**Introduction**

Birth outcomes like birth weight, length and gestational timing are strong predictors of both short- and long-term health. For example, early gestational age at birth predicts the two largest causes of death in premature infants: underdevelopment of mature organs and bronchopulmonary dysplasia, a chronic lung disease that damages alveolar tissue [1, 2]. In addition, the field of the Developmental Origins of Health and Disease (DOHaD) has established that being born small also predicts elevated long-term risk for developing respiratory conditions like idiopathic lung disease and chronic metabolic diseases like hypertension, diabetes, and other cardiovascular diseases [2-6]. Experimental work with animal models shows that restricting prenatal nutrition, or imposing acute stress during pregnancy, replicates many of these long-term outcomes in offspring, showing that gestational conditions can have lasting effects on health in the next generation [7, 8].

While nutrition has received broadest attention for its role in fetal growth, there is growing evidence that the mother’s physiology and metabolism, including systems like stress physiology and inflammation, can impact fetal growth and development operating through effects on gestational conditions like nutrient delivery, oxidative stress or exposure to metabolic or other hormones [9]. As a result, disturbances in the normal levels and amounts of exposure of these biological effectors may produce altered structure, function, and adverse outcomes [10]. As a common example, dysregulation of the hypothalamic-pituitary axis (HPA) during pregnancy is associated with increased levels of maternal cortisol, which elevates risks for premature delivery and low birth weight and can cross the placenta to have direct “programming” effects on fetal metabolism and physiology [11, 12]. Hypertension has been shown to lead to lower birth weights, likely operating through factors like altered blood flow, along with the common co-occurrence of elevated inflammatory cytokines that can suppress growth [13, 14]. Conversely, dysregulated glucose homeostasis, as reflected in uncontrolled diabetes during pregnancy, increases delivery of glucose across the placenta, and can lead to larger than expected newborns with elevated risk of developing obesity and diabetes in as adults [15, 16].

A newly-described set of tools called epigenetic clocks have recently been shown to reflect various domains of maternal physiology and metabolism, and thus could be useful for gauging the intergenerational impacts of chronic maternal physiologic and metabolic dysregulation. Epigenetic clocks are calculated using predictable age-related changes in the epigenome – particularly DNA methylation (DNAm), the methylation of cytosine-phosphate-guanine (CpG) sites on DNA. Although commonly-used epigenetic clocks are notable for their ability to predict one’s chronological age, individuals who appear older epigenetically than their chronological age, a state known as epigenetic age acceleration (EAA), tend to have increased risk for future mortality and to have shorter expectancies. Other clocks have been trained on suites of clinical markers and have been shown to be particularly powerful predictors of life expectancy and the pace of biological aging.

Since epigenetic clocks can be trained on effectively any set of metabolic/physiological processes or states, they are a powerful tool to characterize these states by providing integrative, summary information on a mother’s metabolic and physiological state and measuring the associated “wear-and-tear” on the next generation. One small study (n = 77) among Californian women demonstrated that advanced maternal epigenetic age is associated with early gestational age at birth and low birthweight in offspring, suggesting that epigenetic age may be predictive of adverse fetal outcomes [17]. To date, little is known about the potential for these measures to predict outcomes in a socioeconomically diverse population with greater rates of adverse fetal outcomes.

In this paper, we analyze several prominently used epigenetic clocks, obtained during pregnancies, in relation to longitudinally collected birth outcomes in the offspring of those pregnancies. Data come from the Cebu Longitudinal Health and Nutrition Survey (CLHNS), a cohort study that has followed a large, diverse sample of women and their offspring in metropolitan Cebu City, Philippines for nearly four decades [18]. The present analyses focus on pregnancies of 330 expecting female young adults and their newborns born between 2009 and 2014. We used four published epigenetic clocks to provide complementary information on the mother’s chronic biological dysregulation, as reflected in the degree of accelerated biological aging. EAA using the Levine-DNAmPhenoAge clock (PhenoAge) has been shown to be highly predictive of cardiovascular disease, a poorer likelihood of being free of disease, and to be afflicted with additional morbidities [19]. Acceleration of the Lu-DNAmGrimAge clock (GrimAge) similarly predicts specific cardiovascular conditions, such as hypertension, Type II diabetes, and overall poorer physical functioning [20, 21]. EAA using both the Hannum-Extrinsic Epigenetic Age Acceleration (Hannum-EEAA) and the Horvath-Intrinsic Epigenetic Age Acceleration (Horvath-IEAA) clocks have predicted all-cause mortality [22-25]. We hypothesized that advanced maternal EAA based upon such indices would predict adverse fetal outcomes, including decreased gestational age and measured weight. We further anticipated a gradient of impact, with skinfolds being most labile and sensitive, followed by weight, length and finally, the most canalized outcome of head circumference.

**Methods**

*Study sample and design*

The study data originate from the Cebu Longitudinal Health and Nutrition Survey (CLHNS), a longitudinal survey of 3,080 infants and their mothers who were recruited during their pregnancies between 1983-1984 in Metropolitan Cebu, Philippines. Out of the 1447 original female cohort infants, 823 were interviewed in a later 2009 survey (at ages 25-26). This additional survey tracked new pregnancies among these women between 2009-14. There were 383 who reported pregnancies (28% with 2-3 pregnancies) within the tracking period, yielding 507 pregnancy episodes. Women were visited in-home during pregnancy for anthropometric and questionnaire assessments, along with collection of a dried blood spot (DBS)—capillary whole blood collected on filter paper—for DNAm measurement. A second visit was arranged soon after delivery to obtain additional information from the mothers and to obtain phenotypic measures of their newborns. Newborn anthropometric outcomes included weight, length, head circumference, arm circumference, abdominal circumference, and five skinfold thicknesses (triceps, subscapular, suprailiac, bicep and calf), which were measured in-home by trained interviewers using standardized procedures [reference later] as soon after birth as possible. All research was conducted under conditions of written informed consent, and with approval of the Institutional Review Boards of Northwestern University (Evanston, Illinois), and the Office of Population Studies Foundation (Cebu, Philippines).

*Variable construction*

A composite score of socioeconomic status was measured as a combination of income, education, and assets. Participants reported their annual income from all sources, including in-kind services, and the sale of livestock or other products by household members during the prior year, which were summed to determine total household income. Incomes were log-transformed. Maternal education (in years) was also reported. Participants also reported on ten assets (coded 0, 1) that were selected to capture population-relevant aspects of social class, including electricity, refrigerators, air conditioners, color televisions, cable tv, tape recorders, electric fans, jeepneys, cars, trucks, and owning their residence. In addition, house construction type (i.e., light, mixed, permanent structure) was coded as 0, 1, and 2, respectively. Thus, asset scores ranged from 0 to 13. A principal components analysis was run on log income and assets, along with maternal education, at sample collection. The first component of 70% of the variation, and individual scores for the top component of variation were used as our measure of SES.

Because women were enrolled in the birth outcome sub-study after they were pregnant, we used height and weight measurements collected during prior surveys to estimate pre-pregnancy BMI. We used 2009 BMI when available, and then used 2007 and 2005 data as necessary. Under the assumption that women will tend to maintain a stable position within the population BMI distribution even as the population mean increases with age, we converted all BMIs to age-specific within-sample Z-scores before pooling into a single pre-pregnancy BMI variable. Supporting the validity of this approach, the correlation between Z-scores for BMI measured in 2005 and 2009 was very high (r=0.84). Offspring gestation age was calculated using the time between the last reported menstrual period and infant birth date. Days pregnant at maternal blood sampling was determined by subtracting the time between the blood sample and infant birth date from gestation age. The median and mean interval (in days) between birth and newborn anthropometry measurements were 3 and 4.5 days, respectively, with a range from 1 to 44 days.

*Sample inclusion criteria*

Women for the present study were selected based on reproductive history and sample availability for DNAm measurement. DNAm was measured for a total of 334 women and only women with complete information for all variables were included. For each woman, the last pregnancy during the 2009-2014 tracking period was used unless inadequate DBS sample remained, in which case a blood sample from an earlier pregnancy was used. Fifteen women were missing pre-pregnancy BMI, 2 women were missing data on offspring developmental outcomes, and DNAm for one woman did not pass quality control, and these women were excluded. Analyses were further limited to women with newborns with gestational ages between 32 and 44 weeks, which excluded 5 very premature births, and 10 late deliveries between 45-53. To minimize impacts of the infant’s environment and growth after birth, analyses of infants were limited to those measured within 2 weeks of birth and adjusted for age at measurement in models (this excluded 4 individuals measured more than 2 weeks after birth). After these exclusions, the final sample with all necessary biological and questionnaire data included 297 women singleton births with complete information.

*DNA methylation sample processing and epigenetic clock calculation*

DNA was extracted from dried blood spots (DBS) using PROTOCOL. 750ng of genomic DNA was treated with sodium bisulfite (Zymo EZDNA, Zymo Research, Irvine, CA, USA), and 160ng of converted DNA was applied to the Illumina Infinium MethylationEPIC BeadChip under standard conditions (Illumina Inc., San Diego, CA). Technicians were blind to any information regarding participant characteristics, and samples were randomly assigned to plate, chip, and row. Background subtraction and color correction were performed using Illumina Genome Studio with default parameters. Data were then exported into R for further analysis. Quality control involved first confirming participant sex and replicate status. This was followed by quantile normalization using lumi on all probes including SNP-associated and XY multiple binding probes. To maximize the number of sites available for the epigenetic age calculator, probes with detection p-values above 0.01 were called NA for poor performing samples only, and were otherwise retained. Horvath’s DNAmAge was calculated using an online calculator (http://labs.genetics.ucla.edu/horvath/dnamage/), designed to be generally robust to cell-type differ- ences associated with age. Background-corrected beta values were processed further using the calculator’s internal normalization algorithms.In all cases, maternal epigenetic age acceleration, which is the residual of epigenetic age on chronological age, was used.

*Statistical analysis*

We ran a sequence of multivariate linear regression models designed to assess relationships between maternal epigenetic age acceleration and two fetal outcomes (gestational age and measured weight after birth). Models predicting postnatal outcomes were adjusted for days after birth of anthropometry measurement, gestational age at birth, offspring gender, composite socioeconomic status score. Because birth outcomes are potentially impacted by the mother’s adiposity, we also adjusted for the mother’s pre-pregnancy body mass index (BMI) z-scores. All statistical analyses were conducted using R.

**Results**

* Sample characteristics table?

Yes. Make a table with mean, sd, median, range (min and max) for maternal age at blood draw, weeks pregnant at blood draw, maternal pre-pregnancy BMI (not z-scores if we have those), maternal education in years (if we have it), smoking behavior (y/n). Also, have a slightly different section below with the same metrics for gestational age, infant weight, sex makeup (proportion of males), average age measured.

* Regression Models Summary table

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Outcome | Predictor | b | SE | β | p |
| Gestational Age | IEAA | 0.17 | 0.23 | 0.04 | 0.47 |
|  | EEAA | -0.16 | 0.29 | -0.03 | 0.58 |
|  | PEAA | -0.03 | 0.19 | -0.01 | 0.87 |
|  | GrimAgeAccel | -0.43 | 0.36 | -0.07 | 0.24 |
|  | DNAm ADM | -0.14 | 0.06 | -0.12 | **0.03** |
|  | DNAm B2M | 0.00 | 0.00 | -0.05 | 0.38 |
|  | DNAm Cystatin C | 0.00 | 0.00 | -0.06 | 0.31 |
|  | DNAm GDF | 0.00 | 0.01 | 0.01 | 0.88 |
|  | DNAm Leptin | 0.00 | 0.00 | -0.17 | **0.004** |
|  | DNAm Smoking Pack Years | -0.04 | 0.18 | -0.01 | 0.81 |
|  | DNAm PAI-1 | 0.00 | 0.00 | -0.01 | 0.85 |
|  | DNAm TIMP1 | 0.00 | 0.00 | -0.03 | 0.60 |
|  | DNAm TL | -9.43 | 7.52 | -0.07 | 0.21 |
| Measured Weight | IEAA | 0.00 | 0.01 | 0.04 | 0.51 |
|  | EEAA | -0.01 | 0.01 | -0.08 | 0.15 |
|  | PEAA | 0.00 | 0.01 | -0.03 | 0.58 |
|  | GrimAgeAccel | 0.02 | 0.01 | 0.09 | 0.10 |
|  | DNAm ADM | 0.00 | 0.00 | 0.08 | 0.14 |
|  | DNAm B2M | 0.00 | 0.00 | 0.00 | 0.95 |
|  | DNAm Cystatin C | 0.00 | 0.00 | 0.02 | 0.67 |
|  | DNAm GDF | 0.00 | 0.00 | 0.01 | 0.86 |
|  | DNAm Leptin | 0.00 | 0.00 | 0.01 | 0.79 |
|  | DNAm Smoking Pack Years | 0.01 | 0.01 | 0.08 | 0.12 |
|  | DNAm PAI-1 | 0.00 | 0.00 | 0.01 | 0.82 |
|  | DNAm TIMP1 | 0.00 | 0.00 | 0.05 | 0.37 |
|  | DNAm TL | 0.21 | 0.22 | 0.05 | 0.34 |

* Any scatter plots similar to Ross?

Yes, I believe we decided to present the same plots as Ross – to give an impression of the relationships (or lack thereof in our case).

**Discussion**

I can help with this. We’re going to start with a quick overview of the background (like 1-2 sentences that summarizes the intro. Why did we do this?). Then we quickly mention Ross et al. “a paper among 75 women found x” or something. Mention the Lancaster paper. We sought to test for the effect of maternal biological age on offspring development and replicate previous research expanded this analysis in a larger, more diverse sample of women in the Philippines. We found nothing for any of the major epigenetic clocks, suggesting that maternal cellular aging is not associated with offspring developmental outcomes.

We can start by highlighting some of the strengths of our study. Yet we found nada for most clocks. There were two exceptions. ADM and leptin clocks. Break down the ADM finding. We will need to go into the biology of ADM – what does it mean and how might it be related to pregnancy/birth outcomes. We will then talk about leptin clock. We can think about some potential biological reasons for the relationship between leptin and gestation. But this could also be an artifact. Because we sought to replicate previous work, and with the understanding that multiple clocks pointing in the same direction would be consistent with accelerated cellular aging in mom affecting offspring development, we did not correct for multiple testing. Our finding with leptin is not enough to support that, and the biological pathways to help explain how leptin would lead to offspring outcomes are not obvious.

**Conclusions**

**References**

1. Escobar, G.J., R.H. Clark, and J.D. Greene, *Short-term outcomes of infants born at 35 and 36 weeks gestation: we need to ask more questions.* Semin Perinatol, 2006. **30**(1): p. 28-33.

2. Patel, R.M., *Short- and Long-Term Outcomes for Extremely Preterm Infants.* Am J Perinatol, 2016. **33**(3): p. 318-28.

3. Barker, D.J., *Birth weight and hypertension.* Hypertension, 2006. **48**(3): p. 357-8.

4. Knop, M.R., et al., *Birth Weight and Risk of Type 2 Diabetes Mellitus, Cardiovascular Disease, and Hypertension in Adults: A Meta-Analysis of 7 646 267 Participants From 135 Studies.* J Am Heart Assoc, 2018. **7**(23): p. e008870.

5. Mohseni, R., et al., *Birth Weight and Risk of Cardiovascular Disease Incidence in Adulthood: a Dose-Response Meta-analysis.* Curr Atheroscler Rep, 2020. **22**(3): p. 12.

6. Gluckman, P.D. and M.A. Hanson, *Living with the past: evolution, development, and patterns of disease.* Science, 2004. **305**(5691): p. 1733-6.

7. Bertram, C.E. and M.A. Hanson, *Animal models and programming of the metabolic syndrome.* Br Med Bull, 2001. **60**: p. 103-21.

8. Langley-Evans, S.C., *Metabolic programming in pregnancy: studies in animal models.* Genes Nutr, 2007. **2**(1): p. 33-8.

9. Entringer, S., et al., *Fetal programming of body composition, obesity, and metabolic function: the role of intrauterine stress and stress biology.* J Nutr Metab, 2012. **2012**: p. 632548.

10. Entringer, S., *Impact of stress and stress physiology during pregnancy on child metabolic function and obesity risk.* Curr Opin Clin Nutr Metab Care, 2013. **16**(3): p. 320-7.

11. Diego, M.A., et al., *Maternal psychological distress, prenatal cortisol, and fetal weight.* Psychosom Med, 2006. **68**(5): p. 747-53.

12. Field, T. and M. Diego, *Cortisol: the culprit prenatal stress variable.* Int J Neurosci, 2008. **118**(8): p. 1181.

13. Entringer, S., C. Buss, and P.D. Wadhwa, *Prenatal stress and developmental programming of human health and disease risk: concepts and integration of empirical findings.* Curr Opin Endocrinol Diabetes Obes, 2010. **17**(6): p. 507-16.

14. LaMarca, B.D., et al., *Inflammatory cytokines in the pathophysiology of hypertension during preeclampsia.* Curr Hypertens Rep, 2007. **9**(6): p. 480-5.

15. Fraser, D., et al., *Factors influencing birth weight in newborns of diabetic and non-diabetic women. A population based study.* Eur J Epidemiol, 1990. **6**(4): p. 427-31.

16. Gillman, M.W., et al., *Maternal gestational diabetes, birth weight, and adolescent obesity.* Pediatrics, 2003. **111**(3): p. e221-6.

17. Ross, K.M., et al., *Epigenetic age and pregnancy outcomes: GrimAge acceleration is associated with shorter gestational length and lower birthweight.* Clin Epigenetics, 2020. **12**(1): p. 120.

18. Adair, L.S., et al., *Cohort profile: the Cebu longitudinal health and nutrition survey.* Int J Epidemiol, 2011. **40**(3): p. 619-25.

19. Levine, M.E., et al., *An epigenetic biomarker of aging for lifespan and healthspan.* Aging (Albany NY), 2018. **10**(4): p. 573-591.

20. Hillary, R.F., et al., *Epigenetic measures of ageing predict the prevalence and incidence of leading causes of death and disease burden.* Clin Epigenetics, 2020. **12**(1): p. 115.

21. Lu, A.T., et al., *DNA methylation GrimAge strongly predicts lifespan and healthspan.* Aging (Albany NY), 2019. **11**(2): p. 303-327.

22. Chen, B.H., et al., *DNA methylation-based measures of biological age: meta-analysis predicting time to death.* Aging (Albany NY), 2016. **8**(9): p. 1844-1865.

23. Breitling, L.P., et al., *Frailty is associated with the epigenetic clock but not with telomere length in a German cohort.* Clin Epigenetics, 2016. **8**: p. 21.

24. Marioni, R.E., et al., *DNA methylation age of blood predicts all-cause mortality in later life.* Genome Biol, 2015. **16**: p. 25.

25. Tekola-Ayele, F., et al., *Sex differences in the associations of placental epigenetic aging with fetal growth.* Aging (Albany NY), 2019. **11**(15): p. 5412-5432.