

Accelerated Epigenetic Gestational Aging in Monochorionic Compared with Dichorionic Twins: A Cord Blood DNA Methylation Study

Leejae Cho

Seoul National University College of Medicine

Ji Hoi Kim

Seoul National University Hospital

Jong Kwan Jun

Ewha Womans University

Soong Deok Lee

Seoul National University College of Medicine

Jong-Lyul Park

Korea Research Institute of Bioscience and Biotechnology

So-hee Kim

Seoul National University Hospital

Joong Shin Park

Seoul National University Hospital

Hwan Young Lee

hylee192@snu.ac.kr

Seoul National University College of Medicine

Seung Mi Lee

Seoul National University Hospital

Research Article

Keywords: DNA Methylation, Epigenesis, Genetic, Gestational Age, Twins, Monozygotic, Chorion, Cord Blood

Posted Date: August 25th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10695741/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Background

Biological maturity at birth does not always align with gestational age, and this discordance can be quantified as gestational age acceleration (GAA) using DNA methylation-based epigenetic clocks. Monochorionic diamniotic (MCDA) and dichorionic diamniotic (DCDA) twin pregnancies differ in placental sharing and hemodynamic environment, but whether these differences influence GAA remains unknown. We analyzed cord blood DNA methylation data from 49 monozygotic twin pairs (26 DCDA, 23 MCDA), estimating GAA using two DNA methylation-based gestational age clocks, to compare GAA between chorionicity groups among full-term neonates.

Results

Both clocks showed good within-pair GAA concordance, and GAA was consistently higher in MCDA than in DCDA pairs. Among full-term neonates, MCDA twins had significantly higher GAA according to one clock than DCDA twins (0.34 ± 0.96 vs. -0.27 ± 0.80 weeks; $p = 0.010$), while the other clock showed a similar directional trend without reaching statistical significance ($p = 0.11$). Regression analysis confirmed that MCDA chorionicity among full-term twins was associated with higher GAA relative to full-term DCDA twins ($\beta = 0.64$ weeks; 95% CI $0.02-1.26$; $p = 0.044$), and this association remained robust in most sensitivity analyses adjusting for clinical and obstetric covariates. Preterm birth was not significantly associated with GAA by either clock.

Conclusions

Among full-term twins, chorionicity was independently associated with GAA, with MCDA twins exhibiting higher epigenetic maturity than DCDA twins, despite having the same gestational age at birth. These findings suggest that the hemodynamic environment associated with placental sharing – rather than gestational age at birth alone – may contribute to epigenetic maturation in twin pregnancies, raising the possibility that chorionicity should be further investigated as a biological variable relevant to neonatal risk stratification.

Background

Gestational age (GA) at birth is a major determinant of neonatal health and long-term developmental outcomes (1, 2). However, infants born at the same gestational age may differ substantially in their biological maturity. DNA methylation patterns change systematically throughout fetal development, enabling the development of epigenetic clocks that estimate biological maturity at birth (3, 4). Based on this concept, two epigenetic clocks have been developed to estimate gestational age from cord blood

DNA methylation: Knight's clock, based on 148 CpG sites from 27K and 450K array data, and Bohlin's clock, based on 96 CpG sites from 450K array data (3, 4).

The discrepancy between DNA methylation-based gestational age (DNAm GA) and clinically recorded gestational age is defined as gestational age acceleration (GAA), with positive values indicating greater epigenetic maturity than expected for a given gestational age and negative values reflecting relative immaturity (3). By capturing individual-level deviations, GAA provides a quantitative measure of neonatal maturation beyond standard clinical assessment (5). Nevertheless, the clinical and environmental factors that determine GAA remain poorly understood, particularly with respect to the intrauterine environment.

In twin pregnancies, chorionicity—the number of chorions and a key determinant of placental architecture—provides a biologically informative model for examining how the intrauterine environment influences fetal epigenetic programming. DCDA twins develop with separate placental circulations, whereas MCDA twins share a single placenta containing vascular anastomoses between the fetal circulations (6). These anastomoses can lead to imbalanced inter-twin blood flow, resulting in divergent hemodynamic conditions between the two fetuses.

Previous studies have shown that chorionicity and intrauterine environmental factors contribute to within-pair variation in DNA methylation (7). In severely growth-discordant monochorionic twins, differences in placental perfusion and fetal growth have been associated with differential placental DNA methylation (8). These prior studies support the biological plausibility that the shared monochorionic placental environment may influence fetal epigenetic maturation.

Despite this biological plausibility, the association between chorionicity and GAA has not been directly examined. Previous studies of GAA have been conducted predominantly in singleton birth cohorts (5, 9). Moreover, twin methylation studies have focused mainly on within-pair methylation variation or inter-twin differentiation rather than chorionicity-related differences in epigenetic gestational aging (7, 10).

In the current study, we aimed to determine whether chorionicity is independently associated with GAA in twin pregnancies and whether this association differs between full-term and preterm twin pregnancies.

Methods

Data description

Cord blood DNA methylation data from a previous cohort of Korean monozygotic twins (10) were obtained from the original authors upon request. The dataset included 26 DCDA and 23 MCDA twin pairs. DNA methylation profiling was performed using the Infinium MethylationEPIC v2 BeadChip array. Raw IDAT files were preprocessed in R using the minfi package (11) with NOOB normalization. Probes with a detection P value > 0.05 in $\geq 25\%$ of samples or with any missing beta values were excluded, and beta values were extracted for subsequent analyses. EPIC v2 probe identifiers were mapped to the

HumanMethylation450 (450K) and HumanMethylation27 (27K) nomenclature using the IlluminaHumanMethylationEPICv2manifest R package to ensure compatibility with published clock coefficients. Gestational age recorded in weeks and days was converted to decimal weeks for all subsequent analyses.

Estimation of DNA Methylation-Based Gestational Age and GAA

DNA methylation–derived gestational age (DNAm GA) was calculated using two previously published epigenetic clocks. Knight's clock was developed using 148 CpG sites from cord blood samples profiled on the 27K and 450K arrays (3), and Bohlin's clock was developed using 96 CpG sites from cord blood samples profiled on the 450K array (4). All calculations were implemented in R using the methylclock package (v1.8.0) (12). Missing CpG values were imputed using the reference median values provided in the package, and clock estimates were calculated only when at least 80% of required CpGs were available. All samples satisfied this criterion and were included in subsequent analyses.

Gestational age acceleration (GAA) was defined as the residual from a linear regression of DNAm GA on clinically recorded gestational age at birth (3, 13). Separate regression models were fitted for each epigenetic clock using the entire study cohort. The resulting residuals represented individual-level deviations between observed and expected DNAm GA for a given gestational age. Positive residuals indicate accelerated epigenetic gestational aging, whereas negative residuals indicate decelerated epigenetic gestational aging.

Within-pair concordance and variability of GAA

Because monozygotic twins share an identical genetic background and much of the prenatal environment, we evaluated the degree of within-pair similarity in GAA. To quantify within-pair concordance, we calculated the intraclass correlation coefficient (ICC), which quantifies the similarity of GAA within twin pairs. Higher ICC values indicate greater similarity within twin pairs. Based on established guidelines, ICC values < 0.50 were interpreted as poor, 0.50–0.75 as moderate, 0.75–0.90 as good, and ≥ 0.90 as excellent concordance (14). ICC was estimated using a one-way random-effects model, treating twin pairs as random effects. Analyses were performed separately for Knight's and Bohlin's epigenetic clocks.

To further characterize within-pair variability, we calculated the absolute within-pair difference in GAA ($|\Delta\text{GAA}|$) for each twin pair and summarized these values using the median and interquartile range. Because twin order was assigned arbitrarily, signed within-pair differences were tested against zero using a one-sample t-test to confirm the absence of systematic directional bias. All calculations were implemented in Python using NumPy (15) and SciPy (16, 17) packages.

Statistical analysis

Between-group comparisons of GAA were performed at the individual level using Welch's t-test or the Mann–Whitney U test, as appropriate. These comparisons are reported as exploratory, given the non-independence of twin pairs; formal inference regarding the association between chorionicity and GAA was based on the cluster-robust regression analyses described below.

Two analytical strategies were employed. First, GAA was compared between preterm (gestational age < 37 weeks) and full-term infants across the entire cohort, irrespective of chorionicity; because gestational age at birth is shared within twin pairs by design, co-twins were always classified into the same group. Second, to examine the potential influence of placental sharing on epigenetic gestational aging, full-term infants were divided into DCDA and MCDA groups, and GAA was compared between the two groups.

Association of GAA with clinical and twin-related characteristics

To evaluate associations of GAA with preterm birth and chorionicity, ordinary least squares (OLS) regression models were fitted using full-term DCDA twins as the reference group. Given the non-independence of observations within twin pairs, cluster-robust standard errors were estimated at the twin-pair level using the Huber–White sandwich estimator (18). All models were adjusted for sex.

To evaluate the robustness of the findings, sensitivity analyses were performed by additionally including six covariates—birth weight, small for gestational age (SGA), maternal diabetes mellitus, maternal hypertension, maternal age, and mode of delivery—in all possible combinations, yielding 64 model specifications.

All statistical analyses were conducted in Python. Regression analyses were performed using the Statsmodels package (19), and group comparisons were performed using the SciPy package (16, 17). Statistical significance was defined as a two-sided p value < 0.05.

Results

Sample Characteristics and Gestational Age Distribution

A total of 49 monozygotic twin pairs were included in this analysis: 26 DCDA and 23 MCDA. Using a gestational age threshold of 37 weeks, 17 pairs were classified as preterm and 32 pairs as full-term. Among full-term pairs, 19 were DCDA and 13 were MCDA. Gestational age at birth ranged from 28.3 to 38.0 weeks (mean, 36.31 ± 2.11 weeks). Preterm infants had a mean gestational age of 34.34 ± 2.60 weeks (range, 28.3–36.9 weeks), and full-term infants had a mean of 37.36 ± 0.28 weeks (range, 37.0–38.0 weeks).

Baseline clinical characteristics were broadly similar between full-term DCDA and MCDA twins (Table 1). No significant between-group differences were observed in gestational age, birth weight, sex, or mode of

delivery. Groupwise comparisons between preterm and full-term infants are presented in Supplementary Table 1.

Table 1
Clinical characteristics of full-term twin pairs stratified by chorionicity

N samples	Full-term DCDA	Full-term MCDA	p
	38	26	
N twin pairs	19	13	
Gestational age (weeks), mean $\pm$ SD	37.34 $\pm$ 0.26	37.39 $\pm$ 0.31	0.603
Gestational age (weeks), range	37.0–37.9	37.0–38.0	
Male sex, n (%)	14 (36.8%)	14 (53.8%)	0.276
Vaginal delivery, n (%)	26 (68.4%)	22 (84.6%)	0.240
Maternal diabetes, n (%)	2 (5.3%)	6 (23.1%)	0.054
Maternal hypertension, n (%)	0 (0.0%)	0 (0.0%)	
SGA, n (%)	0 (0.0%)	3 (11.5%)	0.062
Birth weight (g), mean $\pm$ SD	2608.55 $\pm$ 190.28	2599.23 $\pm$ 308.97	0.989
DCDA, dichorionic–diamniotic; MCDA, monochorionic–diamniotic; SGA, small for gestational age; SD, standard deviation. Continuous variables are presented as mean $\pm$ SD; categorical variables as count (%). Between-group comparisons were performed using Welch's t-test for continuous variables and Fisher's exact test or chi-squared test for categorical variables, as appropriate.			

Performance of DNA Methylation-Based Gestational Age Clocks

Following DNA methylation profiling and preprocessing, probes were excluded based on detection p-value and missingness criteria as described in Methods, after which 719,253 CpG sites that could be mapped to legacy probe identifiers were retained for clock-based analyses. Beta-values extracted from the preprocessed data were used to estimate DNAm GA using Knight's and Bohlin's clocks. Missing CpG sites (Knight: 9/148, 6.1%; Bohlin: 17/96, 17.7%), consistently absent across all samples, were imputed by median substitution, and clock-derived gestational age was successfully calculated for all 98 samples.

Both epigenetic clocks showed strong correlations with clinically recorded gestational age. Knight's clock showed a Pearson correlation coefficient of $R = 0.85$, with a MAE of 1.64 weeks and an RMSE of 1.87 weeks, whereas Bohlin's clock showed stronger agreement with clinically recorded gestational age, with $R = 0.94$, a MAE of 1.25 weeks, and an RMSE of 1.58 weeks (Fig. 1). Both clocks showed good performance despite incomplete coverage of the CpG sites required for each clock. Among full-term twins, the mean DNAm GA estimated by Knight's clock was significantly higher in MCDA than in DCDA

twins (35.94 ± 0.87 vs. 35.29 ± 0.79 weeks, $p = 0.010$), whereas no significant difference was observed using Bohlin's clock (38.31 ± 0.38 vs. 38.08 ± 0.53 weeks, $p = 0.178$).

Within-pair concordance of gestational age acceleration

Within-pair concordance of GAA was evaluated using the intraclass correlation coefficient (ICC). Substantial within-pair concordance was observed for both epigenetic clocks. The ICC for Knight GAA was 0.760 (95% CI, 0.612–0.857), while Bohlin GAA showed even higher concordance, with an ICC of 0.879 (95% CI, 0.796–0.930). According to established interpretive guidelines (14), both clocks demonstrated good within-pair concordance.

When stratified by chorionicity, within-pair concordance was higher in MCDA than in DCDA pairs for both clocks, with a more pronounced difference for Knight GAA (ICC, 0.817 vs. 0.666) than for Bohlin GAA (ICC, 0.898 vs. 0.860) (Table 2). Signed within-pair GAA differences showed no systematic directional bias for either clock (Supplementary Fig. 1).

Table 2
Intraclass correlation and within-pair variability of gestational age acceleration by chorionicity

Clock	Group	N pairs	ICC	95% CI	Interpretation ^a	Median ΔGAA (weeks)	IQR (Q1– Q3)
Knight GAA	All pairs	49	0.760	0.612– 0.857	Good	0.478	0.214– 0.821
	DCDA	26	0.666	0.387– 0.834	Moderate	0.432	0.191– 0.807
	MCDA	23	0.817	0.620– 0.918	Good	0.478	0.297– 0.807
Bohlin GAA	All pairs	49	0.879	0.796– 0.930	Good	0.179	0.079– 0.311
	DCDA	26	0.860	0.715– 0.934	Good	0.224	0.073– 0.320
	MCDA	23	0.898	0.778– 0.955	Good	0.165	0.092– 0.257

^a ICC interpretation based on Koo & Li (2016): < 0.50, poor; 0.50–0.75, moderate; 0.75–0.90, good; ≥ 0.90, excellent.

GAA, gestational age acceleration; ICC, intraclass correlation coefficient; |ΔGAA|, absolute within-pair difference in gestational age acceleration; IQR, interquartile range; CI, confidence interval; DCDA, dichorionic–diamniotic; MCDA, monochorionic–diamniotic. ICC was estimated using a one-way random-effects model, treating twin pairs as random effects. ICC values ≥ 0.75 are shown in bold.

Comparisons of Gestational Age Acceleration

Neither epigenetic clock showed a statistically significant difference in GAA between preterm and full-term infants (Fig. 2A). Using Knight's clock, mean GAA was 0.04 ± 1.06 weeks in preterm infants ($n = 34$) and -0.02 ± 0.91 weeks in full-term infants ($n = 64$) ($p = 0.80$). Bohlin's clock yielded a mean GAA of 0.01 ± 0.43 weeks in preterm infants and -0.00 ± 0.49 weeks in full-term infants ($p = 0.91$).

Among full-term infants, however, GAA was higher in MCDA than in DCDA twins for both epigenetic clocks (Fig. 2B). This difference was statistically significant for Knight GAA (DCDA: -0.27 ± 0.80 weeks, $n = 38$; MCDA: 0.34 ± 0.96 weeks, $n = 26$; $p = 0.010$), while Bohlin GAA showed the same directional pattern but did not reach statistical significance (DCDA: -0.09 ± 0.48 weeks; MCDA: 0.12 ± 0.49 weeks; $p = 0.11$).

Regression analyses of gestational age acceleration

The association between chorionicity and GAA was further evaluated using OLS regression models with full-term DCDA twins as the reference group. All models were adjusted for sex, and cluster-robust standard errors were estimated at the twin-pair level.

Because of the limited number of preterm twin pairs, preterm MCDA and DCDA twins were analyzed as a single group. Preterm birth was not significantly associated with GAA compared with full-term DCDA twins (Knight: $\beta = 0.34$ weeks, 95% CI -0.25 to 0.93 , $p = 0.25$; Bohlin: $\beta = 0.10$ weeks, 95% CI -0.19 to 0.38 , $p = 0.50$). In contrast, full-term MCDA twins showed significantly higher Knight GAA than full-term DCDA twins ($\beta = 0.64$ weeks; 95% CI, 0.02 to 1.26 ; $p = 0.044$). A similar positive association was observed for Bohlin GAA ($\beta = 0.20$ weeks), although it did not reach statistical significance ($p = 0.23$) (Fig. 3).

Sensitivity analyses across 64 covariate combinations confirmed the robustness of the primary findings (Fig. 4). The association between full-term MCDA status and Knight GAA remained significant in 52 of 64 models (β range, 0.60 – 0.73 weeks). All 12 non-significant models included mode of delivery as a covariate (p range, 0.052 – 0.060). Preterm birth remained positively but not significantly associated with GAA across all sensitivity analyses for both epigenetic clocks (Table 3).

Table 3
Summary of sensitivity analyses across 64 covariate model specifications

Outcome	Contrast	N significant ($p < 0.05$) / 64	β range (weeks)	P value range	Note
Knight GAA	Preterm vs Full-term DCDA	0 / 64	0.34–0.45	0.166–0.271	Consistently positive, non-significant
	Full-term MCDA vs Full-term DCDA	52 / 64	0.60–0.73	0.027–0.060	12 non-sig models all included vaginal delivery ($p = 0.052$ – 0.060)
Bohlin GAA	Preterm vs Full-term DCDA	0 / 64	0.08–0.16	0.259–0.603	Consistently positive, non-significant
	Full-term MCDA vs Full-term DCDA	0 / 64	0.15–0.23	0.198–0.428	Consistently positive, non-significant

GAA, gestational age acceleration; DCDA, dichorionic–diamniotic; MCDA, monochorionic–diamniotic; β , ordinary least squares regression coefficient. Sex was included in all models; cluster-robust standard errors were applied at the twin-pair level. The reference group was full-term DCDA twins. Sensitivity models sequentially and combinatorially added the following covariates to the base model: birth weight, small for gestational age, maternal diabetes mellitus, maternal hypertension, maternal age, and mode of delivery. No correction for multiple comparisons was applied, as sensitivity analyses were intended to assess robustness rather than to test independent hypotheses.

Discussion

Summary of findings

In this study, we evaluated cord blood–based GAA in a Korean monozygotic twin cohort using two established epigenetic clocks. Both clocks showed substantial within-pair concordance, with higher concordance in MCDA than in DCDA pairs. Among full-term twins, MCDA twins had higher GAA than DCDA twins for both clocks. The difference was statistically significant for Knight GAA and remained significant in sex-adjusted regression analysis with cluster-robust standard errors at the twin-pair level ($\beta = 0.64$ weeks; 95% CI, 0.02–1.26; $p = 0.044$). Bohlin GAA showed the same direction of association but did not reach statistical significance. Preterm birth was not significantly associated with GAA for either clock. These findings suggest a chorionicity-associated difference in epigenetic gestational aging among full-term monozygotic twins.

Epigenetic clocks as measures of fetal biological maturity

DNA methylation-based epigenetic clocks were originally developed to estimate gestational age from cord blood, capitalizing on the systematic changes in CpG methylation that occur throughout fetal

development (3, 4). While their initial application was largely technical — providing a DNA methylation–based estimate of gestational age — subsequent research has reframed GAA as a biologically meaningful measure of fetal maturation. Knight et al. originally proposed that GAA may reflect differences in physiological development, such that neonates with higher GAA show a level of epigenetic maturity that exceeds what would be expected for their chronological gestational age (3). Associations between GAA and perinatal variables have been reported in subsequent cohort studies, though the direction and magnitude of these associations have varied, suggesting that GAA may capture aspects of fetal programming that are sensitive to the specific characteristics of the intrauterine environment (5, 9).

In twin pregnancies, epigenetic clocks provide a useful framework for examining variation in fetal epigenetic maturation while minimizing differences in genetic background. Comparison of monozygotic twins according to chorionicity may therefore help clarify whether placental characteristics are associated with GAA. In the present study, GAA differed between MCDA and DCDA twins at term, with a significant association observed for Knight GAA and a similar direction of association for Bohlin GAA. These findings suggest that chorionicity may contribute to variation in epigenetic gestational maturation at birth.

Hemodynamic differences between MCDA and DCDA twins as a basis for epigenetic divergence

The structural basis for the chorionicity-associated difference in GAA may be related to the fundamental hemodynamic differences between monochorionic and dichorionic placentation. In dichorionic pregnancies, placental vascular anastomoses between co-twins are virtually absent, and each twin develops with an independent placental circulation (6). In contrast, monochorionic placentas are characterized by inter-twin vascular anastomoses, including arterio-arterial, veno-venous, and arteriovenous connections, which provide pathways for blood flow between the placental circulations of the two fetuses (6). The hemodynamic consequences of these connections depend on their number, type, and balance. In clinically significant forms of unbalanced inter-twin transfusion, such as TTTS, altered volume loading can lead to marked fetal cardiovascular adaptation and dysfunction and may also affect perfusion of other fetal organs, including the brain (20). These observations demonstrate that the monochorionic placental circulation has the capacity to influence fetal cardiovascular and end-organ physiology, although they do not establish that comparable disturbances are present in all uncomplicated MCDA pregnancies.

Placental epigenetic studies provide indirect evidence that variation in the monochorionic placental environment may also be accompanied by altered DNA methylation. In severely growth-discordant monochorionic twins, differential placental methylation has been identified at genes involved in fetal growth and development (8). Although the relevance of these findings to uncomplicated MCDA pregnancies and cord-blood–based GAA remains uncertain, they support the possibility that sustained differences in placental perfusion and fetal hemodynamics may influence fetal epigenetic programming. The higher GAA observed in full-term MCDA twins in the present study may therefore reflect an

epigenetic correlate of the distinct circulatory conditions associated with monochorionic placentation. This interpretation is indirect and requires confirmation using concurrent measurements of placental vascular architecture, fetal cardiovascular function, and DNA methylation.

Clock-specific differences in the association with chorionicity

The association between chorionicity and GAA was significant for Knight GAA but not for Bohlin GAA. This clock-specific difference warrants consideration.

The two clocks differ substantially in their development. Knight's clock was trained on 148 CpGs selected from six independent cohorts comprising approximately 1,434 neonates of diverse genetic ancestries, using both cord blood and blood spot samples across a wide range of gestational ages (3). In contrast, Bohlin's clock was derived from 96 CpGs within a single, homogeneous cohort of 1,753 Norwegian newborns (4). It was optimized primarily for gestational age prediction accuracy rather than biological breadth.

These differences in training population, CpG selection, and modeling strategy may partly account for the differing effect estimates. Differences in CpG coverage, probe imputation, and statistical power may also have contributed.

Importantly, across all 64 sensitivity models, the estimated association remained positive for both clocks (Knight GAA β range, 0.60–0.73 weeks; Bohlin GAA β range, 0.15–0.23 weeks). The estimates were directionally consistent across both clocks, although the magnitude and statistical strength of the association differed. This consistency supports the possibility of a shared underlying association, although a clock-specific effect or methodological influence cannot be excluded.

Clinical interpretation of GAA in twin pregnancies

Previous studies have examined GAA in relation to preterm birth and other perinatal characteristics, although the reported associations have varied across study populations and epigenetic clock models (5, 9, 21). In the present study, GAA did not differ significantly between preterm and full-term twins for either clock, whereas full-term MCDA twins showed higher GAA than full-term DCDA twins. This pattern suggests that the observed difference in GAA was not attributable to gestational age at delivery alone. However, because preterm and full-term infants differed in other clinical characteristics, residual confounding cannot be excluded.

Chorionicity defines distinct placental and circulatory conditions in twin pregnancies. Unlike DCDA twins, MCDA co-twins share a single placenta containing inter-twin vascular anastomoses that connect the two placental circulations (6). These anatomical and hemodynamic differences are also reflected clinically in the differing patterns of fetal and neonatal risk reported between otherwise uncomplicated monochorionic and dichorionic twin pregnancies (22). In this context, the higher GAA observed in full-

term MCDA twins represents an additional epigenetic difference between monochorionic and dichorionic twin pregnancies. The biological and clinical relevance of this difference remains to be determined.

Strengths and limitations

This study has several strengths. The use of a monozygotic twin cohort stratified by chorionicity provides a biologically informative framework for examining chorionicity-associated differences in epigenetic gestational aging. Zygosity was confirmed for all twin pairs in the original study from which this cohort was derived, thereby reducing variation attributable to genetic background (10). The application of two independently developed epigenetic clocks with distinct CpG sets enabled comparison of the direction and magnitude of the observed associations across different clock models. In addition, sensitivity analyses across multiple covariate combinations, together with cluster-robust standard errors at the twin-pair level, allowed assessment of the stability of the findings under alternative model specifications.

Several limitations should also be considered. First, the sample size was limited, particularly after stratification by chorionicity and gestational age, which reduced statistical power and may partly explain why the association was significant for Knight GAA but not for Bohlin GAA. Second, although information on twin-to-twin transfusion syndrome and selective fetal growth restriction was available, the number of affected pregnancies was insufficient to examine whether the severity of monochorionic placental complications was associated with GAA. The proposed role of placental hemodynamics therefore remains inferential because placental vascular architecture and fetal cardiovascular function were not directly evaluated. Third, a subset of CpG sites required for both clocks was unavailable and was imputed, with a greater proportion of missing probes for the Bohlin clock; this may have affected clock performance and between-clock comparisons. Fourth, the cohort was derived from a single tertiary center in Korea, which may limit generalizability to other populations and clinical settings. Finally, the cross-sectional assessment at birth does not establish whether the observed GAA differences persist after the neonatal period or are associated with subsequent developmental outcomes.

Conclusions

This study identified a chorionicity-associated difference in cord blood–based epigenetic gestational aging among full-term monozygotic twins. Our findings suggest that monochorionic and dichorionic twin pregnancies may differ epigenetically even at similar clinically recorded gestational ages.

Future research should move beyond replication to determine the source and significance of this difference. Studies designed to link GAA with quantitative features of monochorionic placentation—including the relative distribution of placental territory, vascular anastomotic patterns, and fetal growth discordance—may help identify which components of the shared placental circulation are most closely associated with epigenetic gestational aging. Longitudinal assessment from birth through infancy would further clarify whether the observed difference is transient, reflects fetal adaptation at delivery, or is associated with subsequent growth and developmental trajectories. Such work will be essential to

establish whether GAA serves primarily as a marker of chorionicity-related placental conditions or has broader relevance to neonatal and developmental outcomes.

Abbreviations

GAA	gestational age acceleration
DNAm GA	DNA methylation-based gestational age
MCDA	monochorionic-diamniotic
DCDA	dichorionic-diamniotic
CpG	cytosine-phosphate-guanine
ICC	intraclass correlation coefficient
IQR	interquartile range
OLS	ordinary least squares
SGA	small-for-gestational-age
TTTS	twin-to-twin transfusion syndrome
MAE	mean absolute error
RMSE	root mean square error
NOOB	normal-exponential out-of-band
IDAT	intensity data file

Declarations

This research was supported by the Seoul National University Hospital (SNUH) Research Fund (grant No. 36-2024-0030) and by the SNUH Research Fund (grant No. 0420260540). This work was also supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (RS-2025-00558188), by the Institute of Information & Communications Technology Planning & Evaluation (IITP) grant funded by the Korean government (MSIT) [NO.RS-2021-II211343, Artificial Intelligence

Graduate School Program (Seoul National University)], and by the Korean National Police Agency (Project Number PR10-01-000-21). The biospecimens and data used for this study were provided by the Biobank of Seoul National University Hospital, a member of the Korea Biobank Network (project No. KBN5_A03).

Ethics approval and consent to participate:

This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. 2411-039-1585). Written informed consent was obtained from all participants in the original study from which this dataset was derived.

Consent for publication:

Not applicable.

Funding:

This research was supported by the Seoul National University Hospital (SNUH) Research Fund (grant Nos. 36-2024-0030 and 0420260540), the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (RS-2025-00558188), the Institute of Information & Communications Technology Planning & Evaluation (IITP) grant funded by the Korean government (MSIT) [No. RS-2021-II211343, Artificial Intelligence Graduate School Program (Seoul National University)], and the Korean National Police Agency (Project Number PR10-01-000-21). The biospecimens and data used for this study were provided by the Biobank of Seoul National University Hospital, a member of the Korea Biobank Network (project No. KBN5_A03). The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript.

Author Contribution

LC and JHK contributed equally as co-first authors. SML and HYL contributed equally as co-corresponding authors. LC and JHK performed data analysis and drafted the manuscript. JKJ and SDL contributed to sample acquisition. SHK and JSP contributed to data acquisition and clinical interpretation. JLP provided the dataset used in this study. HYL and SML conceived and supervised the study and critically revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements:

Not applicable.

Availability of data and materials:

The cord blood DNA methylation dataset analyzed in this study was originally reported by Cho MJ et al. (Forensic Sci Int Genet. 2025;79:103307) and is available from the corresponding authors upon reasonable request.

Competing interests: The authors declare that they have no competing interests.

References

1. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet*. 2012;379(9832):2162–72.
2. Saigal S, Doyle LW. An overview of mortality and sequelae of preterm birth from infancy to adulthood. *Lancet*. 2008;371(9608):261–9.
3. Knight AK, Craig JM, Theda C, Baekvad-Hansen M, Bybjerg-Grauholm J, Hansen CS, et al. An epigenetic clock for gestational age at birth based on blood methylation data. *Genome Biol*. 2016;17(1):206.
4. Bohlin J, Haberg SE, Magnus P, Reese SE, Gjessing HK, Magnus MC, et al. Prediction of gestational age based on genome-wide differentially methylated regions. *Genome Biol*. 2016;17(1):207.
5. Girchenko P, Lahti J, Czamara D, Knight AK, Jones MJ, Suarez A, et al. Associations between maternal risk factors of adverse pregnancy and birth outcomes and the offspring epigenetic clock of gestational age at birth. *Clin Epigenetics*. 2017;9:49.
6. Zhao D, Lipa M, Wielgos M, Cohen D, Middeldorp JM, Oepkes D, et al. Comparison Between Monochorionic and Dichorionic Placentas With Special Attention to Vascular Anastomoses and Placental Share. *Twin Res Hum Genet*. 2016;19(3):191–6.
7. Gordon L, Joo JE, Powell JE, Ollikainen M, Novakovic B, Li X, et al. Neonatal DNA methylation profile in human twins is specified by a complex interplay between intrauterine environmental and genetic factors, subject to tissue-specific influence. *Genome Res*. 2012;22(8):1395–406.
8. Roifman M, Choufani S, Turinsky AL, Drewlo S, Keating S, Brudno M, et al. Genome-wide placental DNA methylation analysis of severely growth-discordant monochorionic twins reveals novel epigenetic targets for intrauterine growth restriction. *Clin Epigenetics*. 2016;8:70.
9. Khouja JN, Simpkin AJ, O'Keeffe LM, Wade KH, Houtepen LC, Relton CL, et al. Epigenetic gestational age acceleration: a prospective cohort study investigating associations with familial, sociodemographic and birth characteristics. *Clin Epigenetics*. 2018;10:86.
10. Cho MJ, Park SJ, Lee HY, Park JL, Lee SM, Kim JY, et al. Genome-wide DNA methylome profiling to differentiate monozygotic twins. *Forensic Sci Int Genet*. 2025;79:103307.
11. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics*. 2014;30(10):1363–9.

12. Pelegí-Sisó D, De Prado P, Ronkainen J, Bustamante M, González JR. methylclock: a Bioconductor package to estimate DNA methylation age. *Bioinformatics*. 2021;37(12):1759–60.
13. Horvath S. DNA methylation age of human tissues and cell types. *Genome Biol*. 2013;14(10):R115.
14. Koo TK, Li MY. A guideline of selecting and reporting intraclass correlation coefficients for reliability research. *J Chiropr Med*. 2016;15(2):155–63.
15. Harris CR, Millman KJ, Van Der Walt SJ, Gommers R, Virtanen P, Cournapeau D, et al. Array programming with NumPy. *Nature*. 2020;585(7825):357–62.
16. Bressert E. SciPy and NumPy: an overview for developers. 2012.
17. Virtanen P, Gommers R, Oliphant TE, Haberland M, Reddy T, Cournapeau D, et al. SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat Methods*. 2020;17(3):261–72.
18. Williams RL. A note on robust variance estimation for cluster-correlated data. *Biometrics*. 2000;56(2):645–6.
19. Seabold S, Perktold J. Statsmodels: econometric and statistical modeling with python. *SciPy*. 2010;7(1):92–6.
20. Rotar IC, Zaharie G, Staicu A, Preda A, Muresan D. Fetal cardiovascular alterations in twin-to-twin transfusion syndrome. *Med Pharm Rep*. 2020;93(1):5–11.
21. Daredia S, Huen K, Van Der Laan L, Collender PA, Nwanaji-Enwerem JC, Harley K, et al. Prenatal and birth associations of epigenetic gestational age acceleration in the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort. *Epigenetics*. 2022;17(13):2006–21.
22. Cheong-See F, Schuit E, Arroyo-Manzano D, Khalil A, Barrett J, Joseph KS, et al. Prospective risk of stillbirth and neonatal complications in twin pregnancies: systematic review and meta-analysis. *BMJ*. 2016;354:i4353.

Figures

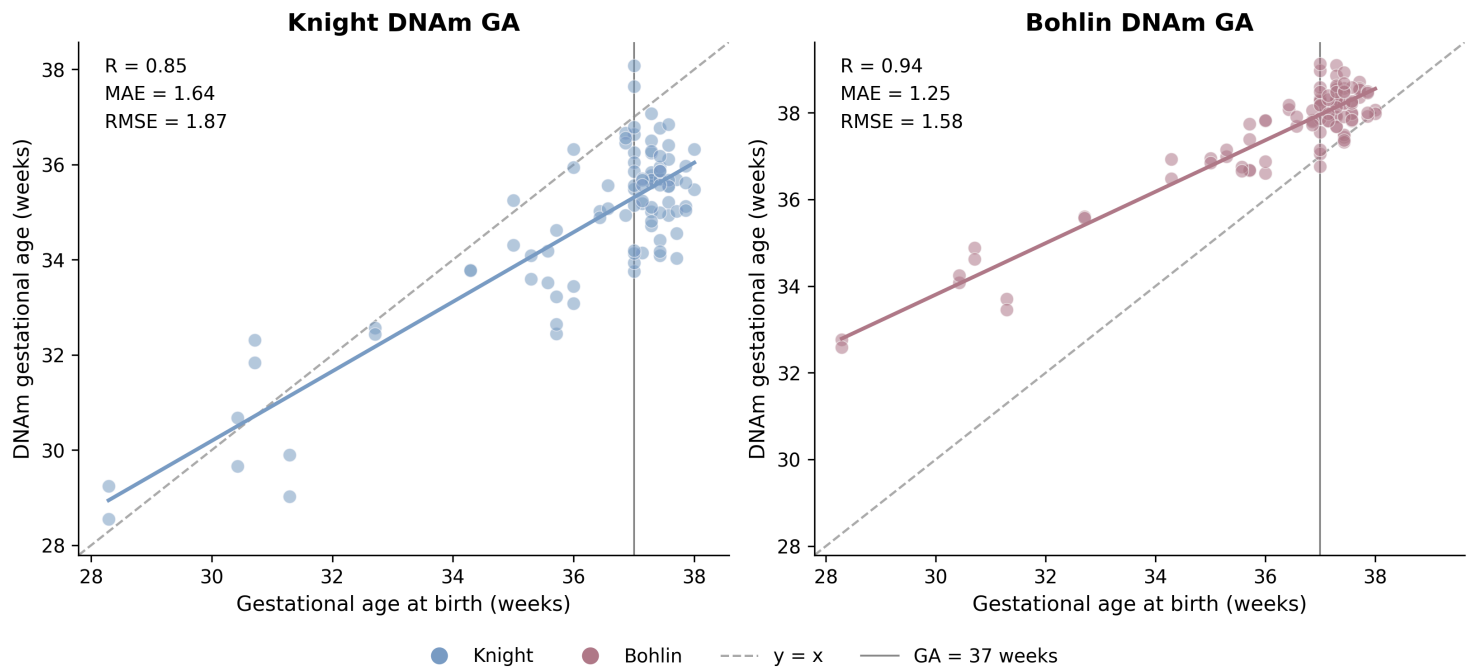
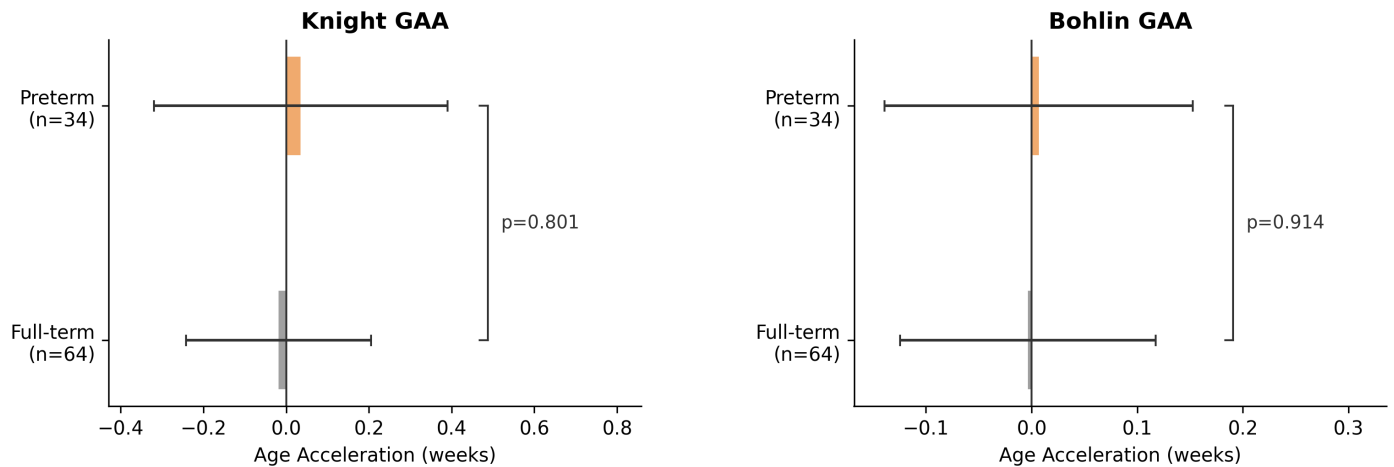


Figure 1

Correlation between epigenetic clock estimates and clinically recorded gestational age.

Scatter plots display DNA methylation-based gestational age (DNAm GA) estimated by Knight's clock (left, blue) and Bohlin's clock (right, red) against clinically recorded gestational age at birth across all 98 cord blood samples. The solid colored line represents the ordinary least squares linear fit; the dashed gray line represents the line of identity ($y = x$); the vertical gray line marks 37 weeks, the threshold distinguishing preterm from full-term birth. Performance metrics are shown within each panel. R, Pearson correlation coefficient; MAE, mean absolute error; RMSE, root mean square error; DNAm GA, DNA methylation-based gestational age.

A.



B.

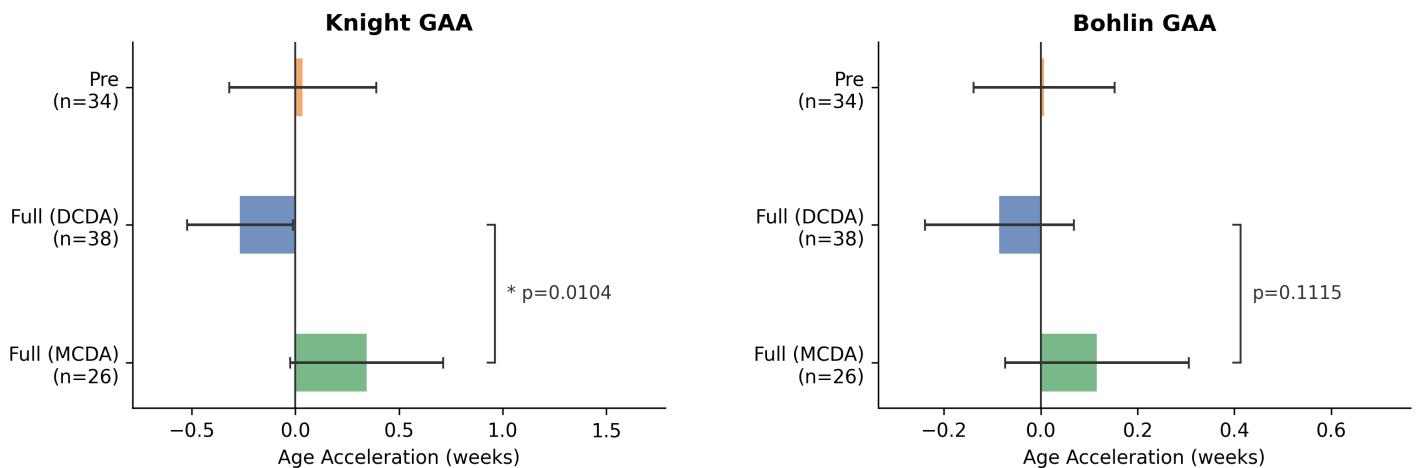


Figure 2

Gestational age acceleration by preterm status and chorionicity.

Horizontal bar plots show mean gestational age acceleration (GAA) with 95% confidence intervals estimated using Knight's clock (left panels) and Bohlin's clock (right panels). (A) GAA compared between preterm (n = 34) and full-term (n = 64) infants across the entire cohort, irrespective of chorionicity. Neither clock showed a statistically significant difference (Knight GAA: p = 0.80; Bohlin GAA: p = 0.91). (B) GAA compared among three groups – preterm (n = 34; DCDA and MCDA combined given the limited number of preterm pairs), full-term DCDA (n = 38), and full-term MCDA (n = 26). Full-term MCDA twins exhibited significantly higher GAA than full-term DCDA twins by Knight GAA (p = 0.010); Bohlin GAA showed the same directional trend without reaching significance (p = 0.11). Between-group comparisons were performed using Welch's t-test or the Mann-Whitney U test according to normality (Shapiro-Wilk test). GAA, gestational age acceleration; DCDA, dichorionic–diamniotic; MCDA, monochorionic–diamniotic.

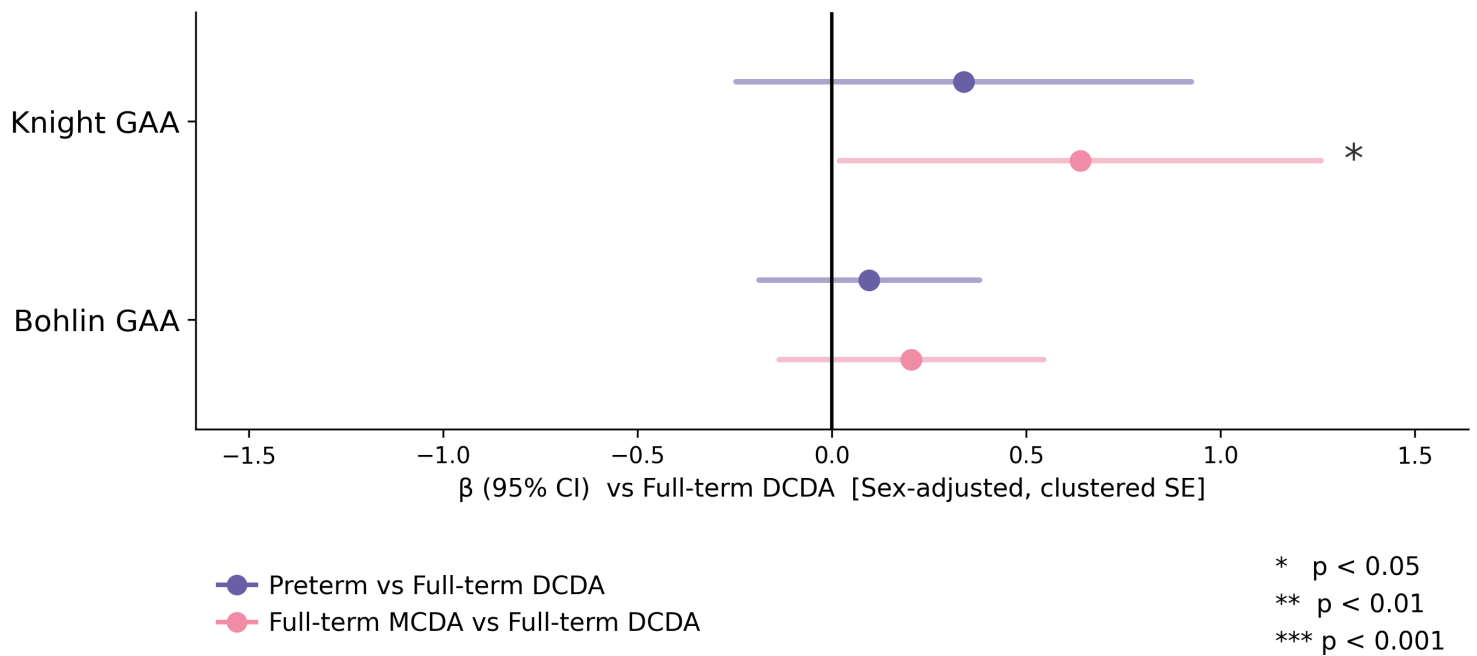


Figure 3

Regression estimates for gestational age acceleration by preterm status and chorionicity.

Forest plots display ordinary least squares β coefficients and 95% confidence intervals for two contrasts — preterm vs. full-term DCDA, and full-term MCDA vs. full-term DCDA — with GAA estimated separately using Knight's clock (top) and Bohlin's clock (bottom). Preterm infants (DCDA and MCDA combined, given the limited number of preterm pairs) were compared with full-term DCDA twins as the reference group. All models were adjusted for sex, with cluster-robust standard errors applied at the twin-pair level ($n = 98$; 49 twin pairs). For Knight GAA, full-term MCDA twins showed significantly higher GAA than full-term DCDA twins ($\beta = 0.64$; 95% CI 0.02–1.26; $p = 0.044$); the preterm contrast was not significant ($\beta = 0.34$; 95% CI -0.25 to 0.93; $p = 0.25$). For Bohlin GAA, neither contrast reached significance (MCDA: $\beta = 0.20$; $p = 0.23$; preterm: $\beta = 0.10$; $p = 0.50$). Asterisk (*) denotes $p < 0.05$. β , ordinary least squares regression coefficient; CI, confidence interval; GAA, gestational age acceleration; DCDA, dichorionic–diamniotic; MCDA, monochorionic–diamniotic.

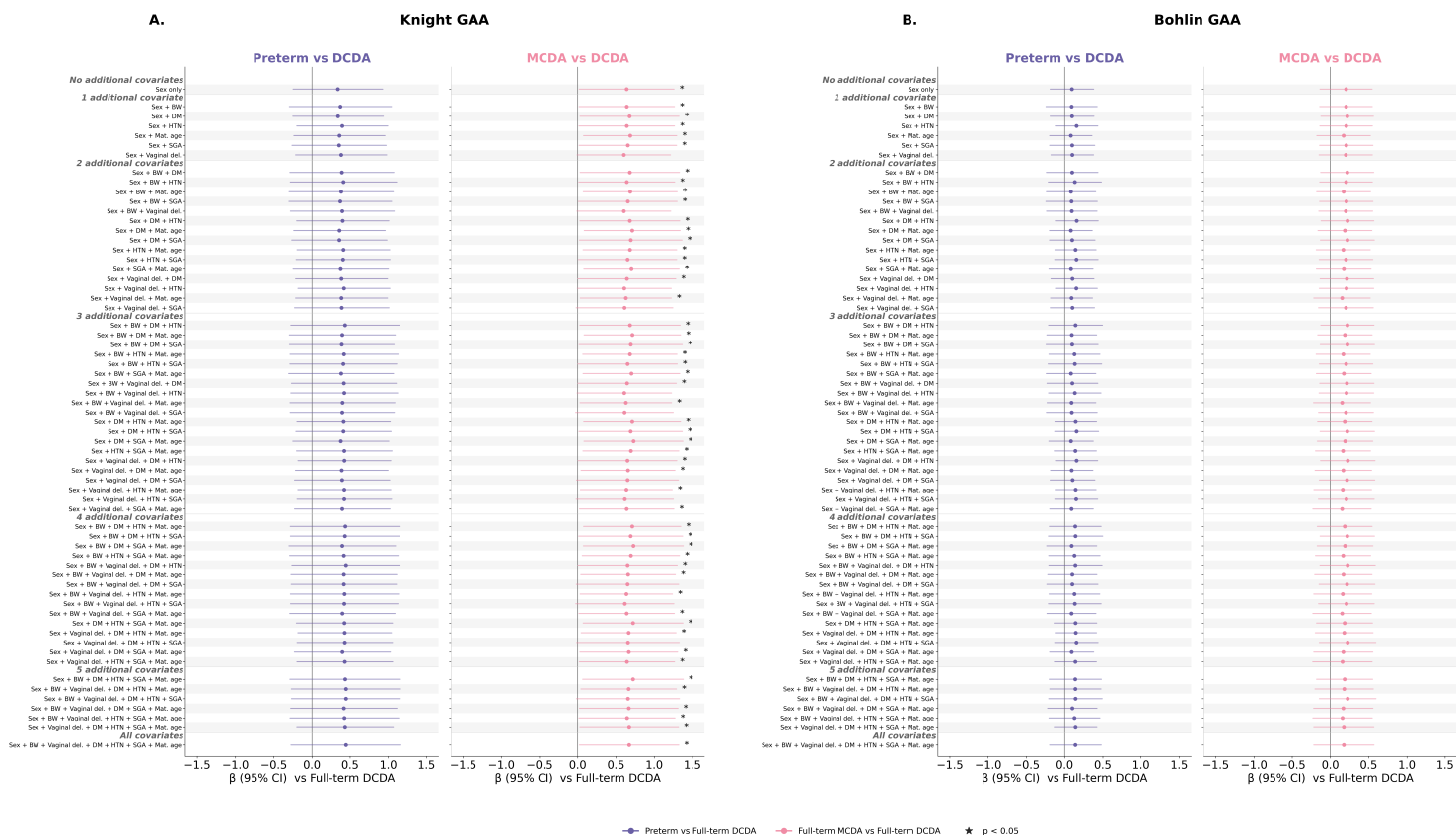


Figure 4

Sensitivity analyses of gestational age acceleration across 64 covariate model specifications.

Each row represents one of 64 covariate combinations derived from birth weight, SGA status, maternal diabetes mellitus, maternal hypertension, maternal age, and mode of delivery, with sex fixed in all models. Forest plots display β coefficients and 95% confidence intervals for two contrasts – preterm vs. full-term DCDA (purple) and full-term MCDA vs. full-term DCDA (pink) – with GAA estimated separately using Knight's clock (left) and Bohlin's clock (right). Preterm infants (DCDA and MCDA combined, given the limited number of preterm pairs) were compared with full-term DCDA twins as the reference group. Cluster-robust standard errors were applied at the twin-pair level. The full-term MCDA vs. full-term DCDA contrast for Knight GAA remained statistically significant in 52 of 64 models; the 12 non-significant models all included vaginal delivery as a covariate. All other contrasts were consistently positive but non-significant across all 64 specifications. Asterisk (*) denotes $p < 0.05$. β , ordinary least squares regression coefficient; CI, confidence interval; GAA, gestational age acceleration; DCDA, dichorionic–diamniotic; MCDA, monochorionic–diamniotic; BW, birth weight; SGA, small for gestational age; DM, diabetes mellitus; HTN, hypertension; Mat. age, maternal age; Vaginal del., vaginal delivery.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [ClinicalEpigeneticsSupplementaryTables.xlsx](#)
- [ClinicalEpigeneticsSupplementaryFigures.pdf](#)
- [SuppFig1DeltaAAviolin.png](#)