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Epigenetic Age Acceleration, Neonatal Morbidities, and Neurobehavioral Responses in Infants

Born Very Preterm

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Abstract Cover Page

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Born Very Preterm

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B.S. Statistics, B.S. Chemistry

University of Georgia

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Abstract

Epigenetic Age Acceleration, Neonatal Morbidities, and Neurobehavioral Responses in Infants Born Very Preterm

By
Uriel Paniagua

Background: Infants born very preterm are at increased risk of experiencing neonatal morbidities and exhibiting nonoptimal neurobehavioral responses. Epigenetic age acceleration is a potential biomarker of developmental maturity that may be useful for this vulnerable population and help identify at-risk neonates for preventative interventions to improve health outcomes.

Methods: To understand how neonatal morbidities and neurobehavioral profiles and responses correlated with age acceleration, we utilized data from 519 infants born <30 weeks gestation participating in the Neonatal Neurobehavior and Outcomes in Very Preterm Infants (NOVI) study. Epigenetic age was estimated using the NEOage epigenetic clocks. Morbidities including bronchopulmonary dysplasia (BPD), severe retinopathy of prematurity, severe brain injury, and serious neonatal infections were combined in an additive risk score ranging from 0-4. We regressed post-menstrual age (PMA) and postnatal age (PNA) acceleration on the neonatal morbidities and neurobehavioral profiles and responses separately, controlling for potential confounders. Interaction with sex was also explored. Parameter estimates using the PedBE and Horvath skin-blood epigenetic clocks were obtained to assess for variability across epigenetic clocks.

Results: Infants with mild BPD had higher PMA age acceleration ($\beta_1 = 0.36$, p-value < 0.01) compared to infants without BPD, and those with moderate-severe BPD had both higher PMA ($\beta_2 = 0.37$, p-value < 0.01) and PNA ($\beta_2 = 0.32$, p-value = 0.047) age acceleration compared to infants without BPD. Infants exhibiting hypertonicity had higher PNA age acceleration ($\beta_1 = 0.40$, p-value = 0.002) compared to those without. In models including interaction, males with moderate-severe BPD had lower PNA age acceleration ($\gamma_2 = -0.64$, p = 0.025) compared to females with moderate-severe BPD. Additionally, in males, increased handling score was associated with lower ($\gamma_1 = -1.09$, p-value = 0.02) PNA age acceleration.

Discussion: Our analyses revealed associations between age acceleration, neonatal morbidities, and neurobehavioral domains among a cohort of very preterm infants, which is consistent with prior research. Future research may consider collecting DNAm at birth and NICU discharge to assess differences among infants surviving and those who succumb to their adverse health outcomes to better establish the utility of age acceleration as a biomarker of neonatal health.

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INTRODUCTION

Preterm birth, defined as birth prior to 37 weeks of gestation, accounts for almost 10% of births in the United States and is the leading cause of death among children under 5 years of age [1] . The final weeks of pregnancy are a critical period of development for organs such as the brains, lungs, and the liver [2], and experiencing this critical window *ex-utero* has consequences. Infants born preterm are predisposed to increased risk of medical complications including bronchopulmonary dysplasia (BPD), severe brain injury, severe retinopathy of prematurity, and serious neonatal infection during their stay at the NICU, and also have a higher predisposition for neurodevelopmental delays that may persist long-term [3, 4]. Preterm births can be further sub-categorized as moderate to late for those born between 32 and 37 weeks of gestation, very preterm for those born between 28 to 32 weeks of gestation, and extremely preterm for those born less than 28 weeks of gestation [5]. As several studies have shown even small differences in gestational time can have significant impacts on neonatal outcomes and long-term neurodevelopmental impairments, these subcategorizations are essential to appropriately evaluate neonatal risk for acute and chronic morbidities [6-9].

Age since conception strongly correlates with organ growth and development. However, even among infants with identical chronological ages, some may be more developmentally mature and others less so. Biological age is an alternative age metric to chronological age, or age since birth, that captures the functional capability of a person or organ. [10]. This age metric may be useful in assessing the health of infants that were born preterm as it may be an indicator of developmental maturity. Useful tools for evaluation of biological age are epigenetic clocks, which have been developed based on DNA methylation (DNAm) and are thought to yield accurate predictions of biological age. Several epigenetic clocks have been developed across

different age groups and human tissue for various indications [11]. Horvath developed the first pan-tissue epigenetic clock by combining publicly available datasets measured on the Illumina 27K or Illumina 450K platforms. The clock utilizes 353 CpGs and has been shown to provide accurate estimates of age across different tissues and cell types [10, 12]. Although estimates of epigenetic age, or biological age, derived using this clock have been shown to highly correlate with chronological age in adolescent and adult populations, the clock yields estimates that are more variable and not as accurate when applied to pediatric populations, as DNAm changes in early life may not be reflective of those later in life [12, 13].

The Pediatric-Buccal-Epigenetic (PedBE) clock was developed to address gaps in DNAm in children and utilizes buccal epithelial cell DNAm at 94 CpG sites [13]. The clock focuses on estimation of chronological age of children ranging from birth to 20 years old and was found to have less variability compared to the Horvath clock when applied to pediatric samples. Though the PedBE clock may yield better estimates than prior epigenetic clocks when applied to pediatric populations, the clock was derived using samples only from individuals that were developing normally between the ages of 0 and 20 years, and therefore may not be as reliable when applied to infants born very preterm, particularly for samples collected during the neonatal period [13].

An epigenetic clock derived by Knight et al. [14] was developed using neonatal cord blood and blood spot samples from infants ranging from 24 to 41 weeks of gestation. They identified 148 CpG sites that yielded estimates of gestational age (GA) consistent with established methods such as ultrasound. This epigenetic clock is useful for estimating age from conception to birth, but it is restricted to blood samples collected at birth.

To further address gaps in methylation clocks for infants born at <30 weeks gestation and for the early postnatal period, four NEOage clocks to predict post-menstrual age (PMA) and postnatal age (PNA) based on DNA methylation across 303-522 CpGs, compatible with the Illumina EPIC and 450k arrays, were derived using buccal epithelial cells obtained from 542 infants enrolled in the Neonatal Neurobehavior and Outcomes in Very Preterm Infants (NOVI) study. PMA refers to time from inception onward, and PNA refers to one's chronological age from birth. Predicted age utilizing the NEOage clocks were found to highly correlate with these infant age metrics during the early neonatal period[15].

Epigenetic clocks such as these have been utilized to gain insights into biological aging through age acceleration, the difference between chronological and epigenetic age. Age acceleration has been found to be associated with increased risk of conditions in adults and adolescents, with age-related conditions including Alzheimer's disease, cardiovascular disease, and cognitive performance shown to be linked to epigenetic age acceleration when using the Horvath pan-tissue clock [12]. In younger populations, differences in epigenetic age acceleration have been found to be linked with prenatal exposures including maternal anxiety, diet, BMI, tobacco smoking status, as well as childhood psychiatric problems [16-20]. However, epigenetic aging studies of preterm infants, particularly during the early neonatal period are limited.

We aim to fill this gap by examining relationships between age acceleration, neonatal morbidities, and neurobehavioral profiles and domains among infants that were born very preterm utilizing the NEOage epigenetic clocks for PMA and PNA compatible with the Illumina EPIC array, and comparing findings with two other epigenetic clocks - PedBE [13] and Horvath Skin-Blood [21], both of which included children's buccal epithelial DNA in their training sets. The NEOage clock for PNA is the only clock to focus on postnatal age predictions in preterm

infants, and was found to yield predicted ages that highly correlated with reported ages [15].

Most research examining epigenetic age acceleration in preterm infants has utilized clocks

derived from samples obtained from older subjects, which are less reliable when applied to

individuals outside of the age range of the population used in clock development. The NEOage

clocks derived from the NOVI cohort are expected to yield more reliable estimates of age

acceleration for these very preterm infants. This paper aims to improve current understanding of

age acceleration during the early neonatal period and its associations with neonatal morbidities

and neurobehavioral profiles and domains.

METHODS

Study Population. The Neonatal Neurobehavior and Outcomes in Very Preterm Infants (NOVI)

Study was conducted at 9 university affiliated NICUs in Providence, RI, Grand Rapids, MI,

Kansas City, MO, Honolulu, HI, Winston Salem, NC, and Torrance and Long Beach CA from

April 2014 through June 2016. These NICUs were also Vermont Oxford Network (VON)

participants. Eligibility was determined based on the following inclusion criteria: 1) birth at <30

weeks post menstrual age; 2) parental ability to read and speak English or Spanish and 3)

residence within 3 hours of the NICU and follow-up clinic. Exclusion criteria included maternal

age <18 years, maternal cognitive impairment, maternal death, infants with major congenital

anomalies, including central nervous system, cardiovascular, gastrointestinal, genitourinary,

chromosomal, and nonspecific anomalies, and NICU death. Parents of eligible infants were

invited to participate in the study when survival to discharge was determined to be likely by the

attending neonatologist. Overall, 704 eligible infants were enrolled. DNAm levels were profiled

from buccal epithelial cells that were collected from study participants at NICU discharge using

Illumina EPIC arrays. After quality-control, pre-processing, and normalization as described in Graw et al. [15], samples from 542 individuals were viable for analysis.

NICU Neonatal Morbidities. To assess the impact of serious neonatal morbidities on age acceleration, we used an adaptation of Bassler et al.'s [3] validated cumulative neonatal morbidity risk score, which was calculated by adding the total number of neonatal health complications including BPD, severe brain injury, severe retinopathy of prematurity, and culture-confirmed infections that the NOVI cohort infants experienced during their stay at the NICU. Neonatal morbidity risk scores ranged from 0-4, although 3 and 4 were grouped into a single category due to a limited number of infants experiencing all four morbidities [22]. The morbidities were also assessed separately to determine individual associations with age acceleration.

NICU Network Neurobehavioral Scale (NNNS). The Neonatal Intensive Care Unit Network Neurobehavioral Scale (NNNS) is a standardized tool commonly used in research examining at-risk infants such as those born preterm [23]. This tool assesses infant neurobehavioral organization, neurological reflexes, motor development, and signs of stress and withdrawal [3] Responses from the infant assessments were converted to 12 domain summary scores including attention, handling, self-regulation, arousal, excitability, lethargy, hypertonicity, hypotonicity, non-optimal reflexes, asymmetric reflexes, quality of movement and stress abstinence. Specifics about the individual domains are detailed in Boukydis et al. [24] Our prior work used latent profile analysis to identify 6 different heterogenous neurobehavioral profiles based on these summary scores, that have been shown to be related to future behavioral and cognitive

performance [22]. The infants in Profile 1 were those with the more optimal responses, whereas those in profile 6 had response patterns that were most predictive of nonoptimal developmental outcomes in childhood. Infants in Profile 5 exhibited the most apparent motor immaturity. Detailed methods of the neurobehavioral profiles are discussed in Everson et al [22]. This paper will focus on comparing the two most atypical neurobehavioral profiles – 5 and 6 – to all other NOVI infants, as well as examining the 12 individual neurobehavioral domains. Hypertonicity and hypotonicity were included as binary predictors as any hypertonia or hypotonia is concerning in infants, while the other 10 domains were assessed as continuous predictors of age acceleration.

Age Metrics. PNA for infants in the NOVI study is the time from birth to tissue collection at NICU discharge. PMA was determined by adding PNA to the estimated gestational age that was obtained using established methods [15].

Estimates of Epigenetic Age. Estimates of PMA and PNA were determined using the NEOage epigenetic clocks compatible with the Illumina EPIC array derived from infants in the NOVI cohort. [15]. To assess for consistency across established clocks, age acceleration was also derived using the Horvath skin-blood [21] and PedBE [13] epigenetic clocks. Epigenetic age acceleration was calculated for all four of these clocks and was defined as the residuals when epigenetic age, or predicted age, is regressed on chronological age in an unadjusted linear model.

Statistical Analyses. To assess relationships between age acceleration and the neonatal morbidity risk score, the individual morbidities, neurobehavioral profiles, and the individual

neurobehavioral domains, we utilized generalized estimating equations (GEE), adjusting for infant sex, site, race, maternal factors including socioeconomic status, obesity status, smoking status, age, and education based on reviews of literature [16, 18, 22, 25], while clustering by family to account for siblings in the cohort. Forest plots were produced to visually assess relationships for some of the key confounding variables. Interaction terms with sex were added to models as a secondary analysis to identify differences in effect by sex. Age acceleration was regressed as a continuous variable and as a binary variable to identify differences in association when comparing infants in the top and bottom quintiles of age acceleration to the remaining cohort. We also performed a sensitivity analysis, examining models with the addition of gestational age as a potential confounder due to the uncertain temporality of age acceleration, neurobehavioral responses and neonatal morbidities experienced during NICU stay. A heatmap of the z-scores comparing the NEOage PMA and PNA clocks, Horvath skin-blood clock, and PedBE clock was produced to compare model results across established clocks. All statistical analyses were conducted in R version 4.1.2. Generalized estimating equations were carried out using the *gee* package, and forest plots were produced using the *ggplot* package.

RESULTS

Study Population. The characteristics of the NOVI cohort have been described in detail elsewhere [22, 25]. Of the 542 infants with estimates of epigenetic age, 519 (95.8%) reported data on the covariates of interest and were included in our analyses. The mean (SD) gestational age among the infants was 27.0 weeks (1.9), mean postnatal age was 12.2 weeks (4.5), and mean post-menstrual age was 39.2 weeks (3.4). Post-menstrual age acceleration ranged from -4.79 to 4.86 weeks, and postnatal age acceleration ranged from -9.57 to 3.82 weeks. A total of 189

(36.4%) infants experienced 1 of the 4 morbidities, 98 (18.9%) experienced 2, and 28 (5.4%) experienced 3 or 4. Across the individual morbidities, 67 infants (12.9%) had a brain injury, 34 (6.6%) had severe retinopathy of prematurity, 101 (19.5%) had a serious infection, 126 (24.3%) had mild BPD, and 143 (27.6%) had moderate-severe BPD. The NNNS Profile 6 had the fewest number of infants with only 35 (6.6%), while Profile 5 consisted of 115 (22.2%) infants.

Continuous PMA Age Acceleration. Infants with mild BPD were observed to have higher PMA age acceleration ($\beta_1 = 0.36$, p-value < 0.01) compared to infants without BPD, as were infants with moderate-severe BPD ($\beta_2 = 0.37$, p-value < 0.01). There were no significant associations between PMA age acceleration and overall neonatal morbidity risk score, though we did observe increasing PMA age acceleration with increased risk score (Table 1, Figure 1A). There were no strong associations with the other neonatal morbidities, neurobehavioral profiles, or the individual neurobehavioral domain summary scores (Table 1). While the association was not found to be statistically significant, infants with mothers whose highest level of education was reported to be below high school, or GED were observed to have lower PMA age acceleration (Figure 1A-B) in the models examining neonatal morbidity risk score and BPD.

Continuous PNA Age Acceleration. Findings from models regressing PNA age acceleration were overall similar to the models regressing PMA age acceleration (Table 1, Figure 1A-D), with one distinction being that infants with hypertonicity were observed to have higher PNA age acceleration compared to infants without hypertonicity ($\beta_1 = 0.40$, p-value $= 0.002$). Additionally, there was not a statistically significant association between PNA age acceleration

and mild BPD, though infants with moderate-severe BPD were observed to have higher PNA age acceleration compared to infants without BPD ($\beta_2 = 0.32$, p-value = 0.047).

Interaction by Sex. Interaction of the primary covariates with sex was explored for the continuous PMA and PNA age acceleration models. With the addition of an interaction term for sex, infants with moderate-severe BPD were still observed to have both higher PMA age acceleration ($\beta_2 = 0.58$, p-value <0.001) and higher PNA age acceleration ($\beta_2 = 0.66$, p-value <0.01) compared to infants without BPD, although males with moderate-severe BPD exhibited a decrease in PNA age acceleration ($\gamma_2 = -0.64$, p = 0.025) compared to females with moderate-severe BPD. Hypertonicity ($\beta_1 = 0.49$, p-value = 0.002) and increasing arousal ($\beta_1 = 0.27$, p-value = 0.04) were associated with higher PNA age acceleration, although there was no statistically significant interaction with sex for the two models. In contrast, while an increasing handling score was not found be associated with a statistically significant change in PNA age acceleration ($\beta_1 = 0.43$, p-value = 0.25), in males, an increasing handling score was associated with a decrease in PNA age acceleration ($\gamma_1 = -1.09$, p-value = 0.02). There were no other significant main effects or interactions for the other models run.

Extremes for PMA and PNA Age Acceleration. The top and bottom quintiles for PMA and PNA age acceleration were examined to assess for differences in effect when comparing infants at opposite extremes of age acceleration to all other infants. Infants with a neonatal morbidity risk score of 1 (OR = 1.68, p-value = 0.047) or 2 (OR = 2.18, p-value = 0.01) were more likely to have a PMA age acceleration in the top quintile of the study cohort compared to infants without any morbidities, with those having a risk score of 2 also being more likely to have a PNA age

acceleration in the top quintile (OR = 2.17, p-value = 0.02). A similar trend was observed when looking at BPD. Infants with mild (OR = 2.34, p-value <0.01) or moderate-severe (OR = 2.49, p-value <0.001) BPD were more likely to have a PMA age acceleration in the top quintile compared to infants without BPD, with those having moderate-severe BPD also being more likely to have a PNA age acceleration in the top quintile (OR = 2.03, p-value = 0.002). Among the neurobehavioral domains, hypertonicity was associated with lower odds (OR = 0.37, p-value <0.001) of having a PNA age acceleration in the bottom quintile, whereas a higher asymmetric reflexes score was associated with higher odds (OR = 1.28, p-value <0.01) of being in the bottom quintile for PNA age acceleration. An increasing asymmetric reflexes score was also associated with lower odds (OR = 0.81, p-value = 0.049) of having a PMA age acceleration in the top quintile, while an increasing excitability score was associated with higher odds of having a PMA age acceleration in the top quintile. There were no statistically significant associations for the other covariates (Table 2).

Adjustment for Gestational Age. Gestational age at birth was added to all models as a sensitivity analysis to assess changes in parameter estimates. In all analyses, gestational age was a significant predictor of PNA and PMA age acceleration (p-values < 0.05). With this adjustment, infants with a neonatal morbidity risk score of 1 were observed to have higher PNA age acceleration compared to infants with no morbidities ($\beta_1 = 0.33$, p-value = 0.02). Infants with a neonatal morbidity risk score of 2 were also observed to have higher PNA age acceleration ($\beta_2 = 0.54$, p-value <0.01), but lower PMA age acceleration ($\beta_2 = -0.30$, p-value = 0.04) compared to infants with no morbidities. Infants with mild (OR = 0.48, p-value = 0.03) or moderate-severe (OR = 0.42, p-value = 0.03) BPD had lower odds of being in the bottom

quintile for PNA age acceleration compared to infants without BPD. Models examining BPD and PMA age acceleration had null associations (p-values > 0.05) when adjusting for GA. Having experienced a serious infection was associated with lower odds of having a PNA age acceleration in the bottom quintile (OR = 0.49, p-value = 0.03) when adjusting for GA. Among the neurobehavioral domains, after adjustment for GA, increasing asymmetric reflexes score was associated with lower PMA age acceleration (β_1 = -0.08, p-value = 0.045). In models that included interaction with sex, increasing asymmetric reflexes score was associated with lower PMA age acceleration among males (γ_1 = -0.18, p-value = 0.01), though the main effect was not statistically significant (β_1 = 0.01, p-value = 0.80). Additionally, increasing excitability score was no longer significantly associated with higher odds of having a PMA age acceleration in the top quintile (OR = 1.11, p-value = 0.09). Other findings were null and/or similar in direction and statistical significance to models omitting gestational age.

Comparison to other Epigenetic Clocks. Models examining PNA age acceleration derived using the Horvath skin-blood and PedBE clocks and the primary covariates, controlling for confounders, were examined for comparison to the NEOage clocks. Figure 2 displays a heatmap of the z-scores for the different models across the four clocks. For the models examining neonatal morbidity risk score and the individual morbidities, the Horvath clock produced z-scores near or greater than 0, in contrast to the NEOage PNA and PedBE clocks. Across the neurobehavioral profiles and domains, the Horvath and PedBE clocks produced estimates that were more comparable to each other than the estimates using the NEOage PNA clock.

DISCUSSION

Our analysis indicated that among a cohort of very preterm infants, mild or moderate-severe BPD was associated with higher PMA and PNA age acceleration, with the effect being different by sex for PNA age acceleration. Hypertonicity and arousal were also shown to be associated with differences in PNA age acceleration. While handling was not a statistically significant predictor of PNA age acceleration, there was a significant interaction by sex. Dichotomizing age acceleration to examine differences in associations at opposite extremes of PMA and PNA age acceleration overall revealed similar trends that were seen with the continuous outcome. Neonatal morbidity risk score, BPD, hypertonicity, asymmetric reflexes, and excitability were found be associated with significantly higher or lower odds of having an age acceleration at one of the outer quintiles. The overall trends in associations with the models examining neonatal morbidity risk score and BPD indicate that BPD may be driving the observed association with the morbidity risk score, as the other morbidities did not have a clear relationship with PMA and PNA age acceleration, as was also observed in Everson et al. [25].

Lower gestational age is associated with increased risk of developing adverse health outcomes, as well as differences in neurobehaviors. However, how age acceleration may tie into this relationship is unclear. To account for this uncertainty, all models were also run with the addition of gestational age as a potential confounder. Overall, adjustment for gestational age resulted in regression parameters that remained consistent with regards to statistical significance and direction compared to models omitting gestational age, though there were some minor differences. In the models examining serious infection, asymmetric reflexes, and excitability, there were changes in statistical significance, though the direction of the association with age acceleration remained the same. However, differences in parameter estimates after adjustment for

gestational age were apparent in infants with a risk score of 2 – these infants had higher PNA age acceleration but lower PMA age acceleration, when compared to infants with no morbidities. Adjustment for gestational age also resulted in null associations of BPD with PMA age acceleration. These notable changes indicate that timing of birth may set a trajectory for epigenetic aging, so adjustment for gestational age in our models may greatly alter the conditional effect of other predictor variables on age acceleration.

A study by Knight et al. [26] found that higher gestational age acceleration was associated with decreased BPD among extremely preterm infants, which is opposite in direction to what was found in our analyses. Though the relationship between age acceleration and BPD described in Knight et al. [26] did not align with our findings, their analysis identified a statistically significant interaction of BPD with sex, with males with BPD having lower age acceleration compared to females with BPD, which is consistent with our observed results. However, their analyses focused on gestational age acceleration, whereas our focus was on PMA and PNA age acceleration that were measured at NICU discharge. In the case of gestational age acceleration and BPD, there is clearer temporality, as gestational age acceleration certainly precedes the experienced morbidities. Thus, their observation that decreased gestational age acceleration was associated with increased risk of BPD may be an indicator of biological prematurity at birth. However, with PNA and PMA age acceleration, it is unclear whether the age acceleration preceded or came after the experienced morbidities. It is possible that our PNA and PMA age acceleration metrics are capturing the stress experienced during the NICU stay, including the NICU environment and the medical procedures that are utilized to treat these morbidities. This would align with other research that demonstrates a relationship between early-life stress, adversity [27, 28], and major medical procedures [29] with age acceleration.

Another explanation for the observed difference may be that Knight et al. [26] focused on extremely preterm infants using an epigenetic clock derived from infants ranging from extremely preterm to term, whereas the NEOage clock used in our analyses was used on and derived from very preterm infants. These findings in extremely preterm infants when examining gestational age acceleration and in very preterm infants when examining PNA and PMA age acceleration indicate that the utility of age acceleration as a biomarker of developmental maturity may not be clear in preterm infants, highlighting the need for further research to better establish the relationship and temporality of gestational age, neonatal morbidities, and age acceleration.

There were no statistically significant associations of the two atypical neurobehavioral profiles with PMA or PNA age acceleration when compared to profiles 1-4. One study examining the same cohort found no significant association of age acceleration when comparing profile 6 to the remaining neurobehavioral profiles [25], which aligns with our current analysis with the PNA and PMA NEOage clocks [22]. However, we did identify statistically significant associations with age acceleration when looking at 5/12 of the individual domains: hypertonicity, asymmetric reflexes, handling, arousal, and excitability. Other research has linked physical and cognitive function [30], which both relate to neurobehavior, with differences in age acceleration. Our findings indicate that neurobehaviors may be predictors of age acceleration even among early neonates.

Analyses examining the PedBE and Horvath-Skin Blood epigenetic clocks revealed considerable differences in z-scores across the models examined. These results were unsurprising, as the PedBE clock was derived from buccal epithelial cells from individuals 0 to 20 years old [13] and the Horvath skin-blood clock was derived from individuals of a wider age range and DNA was extracted from a wider array of samples [21]. Correlations between

predicted and actual PMA and PNA were also considerably lower using these two epigenetic clocks when applied to the NOVI cohort [15]. These findings highlight the potential inaccuracy associated with attempting to apply an epigenetic clock derived from samples outside the age range used in the algorithm to derive the clock, as well as the utility of the NEOage clocks specifically targeted towards preterm infants and neonatal age metrics.

Though our analyses revealed some associations between age acceleration, neonatal morbidities, and neurobehavioral domains, given the rapidly changing and intricate biological mechanisms occurring in infants, our findings may not be as readily applicable if extrapolated to infants not born very preterm. As there have not been other studies done utilizing an epigenetic clock derived from preterm infants, there is no epigenetic clock that the NEOage clocks may properly be compared to, so we are unable to perform replications of analyses with other datasets. Another important limitation of our findings is that DNA samples were collected shortly before infants were discharged from the NICU, and to be included in the study infants had to survive to NICU discharge, introducing potential survivorship bias into our results as there are clearly differences in health outcomes among those who survived and those who became deceased.

Further research may consider collecting DNAm at birth and NICU discharge to assess for differences among infants surviving and those who succumb to their adverse health outcomes, in addition to allowing for better establishment of the temporality of age acceleration and neonatal morbidities. Doing so may improve our understanding of the use of age acceleration as a biomarker of developmental maturity for this vulnerable population and potentially identify at-risk neonates for preventative interventions to improve health outcomes.

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TABLES AND FIGURES

Table 1. Regression Coefficients and p-values for models examining associations between neonatal morbidity risk score, individual morbidities, neurobehavioral profiles and domains, and PMA and PNA age acceleration.

	Post-Menstrual Age Age Acceleration						Postnatal Age Age Acceleration					
	β_x	p	β_x	p	γ_x	p	β_x	p	β_x	p	γ_x	p
Neonatal Morbidity Risk Score												
1 vs 0	0.17	0.13	0.15	0.35	0.03	0.90	0.19	0.17	0.32	0.12	-0.23	0.38
2 vs 0	0.18	0.25	0.24	0.30	-0.11	0.70	0.26	0.16	0.55	0.07	-0.50	0.17
3+ vs 0	0.35	0.13	0.38	0.20	-0.05	0.91	-0.16	0.51	-0.004	0.99	-0.27	0.59
Bronchopulmonary Dysplasia												
Mild vs Absent	**0.36**	**<0.01**	0.31	0.15	0.08	0.75	0.06	0.71	0.18	0.56	-0.20	0.56
Moderate-Severe vs Absent	**0.37**	**<0.01**	**0.58**	**<0.001**	-0.40	0.09	**0.32**	**0.047**	**0.66**	**<0.01**	**-0.64**	**0.025**
Serious Neonatal Infection												
Present vs Absent	-0.01	0.94	-0.02	0.91	0.02	0.93	0.19	0.21	0.33	0.15	-0.26	0.38
Severe Retinopathy												
Present vs Absent	0.22	0.29	0.10	0.72	0.26	0.53	0.05	0.85	-0.04	0.89	0.20	0.71
Severe Brain Injury												
Present vs Absent	-0.09	0.60	-0.16	0.56	0.13	0.70	-0.29	0.20	-0.42	0.28	0.25	0.58
NNNS Profile												
Profile 5 vs Profiles 1-4	-0.002	0.99	-0.08	0.70	0.13	0.58	0.14	0.41	0.15	0.54	-0.02	0.95
Profile 6 vs Profiles 1-4	0.03	0.89	0.08	0.82	-0.07	0.86	0.30	0.26	0.18	0.66	0.19	0.68
NNNS Summary Scores												
Hypertonicity	0.10	0.38	0.22	0.18	-0.22	0.30	**0.40**	**0.002**	**0.49**	**<0.01**	-0.16	0.51
Hypotonicity	0.04	0.74	-0.25	0.23	0.48	0.08	0.06	0.72	-0.26	0.42	0.51	0.15
Nonoptimal Reflexes	0.02	0.48	-0.002	0.95	0.04	0.39	0.003	0.93	-0.02	0.73	0.04	0.52
Asymmetric Reflexes	-0.06	0.18	0.006	0.92	-0.12	0.13	-0.09	0.07	-0.02	0.80	-0.14	0.15
Quality of Movement	0.02	0.80	0.08	0.50	-0.10	0.49	-0.03	0.71	0.09	0.48	-0.20	0.21
Attention	-0.07	0.07	-0.07	0.19	-0.005	0.95	0.01	0.75	0.008	0.90	0.01	0.86
Handling	0.006	0.98	0.32	0.27	-0.55	0.15	-0.18	0.43	0.43	0.25	**-1.09**	**0.02**
Arousal	0.06	0.46	0.19	0.09	-0.24	0.13	0.10	0.27	**0.27**	**0.04**	-0.31	0.08
Excitability	0.02	0.33	0.07	0.05	-0.07	0.12	0.05	0.15	0.05	0.27	-0.009	0.88
Lethargy	0.006	0.81	-0.004	0.90	0.02	0.68	-0.03	0.30	-0.04	0.33	0.02	0.65
Regulation	-0.04	0.56	-0.04	0.67	-0.001	0.99	-0.08	0.41	0.04	0.79	-0.22	0.15
Stress Abstinence	0.11	0.89	0.43	0.70	-0.52	0.70	0.15	0.88	0.74	0.59	-0.97	0.54

*Models were adjusted for sex, site, maternal education, maternal obesity status, maternal smoking status, maternal age, and site, and clustered by family to account for siblings. Each exposure was assessed in a separate model. Shaded columns indicate models that included interaction with sex. β_x denotes the regression coefficients for the main effects. γ_x denotes the regression coefficients for the interaction terms with sex. p denotes the p-value for the different parameter estimates.

Table 2. Odds Ratios (OR) and p-values for models examining associations between neonatal morbidity risk score, individual morbidities, neurobehavioral profiles and domains, and PMA and PNA age acceleration.

	Post-Menstrual Age Age Acceleration				Post-Natal Age Age Acceleration			
	Top Quintile		Bottom Quintile		Top Quintile		Bottom Quintile	
	OR	p-value	OR	p-value	OR	p-value	OR	p-value
Neonatal Morbidity Risk Score								
1 vs 0	**1.68**	**0.047**	1.15	0.59	1.48	0.16	0.90	0.70
2 vs 0	**2.18**	**0.01**	0.74	0.40	**2.17**	**0.02**	0.51	0.07
3+ vs 0	1.65	0.35	0.53	0.31	1.14	0.81	1.55	0.34
Bronchopulmonary Dysplasia								
Mild vs Absent	**2.34**	**<0.01**	0.58	0.07	1.50	0.17	0.84	0.54
Moderate-Severe vs Absent	**2.49**	**<0.001**	0.69	0.21	**2.03**	**0.02**	0.81	0.49
Serious Neonatal Infection								
Present vs Absent	1.19	0.54	1.07	0.81	1.54	0.12	0.62	0.12
Severe Retinopathy								
Present vs Absent	1.21	0.68	0.82	0.69	0.78	0.62	1.26	0.62
Severe Brain Injury								
Present vs Absent	0.92	0.81	0.95	0.89	1.03	0.92	1.24	0.50
NNNS Profile								
Profile 5 vs Profiles 1-4	0.82	0.53	1.48	0.21	1.28	0.42	1.11	0.71
Profile 6 vs Profiles 1-4	1.18	0.73	1.06	0.91	1.13	0.79	0.48	0.20
NNNS Summary Scores								
Hypertonicity	1.13	0.66	0.83	0.51	1.27	0.36	**0.37**	**<0.001**
Hypotonicity	1.11	0.73	0.95	0.87	1.30	0.36	0.85	0.61
Non-Optimal Reflexes	0.98	0.71	1.05	0.42	0.98	0.80	1.00	0.91
Asymmetric Reflexes	**0.81**	**0.049**	1.02	0.86	0.92	0.37	**1.28**	**<0.01**
Quality of Movement	0.93	0.70	1.03	0.89	0.85	0.34	0.85	0.33
Attention	0.96	0.62	1.06	0.49	1.05	0.63	1.02	0.86
Handling	1.59	0.29	1.42	0.43	0.71	0.48	1.13	0.78
Arousal	1.28	0.19	0.93	0.66	1.15	0.43	0.80	0.27
Excitability	**1.13**	**0.04**	0.96	0.48	1.03	0.64	0.93	0.22
Lethargy	0.94	0.34	1.00	0.96	0.94	0.28	1.03	0.60
Self Regulation	0.85	0.34	0.92	0.59	0.98	0.90	1.03	0.87
Stress Abstinence	2.21	0.67	0.27	0.50	0.16	0.33	0.25	0.49

*Models were adjusted for sex, site, maternal education, maternal obesity status, maternal smoking status, maternal age, and site, and clustered by mother to account for twins.
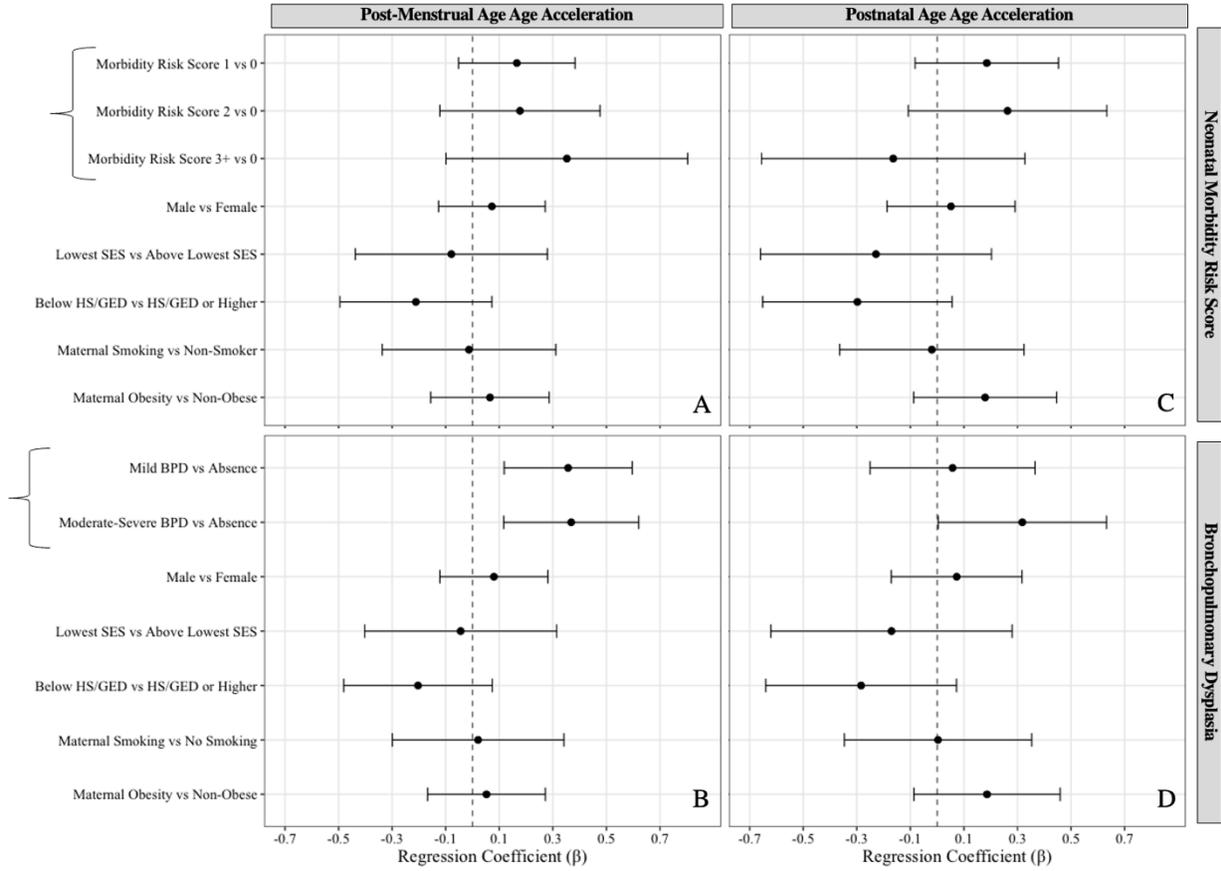
Figure 1. Forest plots of regression parameter estimates for PMA and PNA age acceleration. Models were also adjusted for site, race/ethnicity, and maternal age, and clustered by family to account for siblings.
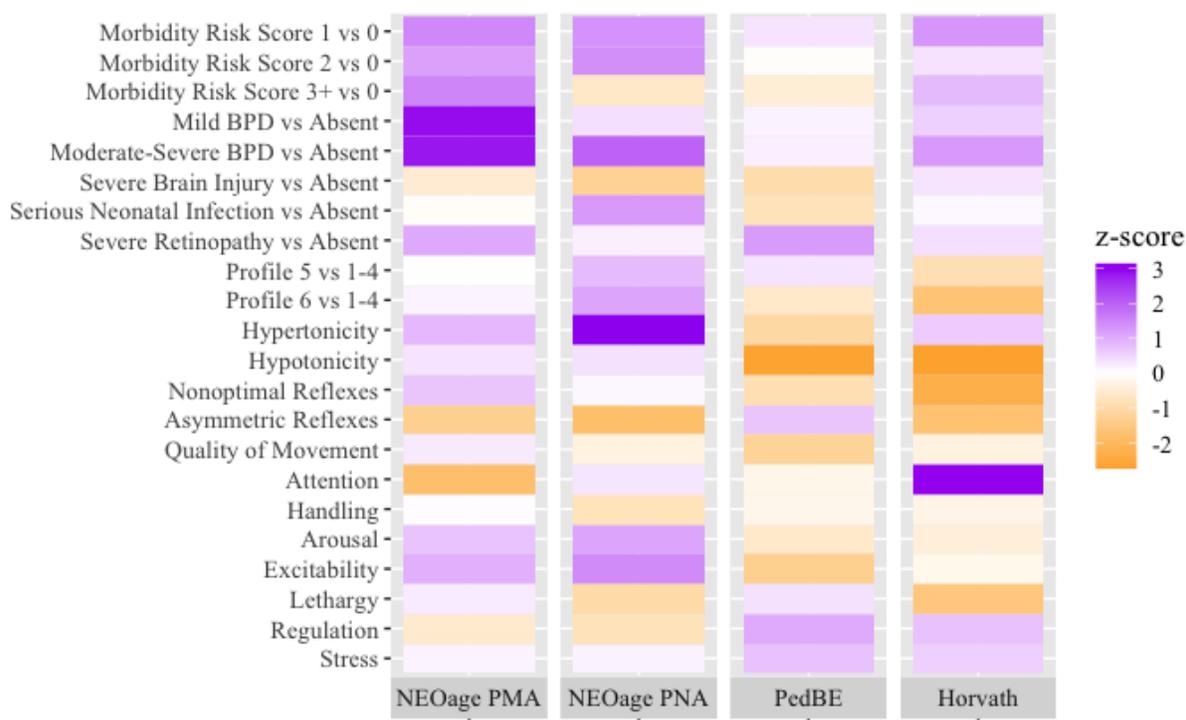
Figure 2. Heatmap of z-scores for age acceleration models across epigenetic clocks. Models

were adjusted for sex, site, maternal education, maternal obesity status, maternal smoking status,

maternal age, and site, and clustered by family to account for siblings.