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Maternal Prenatal Anxiety and the Fetal Origins of Epigenetic Aging

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ABSTRACT

BACKGROUND: The fetal origins of mental health is a well-established framework that currently lacks a robust index of the biological embedding of prenatal adversity. The Pediatric-Buccal-Epigenetic (PedBE) clock is a novel epigenetic tool that associates with aspects of the prenatal environment, but additional validation in longitudinal datasets is required. Likewise, the relationship between prenatal maternal mental health and the PedBE clock has not been described.

METHODS: Longitudinal cohorts from the Netherlands (Basal Influences on Baby Development [BIBO] n = 165) and Singapore (Growing Up in Singapore Towards Healthy Outcomes [GUSTO] n = 340) provided data on prenatal maternal anxiety and longitudinal assessments of buccal cell–derived genome-wide DNA methylation assessed at 6 and 10 years of age in BIBO, and at 3, 9, and 48 months of age in GUSTO. Measures of epigenetic age acceleration were calculated using the PedBE clock and benchmarked against an established multi-tissue epigenetic predictor.

RESULTS: Prenatal maternal anxiety predicted child PedBE epigenetic age acceleration in both cohorts, with effects largely restricted to males and not females. These results were independent of obstetric, socioeconomic, and genetic risk factors, with a larger effect size for prenatal anxiety than depression. PedBE age acceleration predicted increased externalizing symptoms in males from mid- to late childhood in the BIBO cohort only.

CONCLUSIONS: These findings point to the fetal origins of epigenetic age acceleration and reveal an increased sensitivity in males. Convergent evidence underscores the societal importance of providing timely and effective mental health support to pregnant individuals, which may have lasting consequences for both mother and child.

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Epidemiological analyses of prospective birth cohorts show that exposure to prenatal maternal anxiety and/or depression confers a twofold increased risk for child emotional and behavioral symptoms and clinical levels of anxiety/depression in early adulthood, effects that cannot be explained by postpartum symptoms or social circumstance alone (1–4). Likewise, neonatal neuroimaging studies support a specific contribution of prenatal maternal distress to infant neurodevelopment (5–8). However, a common feature of such studies is the marked variation at the level of the individual child, including evidence of sex-specific effects (9–11). Such findings underscore the importance of identifying an informative biomarker that quantifies the likely impact of prenatal distress on child development.

Epigenetic clocks, which derive estimates of biological age from measures of DNA methylation, have emerged as clinically relevant biomarkers that predict adverse health outcomes in adults (12). Epigenetic age acceleration, defined as increased epigenetic age in relationship to chronological age, associates with a range of adverse outcomes in adults (12). Chronic stress also predicts epigenetic age acceleration, likely mediated by glucocorticoids (13). In pediatric cohorts, psychosocial adversity associates with accelerated epigenetic aging derived from a well-established multi-tissue estimator (the Horvath clock) (14,15). However, the large error in epigenetic age estimates provided by the Horvath clock (~3.6 years) limits the sensitivity of such analyses in children.

The Pediatric-Buccal-Epigenetic (PedBE) clock is a novel epigenetic clock that provides an accurate estimate of chronological age in children from an easily accessible biosample (buccal cells) (16). Here, we sought to further characterize the PedBE clock, to determine if the PedBE clock is sensitive to variation in prenatal maternal mental health, if such effects are sex-specific, and the relevance for child mental health. We also test if the PedBE is sensitive to glucocorticoid exposure (17). We do so in 2 independent cohorts, which provide repeated measures of DNA methylation data, paired child genotypes, and detailed measures of the prenatal environment.

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METHODS AND MATERIALS

Participants

Table 1 provides an overview of the cohorts used for these analyses. Basal Influences on Baby Development (BIBO) (in Dutch: Basale Invloeden op de Baby Ontwikkeling) is a community-based cohort from the Netherlands. Ethical approval for the BIBO study was granted by the Ethical Committee of the Faculty of Social Sciences, Radboud University, Nijmegen. The Growing Up in Singapore Towards Healthy Outcomes (GUSTO) cohort recruited pregnant women over the age of 18 years across 2 hospital sites in Singapore (18). Ethical approval for the GUSTO study was granted by governing hospital boards. Fully informed consent, and child assent where applicable, was obtained from all participants included in this study.

Maternal Mood

We used the State-Trait Anxiety Inventory (STAI) to measure maternal symptoms of anxiety (19). Prenatal assessments were collected at 37-week gestation in BIBO and between 26 and 28 weeks of pregnancy in GUSTO. In BIBO, postnatal assessments of maternal anxiety were assessed using the State subscale only at 6 and 8 years postpartum, while both state and trait measures were available at 3 months postpartum in the GUSTO cohort. We focus our analyses on maternal trait anxiety (STAI), where available, as a more stable index of maternal anxiety and performed sensitivity analyses using measures of state anxiety. Clinically significant trait anxiety was defined using an established threshold (STAI $>$ 40) (20).

Maternal symptoms of depression were assessed using the Edinburgh Postnatal Depression Scale (EPDS) (21) across both cohorts. In the BIBO cohort, the EPDS was administered at 37 weeks of pregnancy and at 6 and 8 years postpartum. In the GUSTO cohort, the EPDS was administered between 26 and 28 weeks of pregnancy and at 3 months postpartum. Clinically significant depression was defined as an EPDS score greater than 12 (21).

Table 1. BIBO and GUSTO Cohort Profiles

Measure	BIBO, *n* = 165	GUSTO, *n* = 340
Female, %	47.3%	49.4%
Gestational Age, Weeks	40.1 (1.3)	38.8 (1.2)
Birthweight, g	3601 (464)	3120 (423)
% Missing	9.1%	6.5%
Ethnicity, %		
Caucasian	100%	–
Chinese	–	56.4%
Indian	–	18.8%
Malay	–	24.8%
Prenatal Anxiety	32.1 (7.2)	37.8 (9.1)
% Missing	4.2%	11.5%
Prenatal Depression	5.4 (3.9)	7.8 (4.6)
% Missing	13.9%	11.5%
Age Time Point 1	6.09 (1.72) years	3.06 (0.25) months
n	145	200
Age Time Point 2	10.10 (0.20) years	9.12 (0.31) months
n	158	307
Age Time Point 3	–	48.79 (1.03) months
n	–	302

Data are mean (SD) or %. Maternal prenatal anxiety was measured using the Trait subscale of the State-Trait Anxiety Inventory. Maternal prenatal depression was measured using the Edinburgh Postnatal Depression Scale. Ethnicity was based on maternal report.

BIBO, Basal Influences on Baby Development; GUSTO, Growing Up in Singapore Towards Healthy Outcomes.

Socioeconomic Status

Level of maternal education (low = secondary school diploma or less vs. high = education past secondary school) served as a proxy for maternal socioeconomic status.

Child Mental Health

In the BIBO cohort, maternal ratings of child mental health symptoms were assessed using the Dutch Child Behavior Checklist (22) at 6, 7, and 10 years of age. Internalizing problems were derived from anxious/depressed symptoms, somatic complaints, and withdrawn behaviors, while externalizing problems included delinquent and aggressive behaviors and attention problems. Maternal ratings of child mental health were assessed using the Child Behavior Checklist at 48 months of age in the GUSTO cohort (23).

Biosampling

Buccal swabs were collected from participants in both cohorts using the Isohelix SK-1S collection kit (Isohelix) and genomic DNA extracted using commercially available kits (see Supplemental Methods).

DNA Methylation Analysis

We used the Infinium MethylationEPIC array (Illumina) to describe genome-wide DNA methylation from buccal cell–derived genomic DNA. Signal extraction from raw image files, quality control, and preprocessing steps were performed using the Minfi package in R (24) (see Supplemental Methods). In the BIBO cohort, 165 unique participants (47.3% female) provided DNA methylation data at 1 or more time points, with data available across both time points for 138 participants (45.7% female). In the GUSTO cohort, 340 participants provided methylation data at 1 or more time points (49.4% female), 322 participants provided data at 2 or more time points (49.4% female), and data across all 3 time points were available for 147 participants (53.7% female). For clarity, we provide the number of unique participants (n_p) included in each analysis together with the number of datapoints (n_d) included in longitudinal analyses.

Epigenetic Age Acceleration

We used the total PedBE clock described by McEwen *et al.* (16) to estimate our primary measure of child epigenetic age. The PedBE clock is derived from DNA methylation at 94 CpGs and shares 1 CpG with the Horvath clock. We used linear regression models to regress chronological age on PedBE-derived epigenetic age estimates and used the resulting residuals as our primary measure of epigenetic age acceleration

(AgeAccPedBE). We used the Horvath clock (22) to calculate estimates of epigenetic age (DNAmAgeHO) and the associated measure of epigenetic age acceleration (AgeAccHO).

Genetic Analyses

We used the OmniExpress + Exome array and the Global Screening Array (Illumina) to describe genetic variation in the GUSTO and BIBO cohorts, respectively (see Supplemental Methods). Measures of population stratification (ancestry) were derived from a principal component analysis of genetic data across both cohorts, with genetic principal component scores considered in all multivariate analyses (see Supplemental Methods).

It is possible that passive genetic transmission from mother to child may confound associations between maternal prenatal mood and child outcomes (25). To test this hypothesis, we performed secondary analyses where child polygenic risk scores for 1) generalized anxiety disorder, 2) major depression, and 3) autism (26–28) were considered in prediction models of child epigenetic age acceleration. Polygenic risk scores were calculated as previously described (29). DNA methylation from at least 1 time point and paired genetic data were available on 159 individuals (47.0% female) in BIBO and 328 individuals (49.7% female) in GUSTO.

DNA Methylation Index of Glucocorticoid Exposure

Building on previous findings based on the Horvath clock (13), we sought to determine if the PedBE clock is sensitive to glucocorticoid exposure. In the absence of direct assessments of glucocorticoid exposure, we made use of a DNA methylation–based biomarker, originally developed using hippocampal stem cells and peripheral blood (17) (see Supplemental Methods). The original glucocorticoid exposure biomarker is a weighted score, where weights correspond to the magnitude of change in DNA methylation (at each CpG) following dexamethasone stimulation in peripheral blood. Given the tissue specificity of these weights, we created an unweighted score defined as the sum of DNA methylation (standardized beta values) across glucocorticoid-sensitive CpGs. DNA methylation data (beta values) from 23 of 24 glucocorticoid-sensitive CpGs were available for analysis and included in our score (Table S1). All scores were adjusted for buccal cell heterogeneity. One participant with a very high (>3 SD above mean) score was excluded from further analyses. We note that both the standardized score and weighted score were significantly and inversely correlated across both cohorts (Figure S1). We calculated this biomarker of glucocorticoid exposure for the first time point for all children, i.e., at 6 years in the BIBO cohort and at 3 months in the GUSTO cohort, and examined concurrent associations with child AgeAccPedBE.

Data Analysis

Repeated measures correlations (30) were performed in R (R Foundation for Statistical Computing) and described the relationship between longitudinal measures of epigenetic age and chronological age. Median standard error estimates were used to describe the accuracy of age prediction from each epigenetic clock. A modified Pittman test (31) was used to assess if variance in epigenetic age acceleration was stable over time.

Longitudinal Prediction Models—Maternal Prenatal Anxiety

A generalized estimating equation (GEE) was used (32) to describe the association between prenatal maternal mood and child epigenetic age acceleration over time. GEE is a generalized linear model that is robust to the correlation structure between dependent variables (33). Genetic principal component scores, sex, time point, gestational age, maternal education, and buccal cell heterogeneity were considered in all models. Participants who provided at least one measure of epigenetic age acceleration, maternal ratings of prenatal anxiety, and data on relevant covariates (see Equation 1) were included in our primary longitudinal analysis (BIBO: n_d = 271/n_p = 140, 47.0% female; GUSTO: n_d = 693/n_p = 288, 49.7% female).

$$\text{EAA} \sim \text{ANX}_{\text{prenatal}} + \text{Sex} + \text{GenPCs} + \text{Time Point} + \text{GA} + \text{MatEdu} + \text{BCC} \quad (1)$$

We also examined the association between maternal anxiety in the postnatal period and measures of child epigenetic age acceleration (equation 2 and equation 3).

$$\text{EAA} \sim \text{ANX}_{\text{postnatal}} + \text{Sex} + \text{GenPCs} + \text{Time Point} + \text{GA} + \text{MatEdu} + \text{BCC} \quad (2)$$

$$\text{EAA} \sim \text{ANX}_{\text{prenatal}} + \text{ANX}_{\text{postnatal}} + \text{Sex} + \text{GenPCs} + \text{Time Point} + \text{GA} + \text{MatEdu} + \text{BCC} \quad (3)$$

Finally, we tested if prenatal maternal anxiety associated with the pace of epigenetic aging, operationalized by testing the interaction between prenatal anxiety and child age in the prediction of in epigenetic age acceleration (Equation 4).

$$\text{EAA} \sim \text{ANX}_{\text{prenatal}} + \text{Sex} + \text{GenPCs} + \text{Age}_{\text{chrono}} + \text{GA} + \text{MatEdu} + \text{BCC} + \text{ANX}_{\text{prenatal}} \times \text{Age}_{\text{chrono}} \quad (4)$$

where EAA is child epigenetic age acceleration, ANX is maternal anxiety, GenPCs is genetic principal component scores, GA is gestational age, MatEdu is maternal education, BCC is buccal cell count, and $\text{Age}_{\text{chrono}}$ is chronological age.

Secondary models considered child polygenic risk scores together with all variables described in equation 1. Finally, given an extensive literature pointing to sex-specific effects of prenatal adversity on child outcomes (31), we repeated our primary analyses stratified by child sex.

Child Internalizing/Externalizing Behaviors

A GEE model was used to assess the association between child epigenetic age acceleration at 6 years of age in the BIBO cohort and longitudinal measures of internalizing and externalizing symptoms from 6 to 10 years of age with complementary analyses performed in the GUSTO cohort using epigenetic age acceleration at 3 or 48 months and Child

Behavior Checklist data at 48 months of age. Analyses were stratified by sex, and all models considered measures of population stratification and age at time of assessment.

RESULTS

Epigenetic age estimates from both predictors were significantly correlated with chronological age in the BIBO cohort (DNAmAgePedBE: $r_{rm} = 0.98$, $p < 2.2 \times 10^{-16}$; DNAmAgeHO $r_{rm} = 0.89$, $p < 2.2 \times 10^{-16}$, $n_p = 159$) and the GUSTO cohort (DNAmAgePedBE $r_{rm} = 0.99$, $p < 2.2 \times 10^{-16}$; DNAmAgeHO $r_{rm} = 0.96$, $p < 2.2 \times 10^{-16}$, $n_p = 328$) (Figure 1). DNAmAgePedBE and DNAmAgeHO estimates were also correlated (BIBO: $r_{rm} = 0.96$, $p < 2.2 \times 10^{-16}$; GUSTO: $r_{rm} = 0.96$, $p < 2.2 \times 10^{-16}$). Median standard errors between predicted and observed age were consistently lower using the PedBE estimator in both cohorts (Figure 1), indicating greater accuracy of the PedBE clock. Similarly, within-person differences between epigenetic age estimates and chronological age were consistently smaller (indicating greater accuracy) using the PedBE estimator (Table 2).

Variance in DNAmAgePedBE increased significantly between 6 and 10 years of age in the BIBO cohort ($z = -6.027$, $p = 1.68 \times 10^{-9}$) and from 3 to 48 months in the GUSTO cohort ($z = -9.332$, $p < 2.2 \times 10^{-16}$). Similar, but less pronounced, change in DNAmAgeHO variance estimates were observed over time (BIBO: $z = -2.488$, $p = 4.86 \times 10^{-4}$; GUSTO: $z = -7.009$, $p = 2.41 \times 10^{-12}$).

Finally, while AgeAccPedBE correlated with AgeAccHO in both cohorts, we observed a significantly higher correlation of epigenetic age acceleration measures in BIBO ($r_{rm} = 0.44$, $p = 4.7 \times 10^{-8}$) versus the younger GUSTO cohort ($r_{rm} = 0.19$, $p = 5.1 \times 10^{-5}$; Fisher Z-transformation: $p < .01$).

Sex Differences

In the BIBO cohort, females showed higher AgeAccPedBE ($t_{156} = 2.97$, $p = .003$, $n_p = 158$) and AgeAccHO ($t_{156} = 2.42$, $p = .02$, $n_p = 158$) than males at 10 years of age (Figure 2A, B). No significant differences were observed at 6 years (AgeAccPedBE: $t_{143} = 1.56$, $p = .12$; AgeAccHO: $t_{143} = -0.66$, $p = .50$, $n_p = 145$) (Figure 2A, B). At younger ages, in the GUSTO cohort, males showed higher AgeAccPedBE than females at 3 months only ($t_{198} = -2.03$, $p = .044$, $n_p = 200$), with no significant difference at 9 months ($t_{305} = -0.13$, $p = .90$, $n_p = 307$) or 48 months of age ($t_{300} = 1.36$, $p = .17$, $n_p = 302$) (Figure 2A). We observed no sex differences in AgeAccHO at 3, 9, or 48 months (all $p \geq .20$) in the GUSTO cohort (Figure 2B).

Bivariate associations between our different measures of child epigenetic age acceleration and additional covariates can be seen in Figures S2 (BIBO) and S3 (GUSTO).

Prenatal Anxiety Predicts Pediatric Epigenetic Age Acceleration

In the BIBO cohort, GEE analyses revealed a significant positive association between prenatal maternal anxiety and child AgeAccPedBE from 6 to 10 years (estimate = 0.023, $p = .01$, $n_d = 271/n_p = 140$), independent of relevant covariates (see equation 1). Likewise, in the GUSTO cohort, prenatal anxiety predicted increased AgeAccPedBE from 3 to 48 months

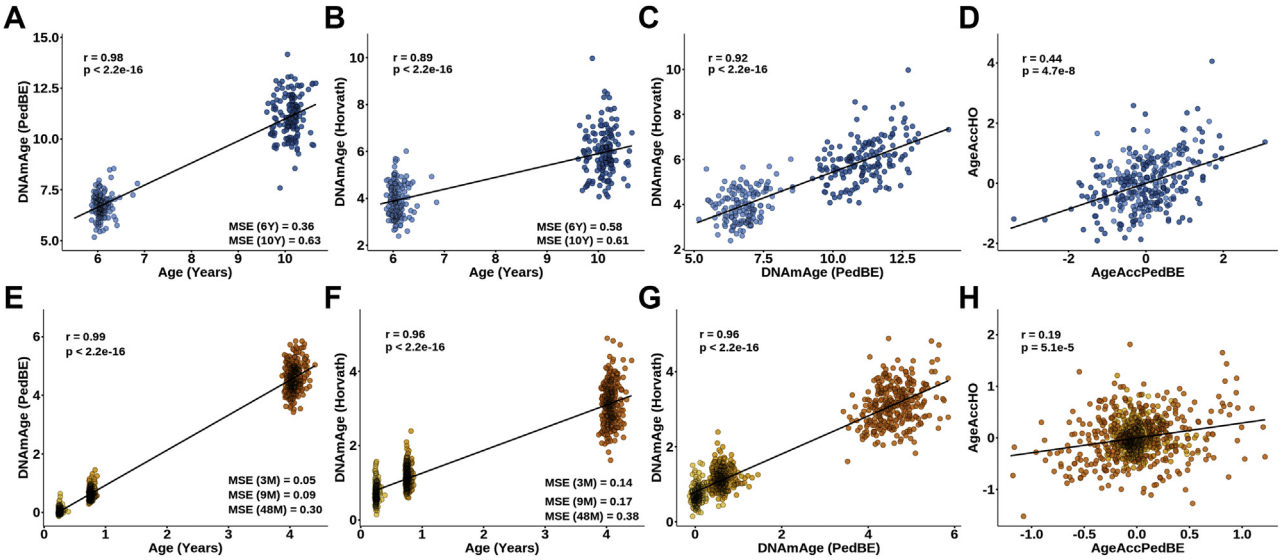

Figure 1. Repeated measures correlations between measures of epigenetic age, chronological age, and epigenetic age acceleration in the BIBO (Basal Influences on Baby Development) (blue) and GUSTO (Growing Up in Singapore Towards Healthy Outcomes) (orange) cohorts. Correlations between chronological age and epigenetic age (DNAmAge) estimated using the Pediatric-Buccal-Epigenetic (PedBE) clock or the conventional Horvath clock are shown together with median standard errors (MSEs) of age prediction from each epigenetic age estimator **(A, B, E, F)**. DNAmAge estimated using the PedBE clock or the conventional Horvath clock are correlated **(C, G)** as are measures of epigenetic age acceleration calculated using the PedBE clock (AgeAccPedBE) or the Horvath clock (AgeAccHO) **(D, H)**.

Table 2. Difference in Predicted Epigenetic Age and Chronological Age Using the Horvath and PedBE Estimators

	Horvath: Mean Difference (SD)	PedBE: Mean Difference (SD)	*p* Value
BIBO			
6 years	−2.162 (0.724)	0.644 (0.585)	5.26×10^{-83}
10 years	−4.138 (0.984)	1.008 (1.003)	1.74×10^{-114}
GUSTO			
3 months	0.478 (0.248)	−0.196 (0.106)	1.71×10^{-93}
9 months	0.409 (0.270)	−0.131 (0.167)	1.18×10^{-93}
48 months	−0.939 (0.531)	0.535 (0.444)	1.14×10^{-130}

Differences derived from epigenetic age less chronological age using estimates that are unadjusted for buccal cell counts. *p* values from paired *t* test.

BIBO, Basal Influences on Baby Development; GUSTO, Growing Up in Singapore Towards Healthy Outcomes; PedBE, Pediatric-Buccal-Epigenetic clock.

(estimate = 0.003, p = .01, n_d = 693/n_p = 288). Similar findings were observed when restricting our analyses to participants providing DNA methylation across all time points (Table S2). A weaker association was observed between maternal prenatal depression and child AgeAccPedBE in the BIBO cohort, while prenatal depression was not significantly associated with child AgeAccPedBE in the younger GUSTO cohort (Table S3 and Figure S4). We note that fewer participants reported clinically relevant symptoms of depression (BIBO = 7%; GUSTO = 14%) than anxiety (BIBO = 12%; GUSTO = 35%) across both cohorts (BIBO: χ^2_1 = 21.56, $p = 3.00 \times 10^{-6}$, n_p = 141; GUSTO: χ^2_1 = 38.53, $p = 5.38 \times 10^{-10}$, n_p = 296).

Prenatal maternal anxiety did not predict child AgeAccHO in the BIBO (estimate = 0.002, p = .80, n_d = 271/n_p = 140) or GUSTO cohorts (estimate = 0.003, p = .08, n_d = 693/n_p = 288). Thus, we focus all subsequent analyses on maternal anxiety and child AgeAccPedBE.

Postnatal Anxiety and Pediatric Epigenetic Age Acceleration

In BIBO, maternal anxiety symptoms averaged between 6 and 8 years after delivery did not predict child AgeAccPedBE (estimate = 0.01, p = .16, n_d = 258/n_p = 132). Similarly, maternal anxiety at 3 months postpartum did not predict AgeAccPedBE in the GUSTO cohort (estimate <0.001, p = .40, n_d = 552/n_p = 233). Similar findings were observed with models that considered maternal prenatal/postnatal state (rather than trait) anxiety (Table S4).

When combined in a single GEE model (see equation 3) prenatal anxiety (estimate = 0.022, p = .01, n_d = 250/n_p = 128) but not postnatal anxiety (estimate = 0.01, p = .57, n_d = 250/n_p = 128) significantly predicted child AgeAccPedBE in the BIBO cohort. In GUSTO, prenatal but not postnatal STAI predicted child AgeAccPedBE at trend level (estimate = 0.003, p = .056, n_d = 536/n_p = 226). Figure 3 describes AgeAccPedBE over time in children born to individuals reporting high versus low anxiety in the prenatal or postnatal period. Finally, we tested if prenatal maternal anxiety predicted an accelerated pace of aging (equation 4). In the BIBO cohort, the effect of maternal prenatal anxiety did not change over time (interaction term p = .186). However, in the GUSTO cohort, a significant interaction between prenatal anxiety and child age was observed (estimate = 0.001, p = .036, n_d = 693/n_p = 288) with a strengthening effect of prenatal anxiety from 3 to 48 months of age. Similar results were obtained when using DNAmAgePedBE as the dependent variable and when using an alternative statistical model (mixed model) (Table S5). Together, these findings suggest that prenatal anxiety may predict an accelerated pace of epigenetic aging in early childhood, a period characterized by dynamic change in DNA methylation (34).

Prenatal Anxiety Predicts AgeAccPedBE in Males but Not Females

In the BIBO cohort, child sex moderated the association between prenatal maternal anxiety and AgeAccPedBE (estimate = 0.004, interaction p = .006, n_d = 271/n_p = 140) (Table 3). Stratified analyses in the BIBO cohort revealed an association between prenatal anxiety and increased AgeAccPedBE in males ($p = 1.6 \times 10^{-4}$, n_d = 143/n_p = 73), but not females (p = .90, n_d = 128/n_p = 67). These findings were consistent in the GUSTO cohort where prenatal anxiety predicted AgeAccPedBE in males (p = .04, n_d = 345/n_p = 146) but not females (p = .21, n_d = 348/n_p = 142) (Table 3). Despite the evidence of sex-specific associations provided by stratified analyses in the GUSTO cohort, we note that the interaction term (prenatal anxiety × sex) was not significant at the cohort level (estimate = −0.002, p = .461, n_d = 693/n_p = 288).

PedBE Age Acceleration and Child Mental Health

Child AgeAccPedBE at 6 years of age predicted longitudinal measures of child externalizing (estimate = 1.33, p = .049, n_d = 405/n_p = 141) but not internalizing problems (p = .93, n_d = 405/n_p = 141) from 6 to 10 years of age in the BIBO cohort. These effects were sex-specific: higher AgeAccPedBE was associated with increased externalizing problems in males (estimate = 1.75, p = .03, n_d = 214/n_p = 75) but not females (estimate = 0.51, p = .64, n_d = 191/n_p = 66). Prenatal anxiety was not significantly associated with externalizing symptoms in males in the BIBO cohort (Table S6). Likewise, AgeAccPedBE was not associated with child internalizing or externalizing symptoms in males or females in the younger GUSTO cohort (all p > .33). Thus, in this study, we were unable to examine candidate mediation pathways between prenatal mood, AgeAccPedBE, and child mental health symptoms.

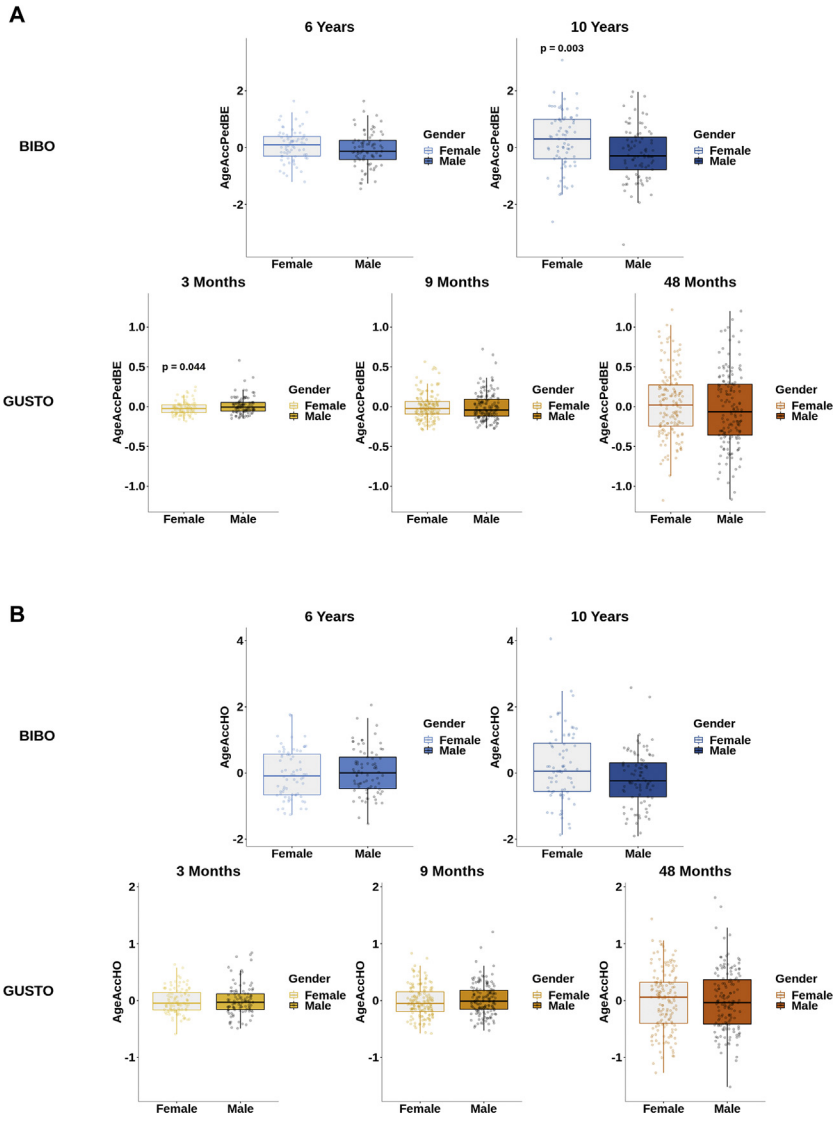

Figure 2. Sex differences in epigenetic age acceleration from infancy to preadolescence. Child epigenetic age acceleration derived from the Pediatric-Buccal-Epigenetic clock (AgeAccPedBE) **(A)** or the conventional Horvath clock (AgeAccHO) **(B)** are shown stratified by child sex in the Basal Influences on Baby Development (BIBO) (blue) and Growing Up in Singapore Towards Healthy Outcomes (GUSTO) (orange) cohorts. *p* values are provided for group differences that reached statistical significance at $p < .05$.

Supplementary Analyses

Obstetric Risk. All multivariate models of child AgeAccPedBE considered gestational age. We note that only 1 child in the BIBO cohort was born preterm at 36 weeks gestational age. However, in the GUSTO cohort, 14 children (10 male, 4 female) providing 29 DNA methylation datapoints were born preterm (range: 33–36 weeks gestational age). We excluded these cases and repeated our primary analyses (see equation 1). Prenatal maternal anxiety remained significantly associated with child AgeAccPedBE (estimate = 0.003, $p = .009$, $n_d = 664/n_p = 274$) (see Table S7). Likewise, the inclusion of birth weight, as a potential indicator of obstetric complications, did not substantively change the magnitude of the association between maternal prenatal anxiety and child AgeAccPedBE (Table S8).

Epigenetic Aging and Polygenic Risk for Psychiatric/Neurodevelopmental Disorders. The association between maternal prenatal anxiety and child AgeAccPedBE was independent of child polygenic risk for anxiety, depression, or autism (Table S9).

A Proxy Measure of Glucocorticoid Exposure Predicts Pediatric Epigenetic Age Acceleration. A DNA methylation index of glucocorticoid exposure (17) predicted AgeAccPedBE in males (BIBO: $r = -0.28$, $p = .01$, $n_p = 76$; GUSTO: $r = -0.28$, $p < .01$, $n_p = 100$) but not females (BIBO: $r = -0.14$, $p = .24$, $n_p = 68$; GUSTO: $r = -0.08$, $p = .41$, $n_p = 99$) across both cohorts. These exploratory analyses point to glucocorticoid exposure/sensitivity as one candidate mechanism that may contribute to the sex-specific findings observed

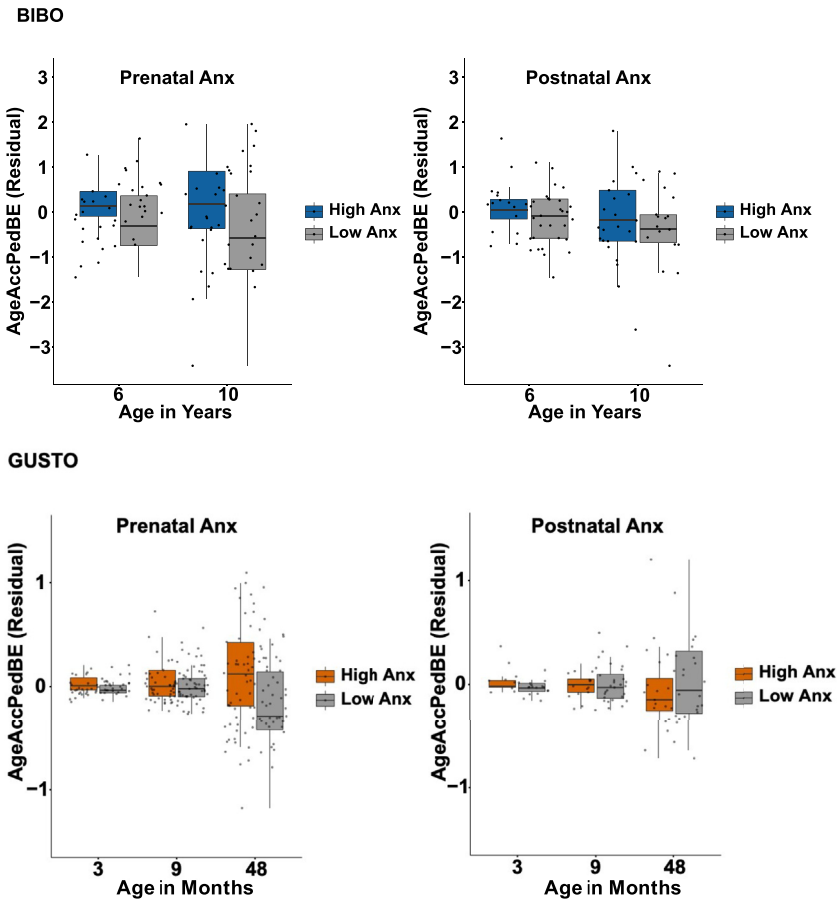

Figure 3. Representative boxplots of child epigenetic age acceleration based on exposure to high or low (mean ± 1 SD) maternal prenatal or postnatal anxiety (Anx). AgeAccPedBE, Pediatric-Buccal-Epigenetic clock–derived epigenetic age acceleration; BIBO, Basal Influences on Baby Development; GUSTO, Growing Up in Singapore Towards Healthy Outcomes.

across both cohorts (Figure S5); however, we note that prenatal anxiety was not significantly associated with this proxy measure of glucocorticoid exposure in either cohort (Table S10).

DISCUSSION

Prenatal maternal anxiety predicted accelerated epigenetic aging across infancy and midchildhood in 2 independent cohorts even after controlling for obstetric, socioeconomic, and child genetic risk factors. The relationship between prenatal maternal anxiety and epigenetic aging varied across individuals, with heightened sensitivity in males. Advanced AgeAccPedBE, in turn, predicted increased externalizing symptoms between 6 and 10 years of age in males from the BIBO cohort only. These findings point to the utility of the PedBE clock as a correlate of the biological embedding of prenatal anxiety and highlight the importance of the prenatal environment for biological aging (34–36).

We showed that the PedBE clock was a more accurate estimator of child age than a conventional multi-tissue epigenetic clock (16) and that variation in epigenetic age acceleration increases across developmental stages (early vs. late childhood). Variability in DNA methylation is a feature of aging, which may arise from decreased fidelity in the transmission of DNA methylation across cycles of cell division (37) or increased molecular damage associated with aging (34). Our data show that variability in AgeAccPedBE may be derived, in part, from exposures in utero. A logical next step is to identify psychosocial factors that buffer or exacerbate such effects, including interventions that target maternal mental health (38).

Our findings highlight a consistent association between prenatal maternal anxiety and child epigenetic age acceleration, with overall weaker associations observed between prenatal maternal depression and child AgeAccPedBE. However, we observed a significantly higher proportion of women reporting clinically relevant anxiety across both cohorts, which may have contributed to differences in effect sizes between prenatal anxiety and depression.

Prenatal maternal anxiety was associated with child epigenetic age acceleration in a sex-specific manner. This finding is consistent with several studies suggesting increased sensitivity of males to the effects of prenatal adversity (10,39–41). Suarez *et al.* report sex-specific associations between prenatal maternal depression and an epigenetic biomarker of gestational age, which, in turn, predicted mental health symptoms in males only (41). This aligns with our own observations that child AgeAccPedBE associated with mental health symptoms in boys but not

Table 3. Sex-Specific Analysis of Maternal Prenatal Anxiety and Child Epigenetic Age Acceleration Measured by the PedBE Clock

	Child AgeAccPedBE			
	Females		Males	
	Estimate	*p* Value	Estimate	*p* Value
BIBO				
Prenatal anxiety	−0.001	.908	0.042	1.58×10^{-4}
Time point (age)	0.022	.434	−0.035	.121
Maternal education	0.345	.100	0.056	.725
Ancestry				
PC1	3.524	.038	0.690	.021
PC2	−2.539	.095	−0.533	.174
Buccal cell count	0.014	.305	<0.001	.944
Gestational age	−0.074	.198	0.045	.358
GUSTO				
Prenatal anxiety	0.002	.206	0.004	.040
Time point, age	−0.001	.206	−0.003	4.19×10^{-4}
Maternal education	−0.014	.731	−0.001	.969
Ancestry				
PC1	−0.038	.948	0.423	.430
PC2	−0.613	.237	0.655	.303
PC3	0.778	.169	0.356	.486
Buccal cell count	−0.022	5.91×10^{-12}	−0.023	5.61×10^{-11}
Gestational age	0.024	.126	0.022	.073

Generalized estimating equation model estimates and *p* values for the association between maternal prenatal anxiety and child epigenetic age acceleration derived from the Pediatric-Buccal-Epigenetic clock (AgeAccPedBE) from the BIBO (top) and GUSTO cohorts (bottom). All models considered time point of sample collection (child age), maternal education level, measures of ancestry derived from genetic principal component (PC) scores, buccal cell heterogeneity, gestational age, and prenatal anxiety assessed using the State-Trait Anxiety Inventory.

BIBO, Basal Influences on Baby Development; GUSTO, Growing Up in Singapore Towards Healthy Outcomes.

girls. Despite these findings, which point to increased male sensitivity following exposure to prenatal adversity, it is important to note that female sex-specific associations have also been reported. Thus, any sex-specific outcomes are likely to arise through the complex interplay of the timing of exposure during pregnancy, the specific outcome, and biological system under study (42).

Our study is not without limitations. First, while our data suggest that child AgeAccPedBE may track sex differences in the timing of puberty with increased age acceleration in females at age 10 years of age, due to the age range of our cohorts, we could not test hypotheses related to child AgeAccPedBE and the timing/tempo of sexual maturation, which is a topic that warrants further study. Second, biosampling did not occur at birth, and so we are unable to discount the possibility that postnatal factors influenced measures of child AgeAccPedBE. We note that measures of postnatal anxiety and postnatal depression did not associate with child AgeAccPedBE, and our models considered sources of obstetric, socioeconomic, genomic, and biological variation, e.g., cellular heterogeneity of biosamples. Third, our cohorts were drawn from largely well-educated community samples from developed countries. It will be important to examine the association between measures of maternal mental health and child AgeAccPedBE across steeper gradients of socioeconomic status and more diverse contexts, e.g., low- and middle-income countries (43). Fourth, our cohorts provided a single prenatal time point, and we could not test if there are trimester-specific effects of prenatal anxiety on child AgeAccPedBE. In addition, the relatively stronger effects we observe in the older BIBO cohort could derive from an unmeasured postnatal exposure (e.g., the quality of the care-giving environment), which correlates with prenatal anxiety and has an impact on epigenetic aging over time. This interpretation and candidate exposures also require further study. Longitudinal cohorts with repeated assessments of maternal/child mental health (that do not rely exclusively on maternal report) paired with broader phenotyping of the early environment and high-frequency biosampling will be needed to address such issues. Finally, direct assessments of prenatal glucocorticoid exposure were not available in either cohort, and our proxy biomarker has not been validated for use in buccal samples. Thus, these findings should be considered preliminary until replicated in a cohort with direct assessments of prenatal glucocorticoid exposure and child AgeAccPedBE. These limitations are offset by several strengths, including the availability of longitudinal measures of DNA methylation with paired genomic data. Likewise, the use of a Caucasian cohort from Europe (BIBO) and a diverse, multiethnic cohort from Singapore (Chinese, Malay, and Indian) suggest that our findings are robust across geographic and sociocultural contexts.

Conclusions

Our findings point to the fetal origins of biological aging and further illustrate how the quality of the prenatal environment can influence multiple aspects of child development. These findings, paired with a wealth of epidemiological studies, underscore the importance of providing timely and effective mental health support to pregnant individuals, which may have lasting consequences from the cellular to the societal level.

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