies have shown sex-specific effects of phthalates,(Berger et al., 2018) as well as increased susceptibility to in-utero exposures in males.(McGill et al., 2022) To identify sex-specific associations, we utilized separate linear regression models at each timepoint for each of the pregnancy average measures of phthalate metabolites, including MBP, MEP, MiBP, MBzP, MCNP, MCPP, and a summary measure of DEHP metabolites, with IEAA or cell-adjusted GAA estimates as the outcome. Models also included child sex, a phthalate*sex interaction, and the *a-priori* selected potential confounders, including maternal poverty category (at or below poverty, poverty - 200%, >200% poverty), parity (0, 1, 2+), maternal age (continuous), maternal smoking (yes or no), and maternal BMI (continuous). Significance of the phthalate*sex interaction term was used to judge the presence of differential association between phthalate metabolites and the epigenetic age measure by sex within the timepoint. Models with evidence for sex interaction, judged by an unadjusted p-value < 0.10, were then run in sex-stratified models with IEAA or GAA as the outcome, with each significant phthalate metabolite measure, and the *a-priori* selected covariates as the independent variables. Models with no evidence for sex-interaction were run in unstratified models with IEAA or GAA as the outcome, with each significant phthalate measure, and the *a-priori* selected covariates as the independent variables, additionally adjusted for child sex. Unadjusted and Benjamini-Hochberg (BH)-adjusted p-values are presented.(Benjamini and Hochberg, 1995) Statistical significance was determined with an BH-adjusted p-value < 0.05, while results displaying an unadjusted p-value < 0.05 but an adjusted p-value of > 0.05 were also discussed as marginal.

Quantile g-computation was then used to estimate the joint association between the prenatal phthalate mixture on IEAA or GAA using the *qgcomp* R package.(Keil et al., 2020) Sex-stratified quantile g-computation models were run at each time point with MBP, MEP, MiBP, MBzP, MCNP, MCPP, and a summary measure of DEHP metabolites as the mixture components, adjusting for the same covariates as the standard linear regression models described above. Significance of the quantile g-computation models was evaluated with the BH-adjusted p-value of 0.05. We report estimates and 95% bootstrap confidence intervals from 1000 iterations for difference in IEAA or GAA for 1-quartile increase in the prenatal phthalate mixture, conditional on covariates. Each individual metabolite's weight was then applied to the corresponding positive and negative scaled effect estimates to visualize each metabolite's contribution to the observed overall mixture effect. Additional sensitivity models were run following the same procedure outlined above but considering separate 13 and 26 week of gestation phthalate exposure measurements, as well as unstratified quantile g-computation models adjusted for sex. All statistical analyses were performed using R version 4.2.0.("R: The R Project for Statistical Computing," n.d.)

3. Results

Overall distributions of relevant covariates were similar in all four timepoints. Mean maternal age at delivery was approximately 26 years, with approximately 95% of the

mothers reporting not smoking during pregnancy. Approximately 62% of mothers were living at or below the poverty line, with approximately 96% living below 200% of the poverty line. At birth the mean gestational age was 39.5 weeks (range: 31 to 42.4 weeks), mean age at the 7-year visit was 7.09 years (range: 6.00 to 8.17 years), 9.24 years (range: 8.92 to 10.66 years) at the 9-year visit, and 14.13 years (range: 13.99 to 15.07 years) at the 14-year visit. A detailed summary of relevant demographics of included participants at each timepoint can be found in Table 1. Gestational age acceleration at birth and intrinsic epigenetic age acceleration measures throughout childhood for the study sample are presented in Figure 1. Cell-adjusted GAA exhibited a median absolute error (MAE) of 3.25 days at birth. The IEAA measures exhibited intermediate variability at age 7 (MAE = 1.141 years), low variability at age 9 (MAE = 0.735 years), and exhibited the highest degree of variability by age 14 (MAE = 1.373 years).

The individual phthalate models examining the influence of each phthalate metabolite on GAA at birth and on IEAA throughout childhood revealed that most associations trended in the negative direction, although most were nonsignificant and imprecise. (Figure 2) In the unstratified models, prenatal MBzP exposure and GAA at birth (β =−0.483 years, 95% CI: 0.947 to −0.019) exhibited a marginal inverse association. Prenatal MBP exposure and IEAA at the 7-year visit (β =−0.179 years, 95% CI: −0.398 to 0.040) and prenatal MBzP exposure and IEAA at the 9-year visit (β = −0.102 years, 95% CI: −0.229 to 0.024) also exhibited some of the strongest, although nonsignificant, inverse associations with IEAA among the unstratified models. Additionally, within the unstratified models the majority of all metabolites exhibited consistent negative associations with GAA and IEAA. Significant phthalate*sex-interactions were observed for DEHP exposure at age 7 ($p_{interaction}$ = 0.027), MBP exposure at age 14 ($p_{interaction}$ = 0.066), and MiBP exposure at age 14 ($p_{interaction}$ = 0.095), and were then estimated via sex-stratified models. Specifically, prenatal DEHP exposure was significantly associated with a decrease in IEAA among males at the 7-year visit (β =−0.623 years, 95% CI: −1.063 to −0.184). (Figure 2c) DEHP exposure was not associated with IEAA among females within this same timepoint. Prenatal MBP exposure (β = 0.291 years, 95% CI: −0.035 to 0.618) and prenatal MiBP exposure (β = 0.176 years, 95% CI: −0.110 to 0.462) were nonsignificantly associated with increased IEAA among females at the 14-year visit, while the same associations among males were weaker and in the negative direction. Effect estimates for the most significant terms from both the sex-stratified models and unstratified models are shown in Table 2, with a full summary attached in the supplement. (Table S3)

The phthalate mixture exhibited a negative marginal association with GAA at birth among males (psi = −1.538, 95% CI: −2.794 to −0.281) in the quantile g-computation models. No other significant associations were observed between the whole phthalate mixture and IEAA in quantile g-computation models. However, the mixture models revealed similar associations regarding the contributions of the individual metabolites. (Figure 3) At birth, MBzP received the largest negative weight among females and exhibited a marginal negative association with GAA in the individual pollutant models. At age 14, MBP received the largest positive weight among females and exhibited a nonsignificant positive association with IEAA among females in the individual pollutant models. Additionally, most quantile g-computation models produced negative overall mixture effect estimates, and most

metabolites produced negative effect estimates in the individual metabolite models. Full quantile g-computation model summaries are presented in the supplement. (Table S4)

In addition to the primary models examining pregnancy-averaged phthalate exposure, we also reran our analysis considering phthalate exposure at 13 and 26 weeks of gestation separately as a sensitivity analysis. When examining 13-week phthalate exposure, only MCNP exhibited a phthalate*sex interaction with GAA at birth and was run in sex-stratified models. 13-week MCNP exposure was significantly associated with a decrease in GAA among males at birth (β = −0.789 years, 95% CI: −1.527 to −0.050), with a positive nonsignificant association among females, while none of the unstratified individual metabolite models exhibited significant associations. (Table S5) In the quantile g-computation models, the 13-week phthalate mixture was associated with decreased GAA at birth among males (psi = −1.649, 95% CI: −2.879 to 0.419) and with decreased IEAA at 7 years among males (psi = −0.603, 95% CI: −1.190 to 0.016). (Table S6) When examining 26-week phthalate exposure, significant phthalate*sex-interactions were observed for DEHP exposure at age 7, MBP exposure at age 14, and MiBP exposure at age 14, which are the same associations found in the main pregnancy-averaged models. 26-week DEHP exposure was significantly associated with decreased IEAA among males at age 7 (β = −0.518 years, 95% CI: −0.848 to −0.188), while none of the remaining stratified models or unstratified individual metabolite models exhibited significant associations. (Table S7) All of the quantile g-computation models exhibited non-significant negative associations between the 26-week phthalate mixture and GAA/IEAA. (Table S8)

4. Discussion

Our study characterized associations between prenatal phthalate exposure and measures of epigenetic age acceleration at birth and throughout early childhood. Our findings suggest that certain prenatal environmental influences on epigenetic aging may only manifest later in childhood. We observed a significant association between prenatal DEHP exposure and decreased IEAA among males at 7-years of age. Additionally, we found a marginal negative association between the whole phthalate mixture and gestational age acceleration in males at birth.

Limited research has attempted to examine outcomes and exposures related to longitudinal changes in epigenetic age acceleration (EAA) in pediatric populations. Unlike in adult populations where EAA is often directly related to biological aging and disease states, EAA in pediatric populations might be more closely related to trajectories of growth and development. One study in the ALSPAC cohort found that a 1-year increase in EAA at age 7 was associated with greater height between the ages of 7–17 years, and also exhibited slower yearly height growth from 7–17 years.(Simpkin et al., 2017) A small study of 94 girls in the GOCS cohort found that increased EAA, measured at Tanner 2 and Tanner 4 timepoints with Horvath’s clock, was associated with decreased time to menarche.(Binder et al., 2018) Another study in the ALSPAC cohort found that positive gestational age acceleration, measured with Bohlin’s gestational age clock, was associated with higher birth weight and length, which attenuated with increasing age, becoming associated with lower average weight by 10 years old.(Bright et al., 2019)

Our findings suggested that prenatal DEHP metabolite exposure is associated with decreased IEAA in males at age 7. DEHP is ubiquitous in the environment due to its presence in plastics and personal care products, and has received particular research focus among phthalates.(Rowdhwal and Chen, 2018) Previous studies has also reported that males may be more susceptible to the epigenetic aging effects of certain prenatal exposures.(McGill et al., 2022) The male-specific influence of DEHP reported in our study is supported by previously reported male-specific reproductive effects of DEHP, linked to the anti-androgenic effect of DEHP.(Radke et al., 2018) Within the CHAMACOS cohort, prenatal exposure to DEP, DBP, and DEHP were positively associated with BMI z-scores and other measures of obesity status at various time points between age 5–12, with similar associations found in boys and girls.(Harley et al., 2017) A further study examining prenatal exposure to HMW phthalates and BPA within the CHAMACOS cohort found that exposure to DEHP and BBzP metabolites were associated with later onset of pubertal markers in girls, and earlier onset of pubertal markers in boys.(Berger et al., 2018) A recent meta-analysis of 6045 participants from 16 cohorts, including CHAMACOS, reported that prenatal exposure to several phthalate metabolites, including some DEHP metabolites, was associated with higher odds of preterm birth.(Welch et al., 2022) Another study in the Rhea pregnancy cohort found that a 10-fold increase in prenatal DEHP exposure was modestly associated with a decrease in waist circumference of 2.6 cm in boys and an increase in waist circumference of 2.14 cm in girls aged 4–6 years old.(Vafeiadi et al., 2018) A recent meta-analysis found that prenatal phthalate exposure, particularly DEHP, was associated with decreased BMI z-score in children, but not associated with body fat percentage.(Lee et al., 2022) This study posited that the underlying mechanism of prenatal DEHP exposure may involve the disruption of muscle growth in early childhood, rather than directly promoting development of body fat mass. In this context, the associations between DEHP exposure and negative IEAA among males at age 7 found in our study could be indicative of this disruption of early-life muscle growth.

Our study also found that the whole phthalate mixture was marginally associated with decreased GAA at birth among boys, and that MBzP was marginally associated with decreased GAA at birth among all participants. Previous studies have found inverse associations between prenatal exposure to several phthalate metabolites, including MBzP, and birth weight.(Ferguson et al., 2022) Additionally, positive gestational age acceleration measures have been found to be associated with larger weight at birth.(Bright et al., 2019) In this context, the marginal negative associations observed between MBzP and the whole phthalate mixture could be indicative of the growth inhibiting influence of prenatal phthalate exposure. However, the causes and consequences of epigenetic aging in children is still relatively poorly understood and warrants further research.

Epigenetics is a key mechanism through which prenatal phthalate exposure can translate to health outcomes later in life. One study involving 336 CHAMACOS participants found that prenatal phthalate exposure was associated with 27 significantly differentially methylated regions in cord blood in genes related to endocrine functions, immune response, fertility, and cancer.(Solomon et al., 2017) Extensive sex-specific differences in DNA methylation patterns have also been reported, with a recent meta-analysis involving 8438 newborns identifying 46979 autosomal CpG sites associated with sex, with most sites continuing

to maintain associations with sex through ages 5.5–10 years old.(Solomon et al., 2022) Interestingly, differentially methylated CpG sites from this study are heavily represented among common epigenetic clocks, with 89 of the 353 CpG sites used by the Horvath clock being among the 46979 autosomal CpG sites associated with sex.

The influence of environmental exposures on epigenetic aging early in life represents a largely unexplored topic, with few other studies seeking out to characterize the influence of prenatal environmental chemical exposures on epigenetic aging early in life. One study of 1173 children in the HELIX cohort evaluating 83 prenatal exposures and 103 early childhood exposures, including 10 phthalate metabolites, found only nominal associations between maternal smoking during pregnancy and EAA at age 7, measured using the Skin & Blood clock.(de Prado-Bert et al., 2021) However, this analysis was limited by an untargeted approach, the reliance of EAA at a single timepoint as the primary outcome for prenatal exposures, and the usage of the Skin & Blood age estimate as the primary epigenetic aging marker. Another study examining the influence of maternal factors on EAA in 613 newborns, measured in cord blood using Horvath's clock, found associations between prenatal tobacco exposure and maternal SES with EAA at birth.(Javed et al., 2016) The influence of other environmental exposures, particularly known and potential developmental toxins, in epigenetic aging early in life represents a key topic for future research.

Our findings are subject to a few relevant limitations. First, our study's relatively small sample size limited the power of our analysis. Although we were still able to detect significant associations within our sample, the majority of models returned null results with relatively wide confidence intervals, highlighting the need to study associations between phthalates and epigenetic aging in additional cohorts. Second, our study sample may affect the generalizability of our results to the general population; most mothers in our study were recent immigrants from Mexico who were of low income and did not complete high school. There is potential for effect modification from social factors or exposure patterns specific to our study population. However, our work contributes to the ongoing necessity of expanding environmental epigenetics research to include more diverse study populations. Third, phthalate exposure was only measured during pregnancy, and it is possible that postnatal and early childhood phthalate exposure may further influence epigenetic aging throughout childhood. Phthalates have relatively short biological halflives so measures of urinary metabolites likely to only reflect recent exposure.(Janjua et al., 2008) Major sources of exposure in the study population are unknown, but a recent study in the same geographical area highlighted personal care products as a potential source of exposure.(Harley et al., 2016) This study utilized pregnancy average phthalate metabolite concentrations obtained from two timepoints during pregnancy, which allowed for a more accurate representation of ongoing phthalate exposure relative to a single measure.

Our study features some notable strengths. We performed the first targeted characterization of the influence of phthalates on DNAm aging early in life, finding evidence that prenatal exposure to common phthalate metabolites is associated with epigenetic age acceleration measures in early childhood. The presence of DNAm data from several timepoints throughout childhood allowed us to highlight that associations between prenatal exposures and epigenetic aging may only manifest during limited age windows. These findings

can help pave the way for further research on the epigenetic aging effects of endocrine-disrupting chemicals and other developmental toxins.

5. Conclusions

Although most tested associations were null, we found evidence that prenatal phthalate exposure, particularly DEHP, may be associated with epigenetic age acceleration in children. The associations between prenatal phthalate exposure and epigenetic aging were influenced by sex and age, with the strongest association observed between DEHP exposure and decreased IEAA among males at the 7-year visit. Our findings suggest that associations between prenatal exposures and epigenetic aging may manifest years following initial exposure, highlighting the importance of studying epigenetic aging in longitudinal cohorts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Characterization of associations between prenatal exposure to phthalates and epigenetic age acceleration measures ranging from birth to adolescence.
- Prenatal DEHP exposure is associated with decreased epigenetic age acceleration among males at 7-years of age.
- Phthalate mixtures are marginally associated with decreased gestational age acceleration in males at birth.

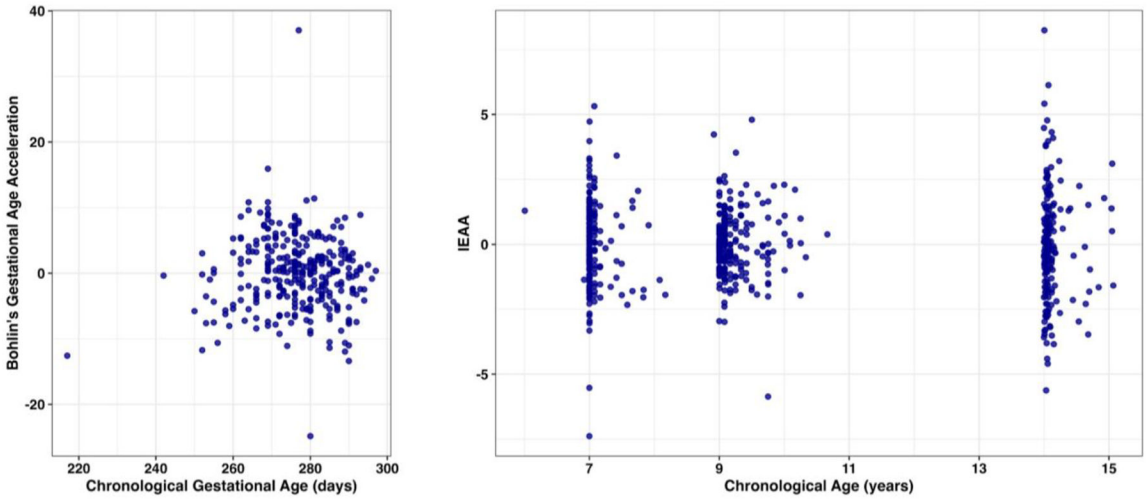

Figure 1:

Cell-adjusted Bohlin's gestational age acceleration (GAA) measures compared to chronological gestational age at birth (left) and Intrinsic epigenetic age acceleration (IEAA) measures compared to chronological age from childhood (right).

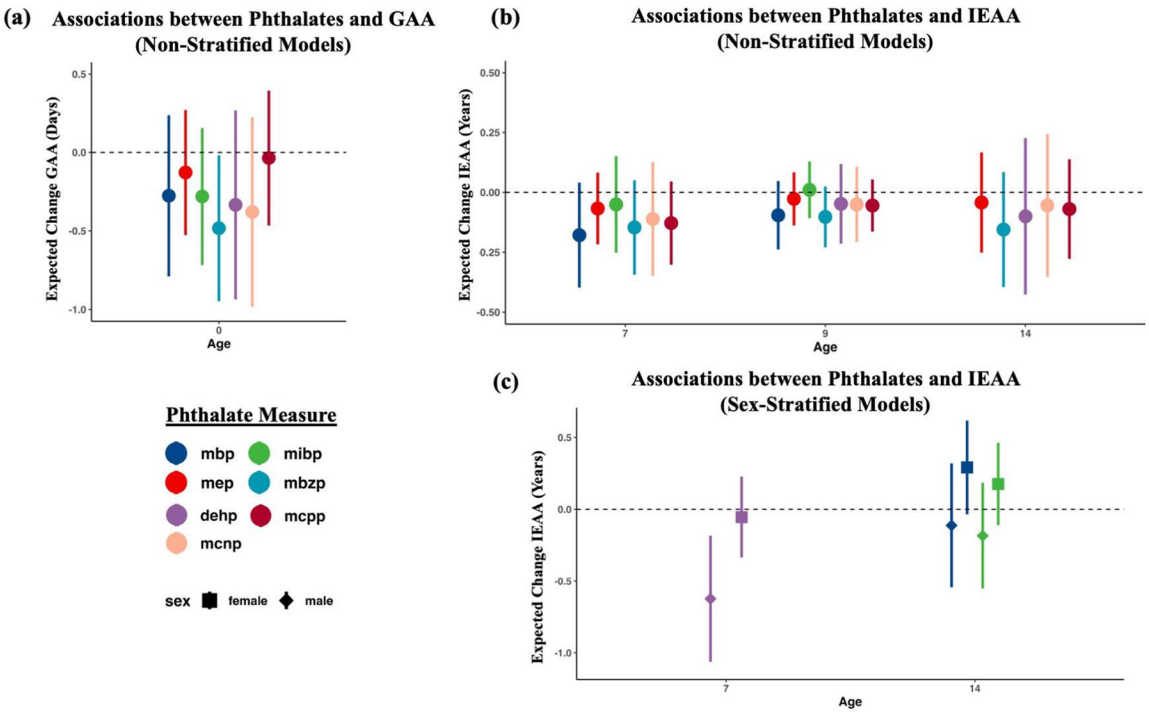

Figure 2:

Model coefficients for the associations between individual phthalate metabolites at each timepoint and epigenetic age acceleration measures. Associations between phthalates and GAA at birth (a) or IEAA in childhood (b) from the non-stratified and sex-adjusted models. Sex-stratified associations between the phthalates and IEAA in childhood among phthalates exhibiting significant sex*phthalate interactions (c). Points represent coefficients and lines represent the 95% CI for the estimate, with color corresponding to phthalate metabolite.

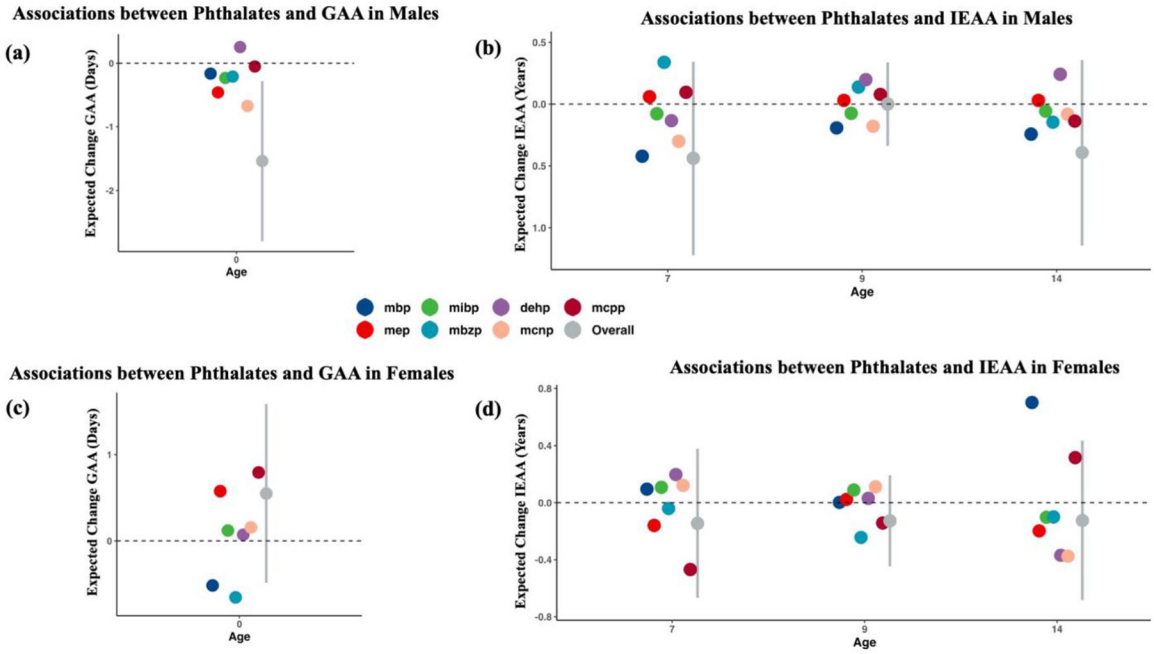

Figure 3:

Quantile q-computation model coefficients from sex-stratified models for the associations between the phthalate mixture and GAA in males (a), IEAA in males (b), GAA in females (c), and IEAA in females (d). Gray points and lines represent the expected change in epigenetic age acceleration measure for a 1 quartile increase in the whole phthalate mixture and corresponding 95%. Colored points represent contributions of individual phthalates, with color corresponding to phthalate metabolite.

Table 1:

Maternal and child sociodemographic characteristics at each timepoint. Summaries presented as count (%) or mean (sd) when appropriate. Sample includes data from n=385 total unique participants from birth to 14 years of age.

	Birth	Age 7	Age 9	Age 14
Sample Size	311	175	219	179
Array	450K	EPIC	450K	EPIC
Child Characteristics				
Sex				
Female	158 (50.8%)	99 (56.6%)	122 (55.7%)	96 (53.6%)
Age (years)	---	7.09 (0.23)	9.24 (0.32)	14.13 (0.21)
Gestational Age (weeks)	39.50 (1.48)	---	---	---
Horvath Age (DNAmAge)	−0.23 (0.16)	9.11 (1.86)	6.04 (1.40)	13.15 (2.44)
IEAA (years)	−0.12 (0.62)	−0.02 (1.70)	0.02 (1.25)	−0.05 (2.14)
Maternal Characteristics				
Maternal Age at Delivery (years)	26.06 (5.03)	26.21 (4.97)	26.65 (5.25)	26.31 (4.86)
Pre-Pregnancy BMI	26.73 (5.21)	27.34 (5.29)	27.49 (5.48)	27.47 (5.73)
Smoking during Pregnancy				
No	294 (94.5%)	168 (96.0%)	211 (96.3%)	172 (96.1%)
Poverty Category				
At or Below Poverty Line	191 (61.4%)	110 (62.9%)	141 (64.4%)	111 (62.0%)
Poverty Line to 200%	109 (35.0%)	60 (34.3%)	67 (30.6%)	63 (35.2%)
>200% Poverty Line	11 (3.5%)	5 (2.9%)	11 (5.0%)	5 (2.8%)
Parity				
0	110 (35.4%)	61 (34.9%)	67 (30.6%)	54 (30.2%)
1	85 (27.3%)	47 (26.9%)	63 (28.8%)	55 (30.7%)
2+	116 (37.3%)	67 (38.3%)	89 (40.6%)	70 (39.1%)

Table 2:

Top phthalate model coefficients and 95% CI for the effect of individual phthalates on epigenetic aging measures within each timepoint from sex-stratified models (top) and sex-adjusted models (bottom). Model covariates included maternal poverty category, parity, maternal age, maternal smoking, child sex, and maternal BMI. Four models with lowest p-values presented for both sex-stratified models and overall models. Model coefficients represent expected change in IEAA (years) or GAA (days), for a 1 unit change in log-transformed SG-adjusted phthalate measures, expressed in μmol/mL for the DEHP summary measure and ng/mL for all other metabolites.

Model	EA Measure	Sex	Age	Metabolite	Beta (95% CI)	Unadjusted p-value	BH-Adjusted p-value
Sex-Stratified							
	IEAA	**Male**	**7**	**DEHP**	**−0.623 (−1.063, −0.184)**	**0.007**	**0.04**
	IEAA	Female	14	MBP	0.291 (−0.035, 0.618)	0.08	0.25
	IEAA	Female	14	MiBP	0.176 (−0.110, 0.462)	0.23	0.46
	IEAA	Male	14	MiBP	−0.184 (−0.552, 0.184)	0.33	0.50
Sex-Adjusted							
	GAA	---	**0**	**MBzP**	**−0.483 (−0.947, −0.019)**	**0.04**	**0.61**
	IEAA	---	7	MBP	−0.179 (−0.398, 0.040)	0.11	0.61
	IEAA	---	9	MBzP	−0.102 (−0.229, 0.024)	0.12	0.61
	IEAA	---	7	MBzP	−0.147 (−0.344, 0.051)	0.15	0.61

Abbreviations: BH, Benjamini-Hochberg