**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.

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**Methods**

*Study population*

The study data originated 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 biomarker measurement. A second visit was arranged soon after delivery to obtain additional information from the mothers and to obtain phenotypic measures of their newborns. This 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).

*Sample inclusion criteria*

Newborn anthropometric outcomes in these analyses included weight, length, head circumference, arm circumference, abdominal circumference, and sum of 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. The median and mean interval (in day) between birth and newborn anthropometry measurements were 3 and 4.5 days, respectively, with a range from 1 to 44 days. 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 17 individuals measured more than 2 weeks after birth). Analyses were further limited to newborns born with gestational ages between 32 and 44 weeks, which excluded 5 very premature births and 2 implausibly late deliveries of around 46 weeks. In addition, only the oldest births for each of the women were included. Finally, any pregnancies with any missing maternal epigenetic data or fetal measurement data were also excluded. After these exclusions, the final sample with all necessary biological and questionnaire data included 299 relatively healthy and unique singletons,

*Maternal covariates*

In each model, the gestational age at birth, fetal age at anthropometric measurement, fetal sex were all adjusted for. Because birth outcomes are potentially impacted by the mother’s adiposity, we also adjusted for the mother’s pre-pregnancy body mass index (BMI). Maternal socioeconomic status was measured using a pregnancy household assets scale reflecting whether the family had electricity, owned their home, owned an air conditioner, refrigerator, TV, vehicle and other appliances assessed, and a measure of household income that tallies all sources of income within the household (Adair et al 2011). 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).

*Genome-wide DNA methylation analysis*

DNA was extracted from whole blood and assayed for DNA methylation by the UCLA Neurosciences Genomics Core using the Illumina Infinium HumanMethylation450 BeadChip (Illumina, Inc., San Diego, CA; 485, 577 CpG sites). DNA methylation data were pre-processed as per standard protocols.

*Calculation of epigenetic clocks*

The epigenetic age of the mothers emanating from their blood sample was calculated using previously established methods (cite later) and algorithms through the online DNA methylation calculator. The four primary epigenetic clocks were *intrinsic epigenetic age acceleration (IEAA), extrinstic epigenetic age acceleration (EEAA), phenotypic epigenetic age acceleration (PEAA), and GrimAgeAccel*. *IEAA* examines the intrinsic biological age of immune cells but does not depend on age-related changes in immune cells in the blood. *EEAA* captures immune cell biological age due to both intrinsic immune cell age and changes in immune cell populations in the blood. *PEAA* is determined using the Levine Method, which uses sites selected due to associations with phenotypic age indicators and chronological age. *GrimAgeAccel* is a marker enriched for DNA methylation sites that are surrogate markers for blood plasma proteins related to mortality. DNAm PAI-1, DNAm ADM, DNAm, B2M, DNAm cystatin C, DNAm GDF, DNAm leptin, DNAm TIMP1, and DNAm smoking pack years serve as these surrogate DNA methylation markers.

*Statistical analysis*

All statistical analyses were conducted using R. We ran a sequence of multivariate linear regression models designed to assess relationships between two fetal outcomes (gestational age and measured weight after birth) and the previously epigenetic age indices. Models predicting postnatal outcomes were adjusted for days after birth of anthropometry measurement, gestational age at birth, offspring gender, socioeconomic status, and pre-pregnancy BMI z-scores.

**Results**

* Sample characteristics table?
* 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?

**Discussion**

**Conclusions**