Associations between Maternal Psychosocial Stress, DNA Methylation, and Newborn Birth Weight Identified by Investigating Methylation at Individual, Regional, and Genome Levels

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ABSTRACT

Stress is known to affect health throughout life and into future generations, but the underlying molecular mechanisms are unknown. We tested the hypothesis that maternal psychosocial stress influences DNA methylation (DNAm), which in turn impacts newborn health outcomes. Specifically, we analyzed DNAm at individual, regional, and genome-wide levels to test for associations with maternal stress and newborn birth weight. Maternal venous blood and newborn cord blood (n = 24 and 22, respectively) were assayed for methylation at ~450,000 CpG sites. Methylation was analyzed by examining CpG sites individually in an epigenome-wide association study (EWAS), as regional groups using variably methylated region (VMR) analysis in maternal blood only, and through the epigenome-wide measures using genome-wide mean methylation (GMM), Horvath's epigenetic clock, and mitotic age. These methylation measures were tested for association with three measures of maternal stress (maternal war trauma, chronic stress, and experience of sexual violence) and one health outcome (newborn birth weight). We observed that maternal experiences of war trauma, chronic stress, and sexual assault were each associated with decreased newborn birth weight ($p < 1.95 \times 10^{-7}$ in all cases). Testing individual CpG sites using EWAS, we observed no associations between DNAm and any measure of maternal stress or newborn birth weight in either maternal or cord blood, after Bonferroni multiple testing correction. However, the top-ranked CpG site in maternal blood that associated with maternal chronic stress and sexual violence before multiple testing correction is located near the *SPON1* gene. Testing at a regional level, we found increased methylation of a VMR in maternal blood near *SPON1* that was associated with chronic stress and sexual violence after Bonferroni multiple testing correction ($p = 1.95 \times 10^{-7}$ and 8.3×10^{-6}, respectively). At the epigenomic level, cord blood GMM was associated with significantly higher levels of war trauma (p = 0.025) and was suggestively associated with sexual violence (p = 0.053). The other two epigenome-wide measures were not associated with maternal stress or newborn birth weight in either tissue type. Despite our small sample size, we identified associations even after conservative multiple testing correction. Specifically, we found associations between DNAm and the three measures of maternal stress across both tissues; specifically, a VMR in maternal blood and GMM in cord blood were both associated with different measures of maternal stress. The association of cord blood GMM, but not maternal blood GMM,

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KEY WORDS: WAR TRAUMA, DEMOCRATIC REPUBLIC OF CONGO, EPIGENETIC CLOCK, VARIABLY METHYLATED REGION, EPIGENOME-WIDE ASSOCIATION STUDY, STRESS EPIGENETICS, SEXUAL VIOLENCE.

Human Biology, Spring 2019, v. 91, no. 2., pp. 117–131. doi: 10.13110/humanbiology.91.2.04.

with maternal stress may suggest different responses to stress in mother and newborn. It is noteworthy that we found associations only when CpG sites were analyzed in aggregate, either as VMRs or as a broad summary measure of GMM.

Psychosocial stress, the physical and psychological stress resulting from social forces, has a well-established relationship to human health (Lehrner and Yehuda 2018; D'Andrea et al. 2011; Massart et al. 2014; Jawaid et al. 2018). Psychosocial stress can result from either chronic stressors, such as daily experiences of unfair treatment (Quinlan et al. 2016), or acute stressors that leave lasting psychological effects, such as exposure to conflict-related violence (Dajani et al. 2018). Understanding how these stress exposures impact biology, and the mechanisms by which this occurs, is key to fully assessing the impact of psychosocial stress.

The molecular mechanisms by which stress affects biology remain to be fully elucidated. In recent years, DNA methylation (DNAm) has garnered increasing attention as a possible mechanism to translate stressful experiences into altered health outcomes. DNAm is a process by which a methyl group is added to a cytosine nucleotide, most commonly one located next to a guanine (called a CpG site). Methylated CpGs are referred to as epigenetic marks, whose presence or absence acts as a genomic regulator that influences cellular biology, development, and disease (Smith and Meissner 2013; Cedar and Bergman 2009; Lister et al. 2009; Roadmap Epigenomics Consortium et al. 2015). Studies have linked DNAm changes to numerous psychosocial stressors, including socioeconomic status, depression, childhood maltreatment, and learned fear experiences tied to posttraumatic stress disorder (PTSD; Braithwaite et al. 2015; Appleton et al. 2013; Cao-Lei et al. 2014; Zovkic and Sweatt 2013; Barfield et al. 2014). There is also evidence that such changes in DNAm may be transmitted to future generations, such as a mother's stressful experience resulting in offspring exposure *in utero* (Braithwaite et al. 2015; Cao-Lei et al. 2014; Mulligan et al. 2012; Heijmans et al. 2008; Tobi et al. 2014).

A primary function of DNAm is to facilitate and maintain cell type differentiation (Houseman et al. 2012), or to aid in producing different cell types among populations of cells with the same genomic sequence. This process of cell differentiation (controlled primarily by gene regulation and partly by DNAm) begins early in embryogenesis, when parental DNAm patterns are erased and new ones are established via a set of proteins known as the methylation complex (Hata et al. 2002; Fatemi et al. 2002). Many of these same proteins then act to maintain, and possibly alter, DNAm patterns throughout development and into adulthood. Changes in DNAm are often associated with changes in gene expression, or the amount of messenger RNA resulting from a gene. The nature of this effect is complex, and although promoter methylation is often associated with decreased expression, the effect of DNAm at other genomic locations, such as in the gene body or at enhancers, can be associated with either increased or decreased gene expression, depending on the specifics of the region (Wagner et al. 2014).

Thus, any changes in DNAm due to psychosocial stress will occur in the context of these larger cell type effects, and the challenge is to detect such changes above and beyond this natural variation. As such, it is generally accepted that the impact of psychosocial stress on DNAm will be small and possibly restricted to specific loci or parts of the genome (Mulligan 2016). It also means that differences in how we conceptualize and analyze DNAm may influence our results. When considering the factors above, there are at least three general possibilities regarding how psychosocial stress may impact DNAm and how DNAm may impact gene expression, resulting in altered health outcomes:

1. Site-specific methylation changes are made at individual CpG sites.
2. Specific genomic regions are affected, meaning groups of CpG sites are altered together in response to a stressor.
3. A generalized response to stress results in changes across the epigenome, altering CpG sites at multiple, disparate locations.

These concepts are not mutually exclusive; combinations are possible. They also stand in

contrast to candidate gene approaches, which we have performed previously and discuss later. Unlike candidate gene approaches that are teleological in nature, due to their selection being based on gene product function, the concepts investigated in this study represent distinct mechanistic possibilities for DNAm itself being affected and are unbiased with respect to gene function. Each concept is associated with specific classes of analytical methods:

(1) The investigation of individual CpG sites is commonly explored using an epigenome-wide association study (EWAS). In an EWAS, each site is tested individually for association with the variables of interest. An EWAS is the most common form of large-scale epigenetic analysis.

(2) Genomic regions can be explored via multiple methods, depending on the nature of the data available (Clukay et al. 2018; Jaffe et al. 2012). One particularly powerful method is the variably methylated region (VMR) technique outlined by Garg et al. (2018). Based on the concept that genomic regions exhibiting higher interindividual variability are more likely to show an association with environment, VMRs are defined as sections of the genome with at least three CpG sites in the upper 5th percentile of variability within the sample, as measured by standard deviation. This technique allows for a more focused approach based upon theory and data, thus increasing power.

(3) Epigenome-wide measures of DNAm can include simple calculations, such as genome-wide mean methylation (GMM), or more complex measures, such as the epigenetic clock developed by Horvath (2013) to measure biological aging or the mitotic age measure developed by Youn and Wang (2018) to measure number of cell divisions. Changes in broad measures, such as GMM, which measures the average methylation level of all autosomal CpG sites (e.g., ~400,000 sites on the Illumina Human-Methylation 450 Bead Chip), capture epigenetic variation across the genome in one measure and can be seen as potential indicators of cellular stress or dysregulation (Carnell and Goodman 2003). The epigenetic clock, a weighted average of ~400 CpG sites throughout the genome associated with age, is an estimate of methylation age calibrated to chronological human aging in those tissues. Initial iterations of the clock were purposefully related to some changes in cell type, as cell composition can change with age (Horvath 2013), but later versions were rendered independent of cell type (Horvath et al. 2016, 2018). Individuals exhibiting increased epigenetic age compared to their chronological age, known as epigenetic age acceleration, may have an increased risk for age-related diseases (Horvath et al. 2016). In contrast, mitotic age estimates reflect cellular turnover or the average number of cell divisions a population of cells has undergone, rather than an individuals' chronological age. Different tissues from the same individual can have significantly different mitotic ages, depending on how quickly cells divide within that tissue. While mitotic age is a recently developed measure, epigenetic age has been previously observed to associate with stress in multiple studies (Zannas et al. 2015; Verhoeven et al. 2018; Fiorito et al. 2017). However, because the precise manner in which DNAm acts in a cell is unknown, both measures may be of interest when studying psychosocial stress.

Here, we continue an ongoing study of the effects of psychosocial stress on mother-infant dyads from the Democratic Republic of the Congo (DRC). Previously, we tested candidate genes and reported associations between maternal stress and DNAm in multiple genes in mother-infant dyads (Kertes et al. 2016, 2017; Montoya-Williams et al. 2018; Rodney and Mulligan 2014). Specifically, we reported associations connecting both maternal stress and birth weight to methylation at *NR3C1*, a gene involved in the stress response, in newborn cord blood (Mulligan et al. 2012; Kertes et al. 2016). We also found associations between DNAm in the methylation complex genes and GMM in mothers, suggesting possible avenues for genome-wide changes in methylation as a result of stress (Clukay et al. 2018).

Collectively, our previous results suggest that DNAm patterns change in response to maternal stress and that associations between DNAm and birth weight also exist. However, the relationship between stress-associated variation in DNAm and birth weight remains unclear. We have continued to use this mother-infant dyad framework to test for associations between maternal stress and methylation variation in mothers and their newborns. Here we used the three methodologies outlined above: EWAS, VMRs in maternal blood, and epigenome-wide summary measures. Methylation was tested for association with two previously constructed

summary variables of maternal stress, war trauma and chronic stress (Kertes et al. 2016, 2017), and an additional variable related to sexual violence due to previous findings indicating the salience of the stressor (Mulligan 2016). Lastly, these measures of methylation were also compared to birth weight as a health outcome.

Materials and Methods

Sample and Data Collection

Sample collection and descriptions of sociocultural data for this project are discussed in detail in Rodney and Mulligan (2014). In brief, this pilot phase of the project recruited 25 mother-infant dyads from HEAL Africa Hospital in Goma, DRC. Recruitment was conducted in the summer of 2010, with mothers being interviewed and blood samples taken shortly after delivery. All women who gave birth during that time period were eligible for inclusion in the study. The study was approved by the Western Institutional Review Board (Olympia, WA; project no. 20100993).

Anthropometric measurements, including birth weight, were collected at the time of birth. Venous blood was collected from mothers shortly after birth, and cord blood was collected immediately after birth. DNA was extracted using QIAamp DNA Mini Kits (Qiagen, cat. no. 51304) per manufacturer specifications. Sociocultural data concerning maternal stress exposures were collected using semistructured interviews and standardized surveys based on Brunet et al. (2001). The beginning of the interview was conversational in nature to build rapport, followed by administration of the survey. Survey instruments were translated into the Congolese dialect of Swahili and modified for use in the DRC to ensure they addressed cultural stressors relevant to the population. A list of questions asked, translated into English, can be found in Supplementary Table S1 (also available on Mendeley Data, http://doi.org/10.17632/wwgw2ct2wv.1).

Measures of Maternal Stress

Summary measures of stress were constructed as described in Kertes et al. (2016). In sum, factor analysis was used to identify two major factors explaining 55% of the variance. All but two questions within those factors contributed to internal consistency; the exceptions were in-law stress and a question regarding cowives, both of which were removed. After processing for internal consistency using Cronbach's α ($\alpha > 0.8$ for each measure after removal of inconsistent questions), the two factors were identified as representing chronic stress and war trauma.

In addition to the two summary stress measures, a third measure of severe sexual violence, reflecting personal experience of rape, was also tested. Sexual violence was commonly used as a weapon of war in the DRC, and we have previously reported associations between this measure and birth weight (Mulligan 2016). We asked mothers whether she had experienced rape in the past or during the pregnancy, whether she had conceived a child previously due to rape, and whether the current pregnancy was due to rape. Each "yes" answer increased the score by 1, resulting in a maximum possible score of 4 and a minimum possible score of 0.

To view all of the sample characteristics, stress measures, and covariates, see Supplementary Table S2 (also available on Mendeley Data, http://doi.org/10.17632/wwgw2ct2wv.1).

Generation and Processing of DNAm Data

DNAm was assayed using the Illumina HumanMethylation 450 Bead Chip per the manufacturer's specifications. The unprocessed methylation data are available on the Gene Expression Omnibus (GEO accession no. GSE54399). Due to space constraints on the chip, only 24 of the dyads were assayed. All internal control and quality probes were run on the chip as part of the standard processing using the meffil packages, including background correction and removal of batch effects. Maternal and cord blood samples were randomized across the chip. Additionally, two cord samples were later found to be contaminated with adult venous blood and were removed from the analysis.

All data processing was performed using R (R Development Core Team 2015), unless otherwise noted. While original processing reported in Rodney and Mulligan (2014) was conducted using the minfi package developed by Aryee et al. (2014), advancements in processing tools, batch correction, and normalization prompted us to reprocess the data for the present study using the meffil package

(Min et al. 2018). Coding and specific parameters used were based on those recommended by the package. Both the code used and the plate layout necessary for running the code can be found in Supplementary File S1 and Supplementary Table S3, respectively (also available on Mendeley Data, http://doi.org/10.17632/wwgw2ct2wv.1). Beta values were normalized using functional normalization via the meffil package. After normalization, CpG sites were masked (i.e., removed) based on recommendations for the African superpopulation by Zhou et al. (2017), resulting in a final total of 412,117 CpG sites.

After processing, cell type proportions for maternal blood and cord blood were estimated separately using the meffil package with reference data sets based on Houseman et al. (2012) and de Goede et al. (2015), respectively. Principal component analysis was performed on cell type proportions and principal components accounting for 95% or more of the variance were included as covariates in all subsequent analyses to account for cell type heterogeneity. This resulted in four cell-type principal components being included as covariates in relation to maternal blood, and five when testing cord blood, due to the increased number of cell types in cord blood (e.g., nucleated red blood cells). Surrogate variables for batch were identified using the meffil.ewas() function, two in maternal blood and three in cord blood, using the option sva = T, in meffil. These surrogate variables were also included as covariates for all analyses related to methylation. Additional covariates for each tissue type are defined below.

Analyses of DNAm

All analyses were conducted in R (R Development Core Team 2015), unless otherwise noted. Associations in maternal blood and cord blood were tested separately for all analyses. Tests of association with maternal blood methylation were corrected for maternal age and tests of association with cord blood methylation were corrected for infant sex. All tests of association with birth weight, regardless of tissue, were corrected for infant sex.

Epigenome-wide Association Study

EWAS analyses were conducted using the meffil.ewas() function native to meffil. For EWAS analyses, the multiple testing threshold was calculated using Bonferroni correction based on the number of individual sites tested ($0.05/412{,}360$ sites $= 1.21 \times 10^{-7}$).

VMR Identification

VMRs in maternal blood were identified using the requirements and scripts described by Garg et al. (2018). CpG sites were marked as part of a VMR using a sliding, 1,000-bp window. Two criteria were required for the CpG sites within a window to be marked as part of a VMR. First, at least three CpG sites within the window were required to be in the top 5% of variability within our sample, as measured by standard deviation. Second, at least half of the total sites within the window needed to fulfill these criteria in order to ensure that a majority of sites within the region actually varied within the sample. The CpG site exhibiting the highest variance within each VMR was then chosen as a representative site and tested for association with the variables of interest using the meffil.ewas() function in meffil. Since separation by sex was necessary for VMR identification in order to prevent confounding, there was insufficient variation among cord blood samples to identify or test VMRs for association.

Epigenome-wide Measures of DNAm

Three summary measures of methylation were calculated in this study: GMM, Horvath's epigenetic clock (Horvath et al. 2018), and mitotic age (Youn and Wang 2018). GMM was calculated as an individual's average level of methylation for all autosomal CpG sites after data processing and filtering. Since GMM is an average across all sites, even small changes in GMM may reflect large shifts in gene regulation. Horvath's epigenetic clock for skin and blood cells, known as the skin and blood clock, reflects changes in the epigenome associated with chronological aging in those tissues. This measure was developed specifically for use in skin and blood cells to compensate for inaccuracies of the pan-tissue clock due to fibroblasts (Horvath et al. 2018). Furthermore, the skin and blood clock has been specifically shown to exhibit higher accuracy and less error when measuring cord blood samples, which our study examines. The measure was calculated for each individual using filtered methylation values and the R code provided by Horvath et al. (2018). Mitotic age was calculated using filtered methylation values and the R code

Table 1. Summary of Maternal (*n* = 24) and Newborn (*n* = 22) Characteristics

Variable	Mean (SD) or *n*
Mothers	
Age	26.9 (5.50)
War trauma	1.58 (2.21)
Chronic stress	6.54 (6.32)
Sexual violence	0.625 (1.09)
Newborns	
Males	12
Females	10
Birth weight (lbs.)	7.00 (1.69)

Table 2. Associations between Maternal Stress and Newborn Birth Weight Using Simple Regression

Maternal Stress Measure	β (lb.)	R^2	*p*-Value
War trauma	−0.49	0.40	9.0×10^{-4}
Chronic stress	−0.18	0.46	3.0×10^{-4}
Sexual violence	−0.91	0.35	2.3×10^{-3}

Data are effect estimates (β), variance explained (R^2), and *p*-value estimates for univariate linear models testing the effect of each exposure or stressor on the outcome, birth weight. Birth weight was not transformed because it was statistically normal, with a Shapiro's *W*-statistic of 0.96 ($p = 0.42$). Beta estimates reflect pounds of change per unit stress.

provided by Youn and Wang (2018). The measure uses a probability model to calculate the average number of cell divisions based on reference data. There is an offset in this measure, meaning that mitotic age values should not be taken as the absolute number of cell divisions. However, the offset is constant across samples, meaning the samples remain internally comparable.

Results

Sample Characteristics

The average age of study participants was 26.9 years old, and average birth weight was 7.00 lbs (Table 1). Stress was estimated using three summary statistics: war trauma, chronic stress, and sexual violence. War trauma, a measure of war-related stressors, including exposure to violent conflict, exposure to sexual violence, and refugee status, had an average score of 1,.58 with a range from 0 to 8. Chronic stress, a measure of daily stressors, including limited access to adequate food, difficulty paying bills, and being ashamed to cry, had an average score of 6.54, with a range from 0 to 18. For sexual violence, seven mothers (29%) had suffered a personal experience of rape, with scores ranging from 0 to 3. This prevalence is comparable to that observed in the United States, where an estimated one in five women will experience rape in their lifetime (Smith et al. 2018).

Associations between Maternal Stress and Newborn Birth Weight

The effect of maternal stress on newborn health outcomes is well known (Lehrner and Yehuda 2018; D'Andrea et al. 2011; Massart et al. 2014; Jawaid et al. 2018). We have previously reported an association between maternal psychosocial stress and newborn birth weight in this sample using slightly different measures of maternal stress (Mulligan et al. 2012). Here we tested three measures of maternal stress (war trauma, chronic stress, and sexual violence) for association with newborn birth weight. All three measures negatively correlated with birth weight and explained between 35% and 46% of the total variation in birth weight (Table 2). These associations constitute the foundation we used to test DNAm as one possible mechanism by which maternal stress impacts health outcomes.

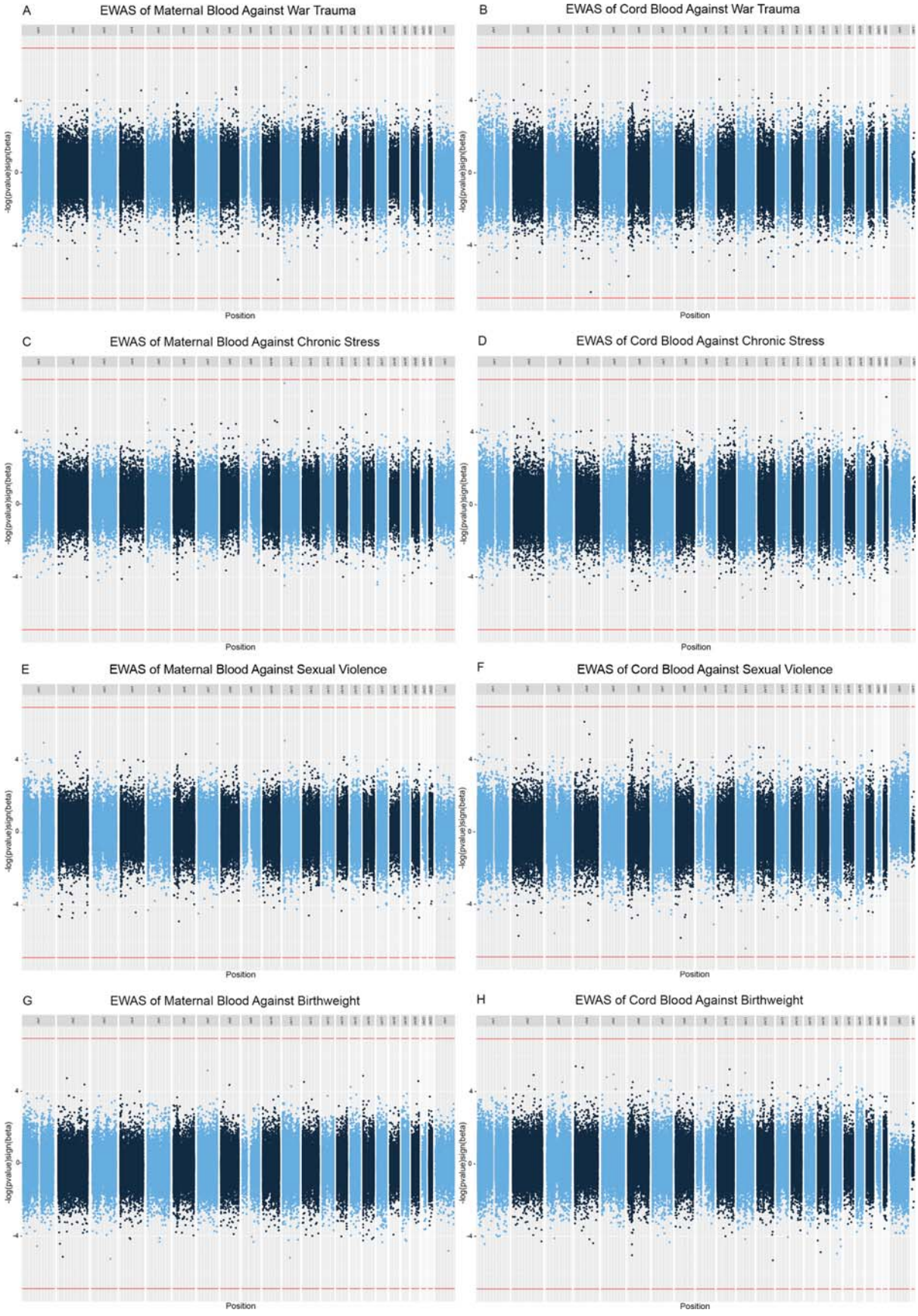
Analysis of DNAm Associations with Maternal Stress and Birth Weight

To test for an effect of maternal stress on DNAm and an effect of DNAm on newborn birth weight, we tested the DNAm data using three different methodologies: (1) EWAS to test individual CpG sites, (2) VMR analyses to test methylation in specific regions, and (3) methylation summary measures to test epigenome-wide changes in methylation.

EWAS as a Measure of Site-Specific DNAm

EWAS was used to test each CpG site individually for association with maternal stress and newborn

FIGURE 1 (*opposite*). Double-sided Manhattan plot of maternal war trauma (A, B), chronic stress (C, D), sexual violence (E, F), and newborn birth weight (G, H) in relation to maternal and cord blood methylation, respectively. Direction of points from zero represents the sign of the β-value for the association. Chromosomes are differentiated by alternating colors and are in numeric order 1–22, followed by the sex chromosomes. Red lines represent the Bonferroni threshold after multiple testing correction (0.05/412,360 CpG sites = 1.21e-7).


A
EWAS of Maternal Blood Against War Trauma
B
EWAS of Cord Blood Against War Trauma
C
EWAS of Maternal Blood Against Chronic Stress
D
EWAS of Cord Blood Against Chronic Stress
E
EWAS of Maternal Blood Against Sexual Violence
F
EWAS of Cord Blood Against Sexual Violence
G
EWAS of Maternal Blood Against Birthweight
H
EWAS of Cord Blood Against Birthweight
-log(pvalue)sign(beta)
Position

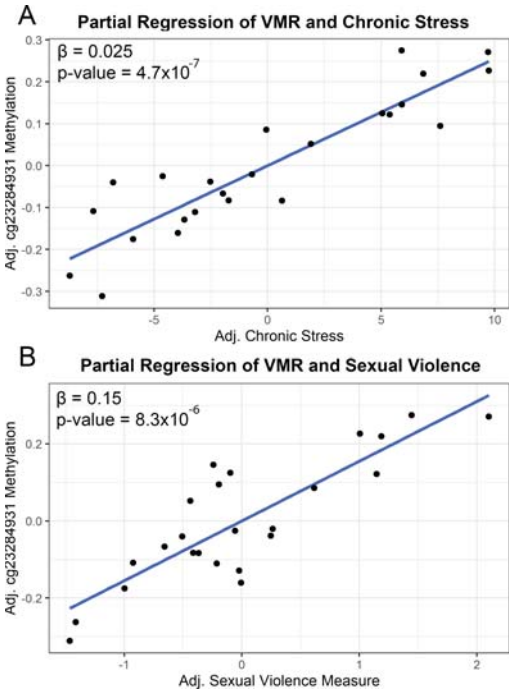


FIGURE 2. Partial regression plots illustrating the relationships between the 150th maternal VMR representative site (cg23284931) and maternal chronic stress (A) and maternal experience of sexual violence (B). All graph values were adjusted for cell composition, batch effects, and maternal age. The significance threshold was calculated using Bonferroni correction based on the total number of VMRs identified (0.05/885 CpG sites = 5.65e-5). The β- and *p*-values are those determined by the model implemented in meffil for the corresponding association.

birth weight. After multiple testing correction (Bonferroni threshold = 1.21×10^{-7}), no CpG sites in maternal or cord blood were individually associated with maternal war trauma, chronic stress, or sexual violence (Figure 1A–F). In addition, no significant associations were identified between birth weight and individual CpG sites in either maternal venous blood or newborn cord blood (Figure 1G,H). The CpG site with an association closest to our confidence threshold (cg23284931; $p = 1.95 \times 10^{-7}$) was observed in maternal blood with maternal chronic stress as the exposure (Figure 1C). The gene associated with this region, *SPON1*, has been previously associated with changes to both DNAm and gene expression in association with PTSD (Mehta et al. 2013), as well as with vitamin D levels, Alzheimer's disease, brain connectivity, and numerous blood protein levels (Manousaki et al. 2017; Sherva et al. 2014; Jahanshad et al. 2013; Sun et al. 2018). This CpG site is also the top-ranked site in maternal blood with maternal experience of sexual violence as the exposure ($p = 8.3 \times 10^{-6}$).

VMR Analysis as a Measure of Regional DNAm

VMR analysis identifies highly variable regions of methylation in which multiple CpG sites are changing within the particular set of samples being tested. This variability increases the likelihood of a region being sensitive to environmental stressors. Since VMR analysis requires separation by sex for VMR identification, there was sufficient sample size to accurately determine variance only in the maternal blood samples.

VMR analysis identified a total of 885 regions in maternal blood (for a list of all VMRs and constituent sites, see Supplementary Table S4; also available on Mendeley Data, http://doi.org/10.17632/wwgw-2ct2wv.1). Of these VMRs, 549 contained CpG sites previously identified in whole-blood VMRs by Garg et al. (2018). Using the CpG site with the highest variability for each VMR as a representative, each VMR was then tested for association with maternal stress and newborn birth weight.

After multiple test correction for the number of VMRs identified (Bonferroni threshold = 0.05/885 = 5.65×10^{-5}), two associations were observed in maternal venous blood with the same CpG site, cg23284931. The VMR represented by CpG site cg23284931 associated with maternal chronic stress with an effect estimate of 0.025 per unit of stress ($p = 1.95 \times 10^{-7}$; Figure 2A) and associated with experience of sexual violence with an effect estimate of 0.15 per point of the sexual violence measure ($p = 8.3 \times 10^{-6}$; Figure 2B). The VMR itself occurs near the *SPON1* gene and consists of 10 CpG sites, all of which are positively correlated with one another (correlation coefficient > 0.45 in all cases). The representative CpG site also exhibited the strongest potential association between the same variables in the EWAS analyses described above: methylation at cg23284931 in maternal blood associated with both chronic stress and experience of sexual violence before multiple testing correction. In the VMR analysis, no tests using birth weight or maternal war trauma met the Bonferroni confidence threshold for significance.

GMM and Methylation Ages as Epigenome-wide Measures of DNAm

Three epigenome-wide measures of DNAm were tested: GMM, the epigenetic clock for skin and blood cells (Horvath et al. 2018), and the mitotic age measure of cell replications (Youn and Wang 2018). These three epigenome-wide measures were generated for both maternal and cord blood and tested separately for association with maternal stress measures (war trauma, chronic stress, and sexual violence) as well as newborn birth weight (Table 3).

Table 3. Associations of Summary Methylation Measures with Maternal Stress and Birth Weight

Methylation Measure	Maternal Stress Measure			Birth Weight
	War Trauma	Chronic Stress	Sexual Violence[a]	
Maternal blood				
GMM (β-value)	4.0×10^{-5} ($p = 0.86$)	5.7×10^{-5} ($p = 0.43$)	1.2×10^{-5} ($p = 0.98$)	5.2×10^{-5} ($p = 0.85$)
Skin and blood clock-age acceleration (years)	0.44 ($p = 0.27$)	0.18 ($p = 0.15$)	0.64 ($p = 0.44$)	–0.32 ($p = 0.50$)
Mitotic age (no. replications)	–4.1 ($p = 0.56$)	0.51 ($p = 0.82$)	–12.7 ($p = 0.38$)	4.07 ($p = 0.63$)
Cord blood				
GMM (β-value)	-4.8×10^{-4} ($p = 0.025$)*	-1.1×10^{-4} ($p = 0.14$)	-7.8×10^{-4} ($p = 0.053$)	5.3×10^{-4} ($p = 0.073$)
Skin and blood clock-age acceleration (years)	5.9e-03 ($p = 0.71$)	2.6e-03 ($p = 0.59$)	0.034 ($p = 0.20$)	–0.028 ($p = 0.16$)
Mitotic age (no. replications)	–9.0 ($p = 0.20$)	0.54 ($p = 0.81$)	–7.9 ($p = 0.55$)	9.7 ($p = 0.31$)

[a]Data for sexual violence associations are presented as mean difference between groups.
*Statistically significant.

Maternal war trauma was associated with cord GMM with an effect estimate of -4.8×10^{-4} per unit of stress ($p = 0.025$; Figure 3A). One suggestive association (p-value between 0.05 and 0.06) was observed between cord GMM and maternal experience of sexual violence, with an effect estimate of -7.8×10^{-4} per unit stress ($p = 0.053$; Figure 3B). Thus, decreased cord GMM was found to be associated with higher levels of maternal war trauma and experience of sexual violence. These associations were not seen with maternal GMM.

No associations were observed between maternal stressors or birth weight and epigenetic age or mitotic age in either maternal or cord blood methylation (Table 3). It is noteworthy that correction for chronological age in maternal blood when calculating epigenetic age acceleration can be accomplished in two ways: by simple subtraction of chronological age from epigenetic age or by including chronological age as a regression variable. Using the subtraction method, we found that epigenetic age was significantly higher than chronological age (Supplementary Figure S1A; mean increase = 4.2 years, $p = 7.1 \times 10^{-6}$). However, the inclusion of maternal age as a regression variable, even as the sole term in the model, followed by taking the residuals to determine maternal age acceleration, completely removed this age acceleration effect (Supplementary Figure S1B; mean increase = –0.093 years, $p = 0.89$). These conflicting results suggest that the increase in epigenetic age seen when subtracting chronological age is not a biologically significant increase in epigenetic age but, rather, an artifact of the epigenetic age calculation, which has been noted in previous studies (Dhingra et al. 2019).

Discussion

This study expands on our previous work investigating the effects of maternal stress on DNAm and newborn birth weight. We have previously observed associations between maternal stress, DNAm, and

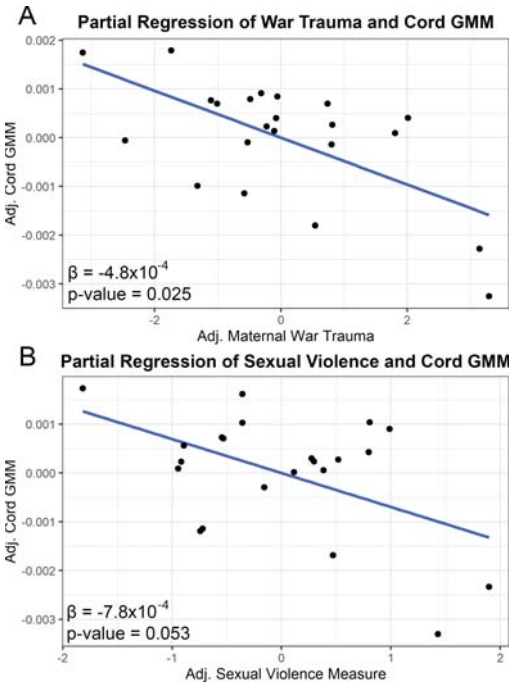


FIGURE 3. Partial regression plot illustrating the relationship of cord blood GMM with maternal war trauma (A) and maternal sexual violence (B). All graph values were adjusted for covariates measuring cell composition, batch effects, and infant sex. Beta, R^2, and p-values reflect those found using a multiple regression including the above covariates.

birth weight using candidate gene approaches (Kertes et al. 2016, 2017; Montoya-Williams et al. 2018; Rodney and Mulligan 2014). We hypothesize that the mechanism by which DNAm is altered may result in different patterns of association among CpG sites, leading us to test for associations between maternal stress, newborn birth weight, and DNAm changes at individual, regional, and epigenome-wide levels. In this study, significant associations were found when analyzing DNAm sites in aggregate at regional and epigenome-wide levels.

Using VMR analysis to test for regional associations, one VMR region, near the *SPONI* locus, was associated with chronic stress and sexual violence in maternal venous blood (Figure 2). In a study by Mehta et al. (2013), the representative CpG site at this VMR locus (cg23284931) and other sites in the region were found to associate with childhood maltreatment. Specifically, they found that individuals with PTSD exhibited higher levels of average methylation in the region compared to controls, similar to our study, which found increased VMR methylation in association with stress and sexual violence. Using microarrays to measure gene expression, Mehta et al. (2013) then verified that the changes in methylation were also associated with expression changes in *SPONI*. While individuals in our sample were not surveyed for PTSD symptoms, our findings are consistent with the premise that specific genomic regions may be targeted for methylation change.

Indeed, other studies support the conclusion that regional methylation patterns are associated with psychosocial stress as well. Previous studies have found altered regions of DNAm in relation to stressors, such as childhood abuse (Shields et al. 2016; Suderman et al. 2014), and mental disorders such as major depressive disorder (Nieratschker et al. 2014). As such, more complex methods of examining regional changes to methylation, such as VMR used here and the bump-hunting method developed by Jaffe et al. (2012), are warranted.

Maternal war trauma appears to influence epigenome-wide DNAm, as measured by cord blood GMM, and birth weight in the same direction. Specifically, higher levels of maternal war trauma were linked with both lower cord blood GMM and lower birth weight. One could speculate that the generalized, epigenome-wide response to maternal stress could be evidence of either dysregulation or multitarget changes in gene expression in preparation for a stressful postnatal environment, as other studies have suggested (Cao-Lei et al. 2014; Suderman et al. 2014). Altered methylation levels could then lead to changes in birth weight, or alternatively, the observed differences in methylation and birth weight could both be independent effects of stress exposure. The lack of a direct association between birth weight and DNAm would seem to favor the interpretation that they are each independently affected by stress.

The associations with GMM reported here differ from those found in previous publications by our group, in which maternal blood, rather than cord blood, GMM correlated with birth weight and stress (Rodney and Mulligan 2014; Clukay et al. 2018). Recent improvements in functional normalization and batch correction using surrogate variable analysis prompted a reprocessing of the data using more advanced techniques (Fortin et al. 2014; Min et al. 2018). The decrease in maternal GMM in association with stress that we previously reported is similar to the decrease in cord GMM in association with stress we report here. In addition, the consistency of our current VMR results with literature, including the direction of the observed effects, lends further confidence to our analysis.

The association of cord GMM with maternal stress stands in contrast to the lack of associations observed when testing two measures of biological age, Horvath's epigenetic clock and mitotic age. While mitotic age is only beginning to be studied in relation to stress, our findings differ from previous studies regarding Horvath's epigenetic clock, which reported age acceleration associated with PTSD in combat veterans, as well as lifetime stress among African Americans (Verhoeven et al. 2018; Zannas et al. 2015). A lack of association in our study could be due to sample limitations, but it is worth noting that Verhoeven et al. (2018) and Zannas et al. (2015) used the subtraction method of correction for chronological age, a method that has come under scrutiny in the work of Dhingra et al. (2019) and this study. As such, our lack of significant associations between maternal stress and biological age could indicate that the effects of stress on human health act through a pathway different from those associated with normal human aging.

The idea that stress may affect groups of CpG sites, as opposed to a single CpG site, is sensible

from a biological perspective. Any effect of stress would occur within the context of an active cell, meaning that not all genomic regions would be equally accessible due to chromatin structure. Factors such as packaging effects, which have been shown to interact with DNAm (Lee and Lee 2012; Hashimshony et al. 2003), could affect the susceptibility of a CpG site to change, possibly explaining differences in signal strength between sites within a given region. For example, the manner in which DNA wraps around histones could lead some sites to be exposed for modification, while others would remain fully or partially shielded by the protein. Furthermore, the high number of base pairs over which histones or groups of histones act would likely mean that any accessible region would likely expose not a single CpG site but, rather, multiple sites.

In addition, some methyltransferase enzymes, which are primarily responsible for the establishment and maintenance of methylation patterns, are processive in nature (Svedruzić 2008). In this context, processivity means that these enzymes tend to methylate multiple CpG sites on a DNA strand before the enzyme detaches from the DNA strand. While methyltransferase enzyme levels generally decrease after birth (Kraiczy et al. 2019; Hu et al. 2008), they nonetheless provide another possible route for modification of CpG sites that could result in groups of sites being altered.

Furthermore, the clustering of methylated and unmethylated regions during the establishment of methylation patterns may mean that multiple CpG sites are associated with a given stressor even if the effect of only a select few is causative. This effect is the epigenetic equivalent of the well-established "hitchhiker effect" seen in genetics, where variants around a causative variant are also associated with a phenotype due to selection and linkage disequilibrium (Kim and Maruki 2011).

In addition to common pathways such as activation of the hypothalamic-adrenal-pituitary axis (Höhne et al. 2014; Eisenlohr-Moul et al. 2018), there are well-documented differences between responses to acute stressors and long-term chronic stressors regarding effects such as immune function (Dhabhar 2008). Our findings in maternal blood that the same VMR was associated with both chronic stress and sexual violence are consistent with the presence of common pathways. Still, the possibility of differing responses based on the nature of the stressor does highlight the importance of examining a variety of stressors, including extreme stressors such as the experience of sexual violence studied here.

The differences between maternal and cord blood observed when testing DNAm in relation to maternal stress (i.e., decreasing methylation in association with cord GMM and increasing methylation in association with a maternal VMR) could be due to multiple factors, including differences in the effect of methylation on gene expression at those sites or differences in developmental stage between mothers and newborns. It is well known that increased methylation can either increase or decrease gene expression, depending on the region affected (Wagner et al. 2014). As such, even though the associations with DNAm may be in opposing directions based on tissue, the downstream effect of such differences on the biological pathway may be similar.

In addition, differences in response based on developmental stage are consistent with current theory. The developmental origins of health and disease hypothesis, first posited by Barker and Osmond (1986), argue for increased developmental plasticity in early development, which can lead to poor health in later life. Recent studies have begun testing changes in DNAm as a possible mechanism for this effect, well summarized in a review by Felix and Cecil (2019). Such a theory would be consistent with our observation of epigenome-wide differences in methylation associated with maternal stress in newborn cord blood but not maternal venous blood. Differing mechanisms based on developmental stage could also explain why the direction of the methylation change observed in the mothers, where increased stress was associated with increased methylation at the significant VMR, differed in direction from those observed in cord blood, where increased stress was associated with decreased GMM.

This study has several limitations. The sample size is small, which could impact our study power to differing degrees, depending on the analysis. The EWAS likely has the least power, followed by the VMR analysis, relative to tests that use summary methylation measures, due to the multiple testing burden. Thus, similar studies with larger sample sizes are needed. However, the fact that

our study found results with both VMR analysis and summary measures after conservative multiple testing correction indicates sufficient power to detect some associations, as well as recommending these techniques as useful tools for future studies with similar sample sizes.

Our study represents a unique examination of DNAm in relation to maternal stress through multiple analytical methods. The findings presented here provide a cautionary note that different means of conceptualizing and analyzing DNAm data can lead to different results, emphasizing the importance of considering the analytical method used in future studies of the human response to stress. Better understanding the mechanisms by which psychosocial stress may translate into epigenetic change should allow for better study design and more targeted questions from an evolutionary perspective.

ACKNOWLEDGMENTS

This project was supported by National Science Foundation grants BCS 1231264 and DGE-1315138, by a University of Florida Research Opportunity Seed Fund award, and by a University of Florida College of Liberal Arts and Science award. We gratefully acknowledge the participation of the women of the Democratic Republic of the Congo and our excellent collaborators, staff, and research facilities at HEAL Africa, Goma, DRC.

Received 3 December 2019; accepted for publication 4 February 2020.

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Supplementary File S1. R Code Used for the Processing of Raw Methylation Data

Go to the following link to view the file: https://digitalcommons.wayne.edu/cgi/viewcontent.cgi?filename=0&article=1160&context=humbiol_preprints&type=additional

Supplementary Table S1. List of Psychosocial Stress Questions Participants Were Asked

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Supplementary Table S2. List of Sample Characteristics, Psychosocial Stress Measures, and Summary Methylation Measures for Each Participant

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Supplementary Table S3. Plate Layout Required for Processing of Raw Methylation Data

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Supplementary Table S4. Maternal VMRs Identified and CpG Sites Associated with Those VMRs

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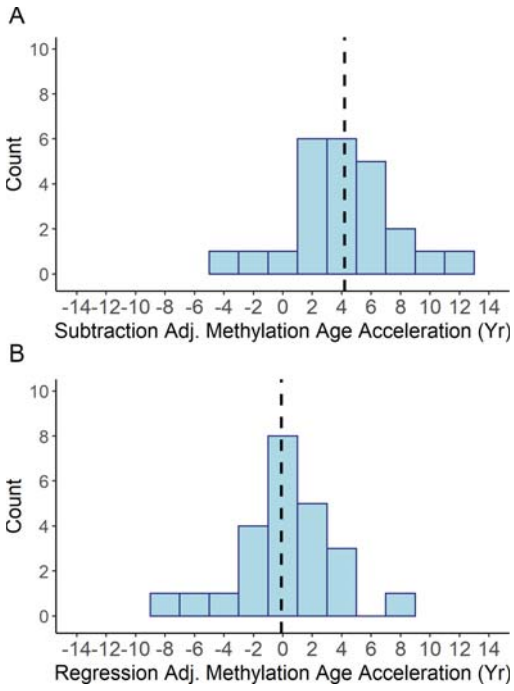

SUPPLEMENTARY FIGURE S1. Distribution of maternal epigenetic age acceleration values when calculated using subtraction of chronological age from epigenetic age (A) and regression of epigenetic against chronological age, with an intercept value set to zero, and extraction of the residuals (B). Dashed lines represent mean values.