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Biomarkers of maternal smoking during pregnancy associated with decreased epigenetic age acceleration at birth

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An abstract of a thesis submitted to the Faculty of the Rollins School of Public Health of Emory University in partial fulfillment of the requirements for the degree of Master of Public Health in Gangarosa Department of Environmental Health
2021

Abstract

Biomarkers of maternal smoking during pregnancy associated with decreased epigenetic age acceleration at birth

By Timothy Tynan

Maternal smoking during pregnancy (MSDP), or the use of smoking tobacco during pregnancy, is a relatively common behavior despite decades of public health recommendations to avoid cigarettes during this time period. MSDP is associated with a wide range of health outcomes, including intrauterine growth restriction, preterm birth, stillbirth, low birth weight, congenital malformations, increased perinatal mortality as well as several other obstetrical disorders. Epigenetic biomarkers of biological aging, known as age acceleration, have been posited as an effective mechanism to measure the health and development of offspring, but few have examined the impact of smoking during pregnancy on offspring epigenetic age.

We aimed to test the relationship between maternal smoking during pregnancy and epigenetic gestational age acceleration (GAA) at birth, using both the Bohlin and Knight epigenetic clocks. The New Hampshire Birth Cohort Study (NHBCS) was utilized and includes data on cord blood DNA methylation, self-report surveys, as well as cotinine and NNAL biomarkers for tobacco use. Study participants consisted of 255 mother-offspring pairs with both epigenetic and exposure data. We used multiple linear regression to test for differences in GAA at birth, associated with self-reported and biomarker-indicated maternal smoking during pregnancy, while adjusting for potential confounders.

We observed statistically significant decreases in GAA_{Knight} with a beta coefficient of -0.894 (95% CI (-1.743, -0.045)) and in GAA_{Bohlin} (beta coefficient of -0.557, 95% CI (-1.087, -0.027)) associated with biomarker-indicated maternal smoking. When adjusting for confounders (maternal age at enrollment, maternal BMI, sex, labor type, and cell types), the observed associations were unperturbed and remained statistically significant for both GAA_{Knight} (beta coefficient of -0.951 (95% CI = -1.752, -0.149) and GAA_{Bohlin} (beta coefficient of -0.567, 95% CI = -1.075, -0.059). Self-reported smoking during pregnancy exhibited the same direction of effect, but the effect sizes were smaller and confidence intervals crossed the null.

Our study provides supporting evidence that MSDP is associated with decreased GAA. These findings were particularly pronounced for when smoking status was determined via higher concentrations of cotinine and NNAL.

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Introduction

Epidemiology of Maternal Smoking During Pregnancy

Maternal smoking during pregnancy (MSDP), or the use of smoking tobacco during pregnancy, is a relatively common behavior despite decades of public health recommendations to avoid cigarettes during this time period. In 2016, 7.2% of women who gave birth in the US smoked cigarettes during pregnancy (*NCHS Data Brief No. 305 February 2018*, 2018). The group with the highest prevalence of smoking during pregnancy was women aged 20-24 (10.7%) (*NCHS Data Brief No. 305 February 2018*, 2018). Women of non-Hispanic American Indian and Alaska Native women had the highest prevalence of MSDP (16.7%) while non-Hispanic Asian women had the lowest prevalence (0.6%) (*NCHS Data Brief No. 305 February 2018*, 2018). Of note, the populations with the highest prevalence are traditionally marginalized populations that have faced adversity throughout their history in the US since colonization. Additionally, states with the highest levels of MSDP are located in the Southern United States with rates of MSDP as high as 25.1% in West Virginia and 18.4% in Kentucky, demonstrating regional variability in this public health problem (*NCHS Data Brief No. 305 February 2018*, 2018).

MSDP is associated with a wide range of health outcomes, including intrauterine growth restriction, preterm birth, stillbirth, low birth weight, congenital malformations, increased perinatal mortality as well as several other obstetrical disorders (National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health, 2014). Effects on offspring developmental characteristics are particularly notable, including impaired fetal growth, and neurodevelopmental deficits in offspring. Specifically, studies have found that MSDP is associated with impaired executive functioning and behavioral problems as well as reduced

cortical grey matter volume and alterations to DNA methylation patterning that is associated with reduced mature neuronal content (Chatterton et al., 2017; Cornelius et al., 2011; Roza et al., 2007). These studies highlight the impact that MSDP has on brain development and subsequent neurodevelopmental abnormalities. MSDP has also been associated with attention deficit hyperactive disorder (ADHD), congenital heart defects, obesity, lung function deficits, and increased systolic and diastolic blood pressure (Berlin & Oncken, 2018; Health, 2014; Thakur et al., 2013).

Epigenetic Mechanisms and Prenatal Exposures

Epigenetic mechanisms, which are molecular features that regulate gene expression but not through changes to the DNA sequence itself, have been proposed as possible mechanisms through which prenatal exposures effect long term health of offspring. DNA methylation, the most thoroughly studied epigenetic mechanism in humans, is a normal physiological process whereby methyl groups are added to DNA in a particular manner which then influences gene activity. CpG sites are locations in the DNA where a cytosine nucleotide is followed by a guanine nucleotide, and these cytosines can be methylated, which can alter the expression-potential of nearby genes. While this is a normal process, increased or decreased DNAm at different locations throughout the genome have been linked to a variety of health problems. Research has shown that epigenetic mechanisms are particularly vulnerable to environmental exposures during pregnancy and early in life (Jirtle & Skinner, 2007; Kundakovic & Jaric, 2017; Perera & Herbstman, 2011). The alteration of these epigenetic mechanisms may have long-term consequences on health that may even be transgenerational and affect offspring phenotypes in future generations (Nilsson et al., 2018). Animal studies demonstrate that prenatal exposure to

various stressors (toxins, drugs, viruses, among others) may cause lasting epigenetic changes in the brain (Bale et al., 2010; Grandjean & Landrigan, 2014; Ross et al., 2015). Additionally, epidemiologic studies have shown that prenatal adversity has been associated with the development of several neuropsychiatric diseases such as schizophrenia, depression, anxiety, and autism. While the exact mechanism of the development of these conditions is not well understood and admittedly complex, prenatal epigenetic alterations have been implicated as a possible etiology (Kundakovic & Jaric, 2017).

Effects of Maternal Smoking During Pregnancy on Epigenetic Mechanisms

Measuring health and development outcomes at birth for babies whose mothers smoked during pregnancy is difficult. Most studies to date have used birthweight and/or premature delivery to understand the adverse effects of MSDP on the newborn. While these are useful indicators of growth restriction and stress during the prenatal period, other indicators will help us further our understanding of the effects of MSDP. Epigenetic biomarkers have been posited as an effective mechanism to carry out this task. Multiple studies have examined the impact of MSDP on the newborn methylome to help elucidate the biological pathways that might be affected by MSDP (Breton et al., 2009; Green & Marsit, 2015; Guerrero-Preston et al., 2010; Joubert et al., 2016). Some of these MSDP-associated changes in methylation patterns are also predictive of health outcomes (Dhingra et al., 2018; Levenson, 2010). Additionally, analysis of DNAm in benign and inflammatory diseases has revealed specific patterns of DNAm that can be used for detection, diagnosis and prediction of outcomes in certain diseases (Levenson, 2010).

Smokers have different DNAm patterns than non-smokers, with related changes in gene expression, across multiple tissues (Tsai et al., 2018) indicating that the epigenetic effects of

smoking are systemic. Even the placental epigenome is affected by tobacco exposure (Morales et al., 2016), with a tendency towards hypomethylation among smoke-exposed placenta (Palma-Gudiel et al., 2019). Epigenome-wide meta-analyses of MSDP have found differential DNAm at hundreds-to-thousands of CpG sites in cord blood (Joubert et al., 2016) and in the placenta (Everson et al., 2021). In cord blood, some of the identified CpGs were located in genes implicated in the genetic studies of respiratory health, neurodevelopmental impairments, and cancers (Joubert et al., 2016). In the placenta, differential DNAm occurs in genes involved in environmental response, inflammation, growth factor signaling, and cardiometabolic outcomes (Everson et al., 2021). Many of the identified blood-based differences in DNAm also appeared to persist into childhood (Joubert et al., 2016) and placenta-based differences in DNAm were strongly associated with birth outcomes such as gestational age and birth size (Everson et al., 2021). Thus, MSDP has a profound impact on epigenetic mechanisms, which may serve as an indicator of how MSDP is affecting the health of newborns.

Epigenetic Age as a Surrogate Measure for Biological Age

The estimation of the biological age of an individual has emerged as a promising area of research in the field of epigenetics. Chronological age is a major risk factor for disease, functional impairments and ultimately mortality. There is, however, significant heterogeneity in the health outcomes of individuals as they age. While some individuals may experience significant impairment or disease in their 60's, others may not experience those same impairments until their 70s or 80s. Herein lies the idea behind biological age, or the notion that the age of an individual's tissues better reflects the age of an individual compared to

chronological age. The search for reliable predictors of biological age has come to the forefront in the past few decades and has resulted in the discovery and development of multiple measures.

Telomere length has been studied for decades as a biological age predictor and was once the gold standard for age-related studies. Some of the more recent research on telomere length has shown that it may not be the most robust predictor to use (Palma-Gudiel et al., 2019). Telomere length has been shown to be influenced by a wide variety of factors including paternal age at time of offspring birth, levels of inflammation, tobacco smoking, physical activity, sex, socioeconomic status, body mass index, multivitamin intake, alcohol consumption, race, hormone replacement therapy, and dietary antioxidants (Mather et al., 2011). Some studies have shown that telomere length may be altered independently by diseases of the immune system, while others have shown that telomere length remains the same or increases with age (Zhang et al., 2017). Many studies do not take these factors into account when performing analyses which may result in confounding. A notable problem with using telomere length as a surrogate for biological age is that there are a wide variety of ways to measure it. These methods vary significantly and are difficult to compare.

DNAm-based (DNAm) estimators of biological age, which have been developed within the last decade, have been shown to be the most precise molecular age-estimators currently in the field. The first "epigenetic clock" was published by Steve Horvath in 2013, which is an algorithm that uses the DNAm at 353 CpG sites to predict the age of an individual, and can do so across numerous different tissues (Horvath, 2013). This means that it does not require any adjustments or offsets when performing analyses and that comparisons of ages of different areas of the body can be made using the same aging clock (Horvath & Raj, 2018). Since the development of this original clock, a number of other epigenetic clocks have been developed that

predict morbidity and mortality in adults (Levine et al., 2018; Lu et al., 2019), confirming that DNAm is able to capture information about the health-related processes of aging. Other epigenetic clocks have been developed that are capable of estimating gestational age from newborn cord blood samples (Bohlin et al., 2016; Knight et al., 2016), opening this field of research up to questions about prenatal exposures and children's health. These developments in epigenetics have created tools that allow us to study how biological age is related to disease or affected by exposures.

Tobacco smoke appears to alter epigenetic aging

Several studies have found associations between tobacco exposure and acceleration of epigenetic age (Beach et al., 2015; de Prado-Bert et al., 2021; Dugué, Pierre-Antoine, 2017; Gao et al., 2016; Javed et al., 2016; Khouja et al., 2018; Simpkin et al., 2016). These studies have consistently found that smoking tends to be associated with increased age-acceleration (**Table 1**).

Table 1. Summary of publications that have examined associations between tobacco use and epigenetic age acceleration.

Author (Year)	**Main Finding**	**Tissue and Timing:**	**Tobacco Exposure**
Javed et al (2016)	Increased Age Acceleration	Cord Blood / Birth	Self-reported
Simpkin et al (2016)	Increased Age Acceleration	Peripheral Blood / Cord blood	Cotinine and Self-reported

Dugue et al (2017)	Increased Age Acceleration	Peripheral Blood / Adult (27-76 years)	Self-reported
Gao et al (2016)	Increased Age Acceleration	Whole Blood / Adult (average age 62)	Self-reported and DNAm Smoking Index
Wu et al (2019)	Increased Age Acceleration of Human Respiratory Organs	Buccal cells, airway cells, esophagus tissue, lung tissue / Adult (23-93 years)	Multiple studies with different assessments
Yang et al (2019)	Increased Age Acceleration	Whole blood / Adult	Self-reported
Khouja et al (2018)	No Association with MSDP	Cord blood / birth	Self-reported
Prado-Bert et al (2021)	Increased Age Acceleration associated with MSDP	Blood samples / Children (mean age of 8.1 years)	Self-reported

Current Gaps in the Research

While a significant number of studies have been conducted on the health effects of MSDP, and on how this exposure affects the newborn methylome, fewer have addressed how MSDP might affect epigenetic age at birth and the influence that may have on health outcomes. Most of these prior studies used epigenetic clocks that were developed to predict age across the life-course. New epigenetic clocks have been developed to specifically examine biological aging

as it relates to gestational age, referred to as gestational age acceleration (GAA) (Bohlin et al., 2016; Knight et al., 2016), and these may be more appropriate for studying the relationships between prenatal exposures and age acceleration at birth. Only Khouja et al. have examined whether MSDP is associated with age acceleration via these gestational epigenetic clocks (Khouja et al., 2018). This is an important gap in the field because MSDP associated differences in epigenetic age could reveal developmental characteristics that are not captured by common birth outcome measures such as birthweight, and that may not be represented in the life-course epigenetic clocks. Studies have found that GAA is associated with increased birth weight and length and that these differences can persist until 9 months of age. These associations reverse at 5 years of age such that greater GAA is associated with lower childhood weight (Bright et al., 2019). Other research has found that epigenetic GAA is associated with various developmental characteristics throughout childhood and adolescence and may be an important index to use as a measure of developmental aging (Simpkin et al., 2016).

Objectives and Hypotheses

In this project, we aim to test the relationship between maternal smoking during pregnancy and GAA at birth. We hypothesize that MSDP is associated with differences in GAA at birth. Secondly, we aim to explore whether dose of exposure (concentration of tobacco biomarkers) is associated with linear increase or decrease in GAA among those that were exposed, using continuous concentrations of cotinine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides (NNAL), which are biomarkers of tobacco use.

Methods

Study Population and Inclusion/Exclusion Criteria

The New Hampshire Birth Cohort Study (NHBCS), a prospective longitudinal cohort study started in 2009, includes data on cord blood DNA methylation, self-report surveys, as well as cotinine and NNAL biomarker measures. The design of the NHBCS has been described before in a 2011 study (Gilbert-Diamond et al., 2011). Our study included 255 NHBCS participants with cord blood DNA methylation and complete exposure data. Eligibility criteria included 18-45 years of age, mentally competent with a singleton parent, English speaking participants whose primary source of residential water was a private well. Women planning to move during their pregnancy were excluded from the study.

Institutional Review Board (IRB) Approval

All participants provided written informed consent before participation according to guidelines set forth by the Committee for the Protection of Human Subjects at Dartmouth. Additional IRB approval was obtained through the Emory University Institutional Review Board and categorized as non-human subjects research.

Cord Blood and DNA Methylation

Cord blood samples were collected upon delivery of the newborn by obstetrical staff members. These samples were processed within 24 hours of collection and then stored at -80° C. DNeasy® blood and tissue kits were used to isolate DNA from the buffy coat fraction. These were bisulfite converted using the EZ DNA Methylation kit (Zymo, Irvine, CA). Epigenome-

wide DNA methylation assessment was carried out on the Infinium HumanMethylationEPIC BeadChip (EPIC), with samples randomized across plates, and includes approximately 860,000 CpG sites (Illumina, San Diego, CA). Microarray processing took place following standard protocols at the Biomedical Genomics Center at the University of Minnesota (Minneapolis, MN). Individual CpG locus methylation status was calculated as the ratio of fluorescent signals from 0 (no methylation) to 1 (complete methylation). Samples and probes with poor detection were excluded, and data were normalized with functional normalization and beta-mixture quantile normalization (Fortin et al., 2014; Teschendorff et al., 2013).

Epigenetic Age Estimation Methods

Two existing epigenetic clocks for gestational age were used to estimate epigenetic gestational age with cord blood DNAm data described above. The Bohlin algorithm predicts gestational age from 96 CpGs that were initially selected by an elastic net regression model (Bohlin et al., 2016). The Knight model found that gestational age can be accurately estimated from DNA methylation of neonatal cord blood and spot samples. This algorithm estimates gestational age using 148 CpG sites selected through elastic net regression in six training datasets and evaluated predictive accuracy in nine testing datasets. They found that DNA methylation gestational age was consistent with gestational age estimates that use established methods (Knight et al., 2016). The primary difference between these two methods is that the Knight method included infants born preterm while the Bohlin method excluded preterm infants. We used the both the Bohlin and Knight algorithms to estimated epigenetic gestational age in our sample. We then estimated GAA by regressing epigenetic gestational age on reported gestational age and extracted the residuals from these models.

Smoking Variables

Maternal cigarette use over the course of pregnancy was collected via self-report with questionnaires, asking for information about history of smoking, current smoking status, and whether the participant smoked during the first and/or second trimester. Additionally, maternal urine specimens were collected between 22-30 weeks of gestation for biomarker analyses. From these urine samples, concentrations of cotinine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides (NNAL) were measured. The cutoff concentration for a positive smoking status using NNAL was 0.1 pmol/ml, while the cutoff concentration for cotinine was 30 ng/ml. In our statistical analyses, described below, we tested for associations with self-reported smoking status via questionnaire, biomarker confirmed smoking status (either cotinine or NNAL greater than the thresholds above), and biomarker confirmed smoking that was congruent with self-report (mother with mismatch between biomarker and self-report were excluded).

Statistical Analysis

We evaluated the relationships between predicted epigenetic age and reported chronological age using scatterplots and Spearman's correlation coefficients. GAA was treated as the dependent variable for all further analyses. We assessed the relationship between GAA and maternal smoking variables using linear regression. We included the following covariates in our regression models to adjust for confounding: maternal age at enrollment, maternal BMI, maternal education level, newborn sex, labor type, and numerous cell types (natural killer cells, CD4 T-cells, CD8 T-cells, granulocytes, monocytes, and red blood cells).

Results

Characteristics of the Study Population

Study participants consisted of 255 mothers above the age of 18 (mean age of 32). 58.8% (n=150) of the newborns were male. The average gestational age at delivery was 39.5 weeks. Labor types included 41.8% (n=105) spontaneous births, 40.6% (n=102) induced births, and 17.5% (n=44) cesarean section births. The percentage of respondents self-reporting their smoking status via survey as "ever smoked" was 11.9% (n=28) and the percentage of respondents reporting "smoked during pregnancy" was 5.8% (n=14). Biomarker data included NNAL and cotinine concentrations from maternal urine samples: 8.2% (n=21) of participants had NNAL concentrations above the lower limit of quantification (LLOQ), while 22.7% (n=58) of participants had cotinine concentrations above the LLOQ. For both NNAL and cotinine concentration, 8.2% (n=21) of participants had concentrations above the minimum threshold for positive smoking status (>30 ng/ml for cotinine and >0.1 pmol/ml for NNAL). Overall, 9% (n=23) of mothers were classified as smokers by at least one of these biomarker thresholds. To account for incongruencies between self-reported smoking status and biomarker status, we created variables indicating whether participants self-report was consistent with their biomarker status. We found that 4.4% (n=13) of participants had biomarker confirmed smoking status but did not self-report as having smoked during pregnancy.

Relationship Between DNA Methylation Age and Chronological Age

Using the Knight algorithm, we found the average predicted gestational age of our cohort to be 38.09 weeks (SD=1.49), while according to the Bohlin algorithm, the average predicted

gestational age of newborns was 41.04 weeks (SD= 1.10). Whereas the average reported gestational age at time of delivery was 39.48 weeks (SD=1.58). Spearman ranked correlation was performed for both Knight and Bohlin algorithms to test the relationship between epigenetic gestational age and reported gestational age (**Figure 1A & 1B**). The rho value for the Knight model was 0.437 (p=2.401e-13), while the rho value for the Bohlin model was 0.633 (p<2.2e-16) demonstrating positive correlations between DNAm predicted and reported gestational ages.

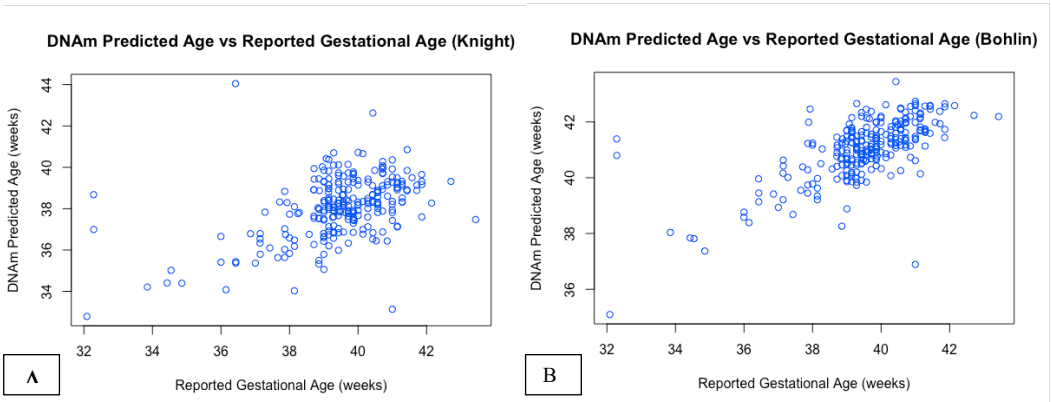

Figure 1: Scatterplots of the relationships between DNAm predicted age (y-axes) and reported gestational age (x-axes) for Knight (A) and Bohlin (B) algorithms

Age Acceleration Associations

We first tested whether GAA was associated with self-reported smoking status (ever vs. never, and smoking during pregnancy vs. no smoking during pregnancy), biomarker-indicated smoking during pregnancy (>30 ng/ml for cotinine and/or >0.1 pmol/ml for NNAL), or concordant self-report and biomarker-indicated smoking status using unadjusted linear regression. We observed a slight decrease in GAA in newborns whose mothers reported smoking ever or smoking during pregnancy, but these confidence intervals for these effects crossed the null (**Table 2**). We then tested whether GAA was associated with biomarker-indicated smoking

status, and again observed lower GAA among smoke-exposed newborns with confidence intervals crossing the null (**Table 2**). Since there was no apparent relationship between GAA with self-reported ever smoking, we focused on biomarker indicated and biomarker concordant smoking status for our secondary analyses.

Table 2. Associations between GAA (via Bohlin & Knight estimators) and maternal smoking status via self-report and biomarker indication.

Smoking Status Determination	**GAA_{Knight}: Slope (95% CI)**	**GAA_{Bohlin}: Slope (95% CI)**
Self-reported ever smoker	-0.08 (-0.60, 0.44)	-0.27 (-0.36, 0.30)
Self-reported MSDP	-0.66 (-1.37, 0.04)	-0.33 (-0.78, 0.12)
Biomarker-indicated MSDP	-0.52 (-1.08, 0.04)	-0.17 (-0.52, 0.18)
Concordant biomarker and self-reported MSDP	-0.89 (-1.74, -0.05)	-0.56 (-1.09, -0.03)

We then examined whether adjustments for potential confounders affected the associations between gestational GAA with biomarker-indicated smoking status. We first examined whether cell types were associated with age acceleration to determine whether some cell proportions should be adjusted for in our regression models. We found that AA was strongly associated with B-cells (Estimate=-12.671, Std Error= 4.605, p=0.006), but not with other cell types. We thus included B-cell proportions as an additional covariate in our adjusted regression models described below. Other confounders were determined a priori, and included maternal age at enrollment, maternal BMI, sex, labor type, and maternal educational attainment as an indicator of socioeconomic status.

We ran four multiple regression models for each of estimate of GAA_{Knight} and GAA_{Bohlin}. First, we adjusted for maternal age at enrollment, maternal BMI, sex, labor type and B-cells, then additionally adjusted maternal educational attainment. We tested for associations with biomarker-indicated MSDP and biomarker-concordant MSDP. All results from these adjusted regression models are reported in **Table 3**, with the most robust results highlighted below. Overall, adjustment for maternal age at enrollment, maternal BMI, sex, labor type, educational attainment, and cell types, improved the associations between MSDP and gestational AA for both exposure definitions and both clocks.

Table 3. Associations between GAA (via Bohlin & Knight estimators) and biomarker indicated smoking during pregnancy with confounder adjustment.

Smoking Status Determination	**GAA_{Knight}: Slope (95% CI)**	**GAA_{Bohlin}: Slope (95% CI)**
Biomarker-indicated MSDP[1]	-0.70 (-1.27, -0.14)	-0.30 (-0.66, 0.06)
Biomarker-indicated MSDP[2]	-0.70 (-1.27, -0.12)	-0.37 (-0.73, -0.01)
Concordant biomarker and self-reported MSDP[1]	-0.95 (-1.75, -0.15)	-0.57 (-1.07, -0.06)
Concordant biomarker and self-reported MSDP[2]	-0.99 (-1.82, -0.15)	-0.74 (-1.27, -0.22)

[1] adjusted for maternal age, maternal BMI, sex, labor type, cell types; [2] adjusted for maternal age, maternal BMI, sex, labor type, cell types, education level

Interestingly, biomarker-concordant smoking status (excluding those whose self-reported smoking and biomarker-indicated smoking status were inconsistent) produced the largest effect sizes and confidence intervals routinely excluded the null for unadjusted and adjusted regression

models. For these biomarker-concordant results, we observed statistically significant decreases in GAA_{Knight} with a beta coefficient of -0.894 (95% CI (-1.743, -0.045)) and in GAA_{Bohlin} (beta coefficient of -0.557, 95% CI (-1.087, -0.027)) in unadjusted models. When adjusting for confounders (maternal age at enrollment, maternal BMI, sex, labor type, and cell types), the observed associations were unperturbed and remained statistically significant for both GAA_{Knight} (beta coefficient of -0.951 (95% CI = -1.752, -0.149) and GAA_{Bohlin} (beta coefficient of -0.567, 95% CI = -1.075, -0.059). Additional adjustment for educational attainment again resulted in stronger effect sizes, for both estimates of GAA and for both definitions of maternal smoking during pregnancy.

Secondary Analyses

We performed exploratory secondary analyses among those with detectable cotinine or NNAL concentrations to explore whether GAA decreases with increasing dose of exposure biomarkers. Since the concentration of biomarkers in study subjects was heavily skewed, log transformations were performed to approximate normality. These analyses were only performed within the subset of participants whose mothers had cotinine (n=58) or NNAL (n=21) concentrations above the LLOQ. Using log-transformed cotinine concentrations, we observed modest decreases in GAA with higher concentrations of the cotinine biomarker (GAA_{Knight} estimate= -0.106, p=0.00861; GAA_{Bohlin} estimate=-0.058, p=0.0363). No statistically significant decrease in AA was observed with NNAL concentration for either AA_{Knight} or AA_{Bohlin}. These results are primarily exploratory given the small sample sizes of mother with detectable biomarker concentrations.

Summary of Results

Overall, our results indicate that GAA was decreased among those newborns who were exposed to tobacco smoke during pregnancy. This was most apparent for biomarker-indicated smoking status, and those who had biomarker confirmed smoking status concordant with their self-reported smoking status. Adjustment for potential confounders did explain our findings, and even increased the strength of our most robust associations. Our exploratory analyses suggest that there may be a relationship between continuous cotinine biomarker concentrations and decreased age acceleration, though these findings need to be explored in larger samples.

Discussion

We aimed to study the relationship between maternal smoking during pregnancy and gestational age acceleration. We found that babies born to mothers who smoke tend to have decreased gestational age acceleration. This relationship was strongest and most consistent for biomarker-indicated and biomarker-concordant definitions of MSDP. When adjustments were made for confounding variables, our findings not only remained statistically significant, but also became stronger. Our results indicated a linear relationship between concentrations of cotinine and gestational age acceleration, though this finding was based on a small sample size.

Several studies have shown epigenetic age acceleration tends to increase with maternal smoking during pregnancy. Simpkin et al found a positive correlation between AA and maternal smoking during pregnancy in both cross-sectional and longitudinal analyses with longitudinal analysis showing a 0.22 year increase in AA per year of life compared to non-smokers (Simpkin et al., 2016). Javed et al similarly found increased epigenetic age acceleration associated with

maternal smoking during pregnancy (Javed et al., 2016). De Prado Bert et al. found that children (~7 years old) whose mothers used tobacco during pregnancy experienced epigenetic age acceleration. This was a dose dependent effect in which longer duration or higher doses of exposure was associated with increased estimates (de Prado-Bert et al., 2021). Whereas Khouja et al found there was no associated between MSDP and GAA (Khouja et al., 2018). Of these studies, only Khouja et al utilized an estimator of GAA (Bohlin et al., 2016; Knight et al., 2016), the other investigators utilized epigenetic clock estimators based on age-associated changes in DNAm after birth (Horvath, 2013; Horvath & Raj, 2018).

Our findings of decreased GAA with maternal smoking during pregnancy are not in alignment with most of these previously reported findings. We believe that this may be due to the novel combination of biomarker data employed in the present study, since most prior studies relied upon self-reported smoking assessments and thus are more prone to misclassification. Additionally, the majority of the previous literature assessed the relationships between epigenetic age acceleration and smoking in adulthood, while only a few have focused on maternal smoking during pregnancy and age acceleration at birth. While age acceleration in adults is likely a marker of accelerated biological aging and associated with degenerative conditions and chronic diseases, the meaning of GAA in newborns is less clear. However, since smoking during pregnancy is known to cause shorter gestational age at birth and increased risk of preterm birth (Soneji & Beltrán-Sánchez, 2019), we posit that the observed smoking-associated decrease in GAA could be related to decreased maturity at birth for a given gestational age. Another study on this topic found that higher GAA may be an indicator of increased maturity at birth, with these babies have larger birth weights, lengths and head circumferences (Khouja et al., 2018), which was confirmed in a separate independent study (Bright et al., 2019). While the clinical and

health significance to the fetus of accelerated gestational age at birth is still unknown, these studies do indicate that it appears to relate to maturity or development.

Our study should be interpreted within the contexts of its strengths and limitations. The most apparent weakness of the present study is the small sample size of smokers within the dataset. This is an important weakness to note as it lowers statistical power and limits our ability to study dose-response with biomarker concentrations. Further studies using a similar combination of exposure variables, especially biomarkers, with larger numbers of smoke-exposed newborns would be of particular benefit. We also performed some analyses with self-reported smoking status. While this measure is subject to misclassification and problems associated with self-reporting information bias (social desirability bias, recall bias, selective recall etc.), it has been shown to be a reliable measure of smoking behavior (Blank et al., 2016; Vartiainen, 2002). It is important to take into account that smoking during pregnancy may be particularly prone to social desirability bias and thus reiterates the importance of verifying this measure with biomarker measures (Boyd et al., 1998). A critical strength of our study is that we did include comprehensive assessment of tobacco use during pregnancy. In addition to self-report, we assessed concentrations of cotinine and NNAL in maternal urine. These biomarkers are particularly robust measures as they are not subject to mis-reported information and allow for exploration of dose response outcomes.

Cotinine is the primary metabolite of nicotine and is often considered the best biomarker of current tobacco smoke exposure (*Biomonitoring Summary | CDC*, 2019). It can be measured in serum, urine, saliva as well as hair and is a preferred marker because it persists in the body longer than nicotine. One drawback of this measure is that non-Hispanic blacks metabolize cotinine more slowly that do non-Hispanic whites, leaving room for discrepancies when comparing cotinine

levels across racial categories. This is unlikely an issue in our study given the lack of racial and ethnic heterogeneity in the NHBCS. Urinary NNAL is a tobacco-specific biomarker and is clearly related to an established lung carcinogen, thereby making it an appropriate measure in tobacco exposure studies (Hecht, 2004). Additionally, this measure is useful for detecting tobacco use for a month or longer and detects more intermittent tobacco exposure than cotinine alone which detects tobacco use in the past 2-4 days (Benowitz et al., 2018). The present study not only incorporated these informative biomarkers but the findings were strongest when using the biomarker thresholds as opposed to self-report. We studied both of the existing estimators for GAA, Bohlin and Knight, in parallel. These are the most appropriate epigenetic clocks for studies of prenatal exposures and cord blood DNA methylation. This is an important difference from the majority of studies of age acceleration associations with MSDP which instead used an epigenetic estimator of age across the entire life course rather than one specific to newborns.

Public Health Significance

While the risks of smoking, including smoking during pregnancy, have been established for many decades, smoking during pregnancy continues to be a common occurrence in many populations throughout the world. The present study offers a unique way to evaluate newborn health via epigenetic age acceleration. While the specific health outcomes are still unelucidated, we offer an important perspective on potential starting points and areas of further research. Previous studies have shown associations between age acceleration and childhood asthma and allergies (Peng et al., 2019) increased BMI, probability of middle-age cardiovascular disease, and increased inflammatory markers (Huang et al., 2019). The health-relevance of GAA requires

additional study, but some evidence suggests that it may correlate with growth and developmental characteristics at birth.

Conclusions and Future Directions

Our study provides supporting evidence that MSDP is associated with decreased GAA. These findings were particularly pronounced for biomarker-indicated and biomarker-concordant definitions of MSDP. While our findings were inconsistent with much of the literature to date, the novel combination of exposure variables using biomarker definitions of MSDP and our focus on gestational epigenetic clocks, rather than life-course epigenetic clocks, likely explains some these discrepancies. Further studies are needed to confirm our observed associations between MSDP and GAA (especially using similar biomarker exposure variables as the present study). Additionally, further studies are needed to better characterize whether health outcomes and developmental characteristics are associated with decreased GAA, and potential points of intervention to prevent adverse health outcomes.

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