Epigenome-Wide Meta-Analysis of Prenatal Maternal Stressful Life Events and Newborn DNA Methylation

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Abstract

Prenatal maternal stressful life events are associated with adverse neurodevelopmental outcomes in offspring. Biological mechanisms underlying these associations are largely unknown, but DNA methylation likely plays a role. This meta-analysis included twelve non-overlapping cohorts from ten independent longitudinal studies (N=5,496) within the international Pregnancy and Childhood Epigenetics consortium to examine maternal stressful life events during pregnancy and DNA methylation in cord blood. Children whose mothers reported higher levels of cumulative maternal stressful life events during pregnancy exhibited differential methylation of cg26579032 in *ALKBH3*. Stressor-specific domains of conflict with family/friends, abuse (physical, sexual, and emotional), and death of a close friend/relative were also associated with differential methylation of CpGs in *APTX*, *MyD88*, and both *UHRF1* and *SDCCAG8*, respectively; these genes are implicated in neurodegeneration, immune and cellular functions, regulation of global methylation levels, metabolism, and schizophrenia risk. Thus, differences in DNA methylation at these loci may provide novel insights into potential mechanisms of neurodevelopment in offspring.

Introduction

Maternal stressful life events during pregnancy can stem from cultural, social, and/or broader environmental experiences. They can impact fetal development[1] depending on their intensity and severity. Typically, maternal stressful life events refer to stressful situations in personal relationships (including physical, sexual, and emotional abuse), work, housing, and social issues[2-4]. Experiencing psychosocial stress can result in physiologic alterations for the mother such as, vascular disorders including hypertension and preeclampsia[2, 5], mitochondrial dysfunction[6], as well as psychiatric implications, most commonly depression and anxiety[7-10]. These maternal stress-induced sequelae may subsequently impact child development, physical and mental health. For example, maternal stress has been linked to preterm birth[2, 5, 11-14], increased asthma risk and poorer lung function[15-18], internalizing and externalizing problems[7, 8, 19, 20], poor cognition[21-24], anatomical and structural alterations of the brain[25, 26], and psychiatric disorders later in life, such as schizophrenia[1, 7, 27] in the offspring. Outcomes affecting both mother and child are influenced by the cumulative nature of maternal stressful life events across the pregnancy. For example, evidence has shown that various types of psychosocial stress in pregnant mothers when experienced concurrently can predict low birth weight[11], lead to higher rates of preterm birth[13, 14]and can precipitate maternal psychopathology, such as depression and anxiety, including pregnancy-specific anxiety[7], thus impacting maternal and fetal outcomes.

Despite the well-documented associations between maternal stressful life events starting in pregnancy and child health, we are only beginning to understand the underlying biological and molecular mechanisms involved. Most prior studies have focused on the hypothalamic-pituitary-adrenal (HPA) axis and the contribution of both maternal and placental cortisol response systems as potential mediators of negative neurodevelopmental outcomes[8]. Some studies suggest that fetal exposure of maternal stress hormones (cortisol, epinephrine) can affect early brain development[8]. Research on the genes involved in the stress response system led to studies exploring epigenetic mechanisms of transcriptional regulation of stress and neurologic function[28]. Epigenetic changes of HPA axis genes, which are heritable modifications in DNA that do not alter the sequence but can result in gene expression changes, have also been linked to adverse child outcomes. Several targeted analyses of DNA methylation have focused on the gene coding for Nuclear Receptor Subfamily 3 Group C Member 1 (*NR3C1)*, also commonly referred to as glucocorticoid receptor (*GR*) gene. For example, DNA methylation profiles of *NR3C1* in blood and saliva of children was associated with neurodevelopmental problems in children whose mothers experienced stress during pregnancy[29, 30]. Radtke et al[31] examined the effects of intimate partner violence during pregnancy reporting increased *NR3C1* DNA methylation in children 10-19 years after birth. While data show that the HPA axis plays a major role in regulating maternal stress response, additional studies in HPA axis genes with inconsistent results have made clear that there are likely other pathways involved in the regulation of maternal stress during pregnancy and its impact on fetal outcomes[1, 8].

While candidate gene analyses are informative, they are limited in scope. Thus, it is critical to investigate genes outside of those contained in the HPA axis system. Very few studies

have utilized an agnostic epigenome-wide approach to examine the associations of maternal stressful life events with infant DNA methylation, and their results have been conflicting. One study using an epigenome-wide association study (EWAS) approach, conducted in a multi-ethnic pregnancy cohort from the United States found maternal stressful life events to be associated with differential placental DNA methylation at several loci involved in cellular metabolic pathways[32]. Contrastingly, a meta-analysis of two epidemiological cohorts of predominantly European ancestry found no signficant associations between prenatal stressful life events and DNA methylation in cord blood[33]. Other agnostic EWASes examining associations with prenatal perceived maternal stress and cord blood DNA methylation[34] or maternal depression during pregnancy and placental DNA methylation[35, 36], also report mixed results. Given the conflicting nature of these studies, likely due to population characteristics and/or choice of tissue, there is a need to conduct larger-scaled meta-analyses to increase power, include populations from different genetic ancestries, as well as cultural settings with different levels of maternal stressful life events, and utilize a single tissue sample source for comparison .

Thus, we studied associations between prenatal maternal stressful life events and epigenome-wide DNA methylation in twelve non-overlapping cohorts (we will refer to these as "cohorts" in the rest of the manuscript) from ten independent longitudinal studies as part of the Pregnancy and Childhood Epigenetics (PACE) Consortium. DNA methylation was measured using cord blood across all cohorts which may provide insight about the cellular and molecular mechanisms involved in programming neurodevelopment[37] and serve as a useful marker for capturing the effects of maternal stress in the fetal environment.

Methods

Participating Cohorts

Twelve European, North American, and South African cohorts from ten independent independent longitudinal studies, all members of the PACE Consortium[38] participated in a coordinated EWAS analysis and subsequent meta-analysis (Figure 1). The participating 5,496 mother-child dyads are described in Table 1. Additional cohort characteristics are described in the Supplementary Methods.

Assessment of Prenatal Maternal Stressful Life Events

Participating cohorts in the PACE consortium provided detailed information on questionnaires used to collect data on a wide range of maternal stressful life events that occurred during pregnancy. Harmonization of cohort-level data resulted in five overlapping prenatal stressor specific domains including: 1) Conflict with family and friends, 2) Physical, sexual or emotional abuse (from now on referred to collectively as "abuse" in the remainder of the manuscript), 3) Death of a close friend or relative, 4) Conflict with partner and, 5) Financial stress. All stressors were self-reported by the mother as yes or no. In order to ensure similar comparisons across cohorts, a composite stress score was derived by summing the number of stressors a mother reported, then dividing by the number of stressor specific domains measured in the cohort. The possible range of composite scores was 0-1 with higher scores representing more stressful life events experienced by the mother. This number is denoted as a cumulative stress score in the remainder of the manuscript. Previous studies have utilized a similar methodology by defining

broad domains of exposure variables based on questionnaire and survey data of cohorts[39-41]. Please see Supplementary Methods for additional details.

Cord Blood DNA Methylation

Cohorts collected umbilical cord blood at birth. DNA methylation was assessed with the Illumina® HumanMethylation450 (450k) or the HumanMethylationEPIC (EPIC) BeadChip assay at Illumina or cohort-specific laboratories. Cohorts performed sample processing, quality control, and normalization as described in the Supplementary Methods. We used normalized, untransformed DNA methylation beta values, ranging from 0 (completely unmethylated) to 1 (completely methylated), after excluding extreme outliers (3 × interquartile range from the 25th and 75th quartile limits). We also excluded probes on the sex chromosomes, polymorphic CpGs which overlap with known single-nucleotide-polymorphisms, probes with cohort-level call-rate <95%, control probes, and cross-reactive probes (targeting repetitive sequences/co-hybridizing to alternate sequences)[42, 43].

Covariates

All cohort-level EWAS analyses were adjusted for child sex, maternal age (continuous), maternal socioeconomic status (see Supplementary Methods for definitions per cohort), and technical covariates, including batch effects, cell type estimates, and ancestry (if available). All EWAS analyses also included adjustments for cell type composition estimated using the Houseman algorithm[44] and the Gervin reference panel for cord blood[45]. In sensitivity analyses, additional adjustments were made for maternal smoking during pregnancy, child birth weight, and gestational age at birth. Maternal smoking and socioeconomic status (described in more detail

by each cohort in Supplemental Methods) were categorized according to cohort data availability, as shown in Table 1. To reduce the effects of population stratification, cohorts with genome-wide genotyping data also adjusted for the top principal components (ALSPAC, MARBLES, MOBA, POSEIDON). Cohorts without genotyping data adjusted for self-reported race/ethnicity or included ancestry-related principal components using EPISTRUCTURE.

Statistical Analyses

Epigenome-Wide Association Studies and Meta-Analyses

Cohort-level EWASes were performed according to a predefined analysis plan. In brief, each cohort utilized robust multiple linear regression (*MASS* R package[46]) to control for potential heteroscedasticity and DNA methylation outliers. Cohorts excluded participants with incomplete data, multiple births, and siblings (one child selected randomly). Models are described in more detail in the Supplementary Methods.

The primary meta-analysis examined the associations between cumulative prenatal maternal stressful life events and DNA methylation at 364,678 CpGs across ten independent longitudinal studies. Probes were annotated to genome build hg19 according to the R package *meffil*[47]. We performed an invariance-weighted fixed effects meta-analysis using METAL[48], to combine the cohort-level EWAS. Cohorts with both 450k and EPIC BeadChip data were included, but only sites available on the 450k array were meta-analyzed. We then calculated cohort- and meta-analysis level genomic inflation factor lambdas (λ) and examined quantile-quantile plots to assess epigenome-wide statistical inflation. Cohort-level results were meta-analyzed at the

University of Cincinnati. A shadow meta-analysis was conducted independently at Erasmus MC to exclude computational errors.

Secondary meta-analyses examined associations between each of the five binary stressor specific domains and DNA methylation, using an otherwise similar approach as in the primary meta-analysis. The five models in this study include: conflict with family or friends, abuse during pregnancy, death of a close friend or relative, conflict with a partner, and financial stress.

Two separate sensitivity analyses were conducted to evaluate potential heterogeneity across studies. We calculated I^2 statistics[49], a measure of heterogeneity ($I^2 \geq 50$) in meta-analyses, and repeated the primary and secondary meta-analyses after including only cohorts 1) with a majority (> 50%) of participants of European ancestry (removed DCHS-450K, DCHS-EPIC, PRISM) and 2) that used the 450k array (removed DCHS-EPIC, ITU, MARBLES). Moreover, in order to confirm that none of the primary meta-analysis CpG sites ($p < 2.40 \times 10^{-7}$)[50] were not confounded by maternal smoking during pregnancy, mean birth weight, and gestational age at birth, we conducted analyses adjusting for these covariates.

Multiple-testing Correction

For all meta-analyses, genome-wide significance was calculated based on the Saffari recommended cutoff for 450k-array-based EWASes, ($p < 2.40 \times 10^{-7}$)[50]. In order to detect differentially methylated regions (DMRs), we utilized the recommended adjusted *p* value (FDR) cutoff of <0.05[51]. A less rigorous cutoff of $p < 5.0 \times 10^{-5}$ was used for the follow-up analyses of BECon and mQTL of our cumulative maternal stressful life events model[52].

Follow-Up Analyses

Differentially Methylated Regions Analyses. DMRs are defined as differentially methylated genomic regions, typically observed in promoter regions[53] To identify DMRs associated with maternal stress during pregnancy, we used the *DMRcate* R package[51]. Settings were adjusted to identify methylated regions according to author's recommended threshold settings in order to avoid false positives. These were specified as within a length of ≤1000 base pairs and *p* associations with significance level of FDR <0.05.

Blood-Brain Epigenetic Concordance (BECon) Analysis. Epigenetic processes are tissue-specific, making the assessment of disease-relevant tissue an important consideration for EWAS. We used a web-based application called BECon (https://redgar598.shinyapps.io/BECon/) to calculate correlation and variability metrics between blood DNA methylation levels of our most significant associations in our primary analysis of cumulative stress ($p < 5.0 \times 10^{-5}$) and three different brain regions; Brodmann area 7 (parietal cortex), Brodmann area 10 (frontal cortex), and Brodmann area 20 (temporal cortex).

Methylation Quantitative Trait Loci (mQTL) Analysis

Given that genetic influence on DNA methylation is common[54, 55], we also conducted mQTL mapping (*cis* and *trans*) of those CpG sites in the cumulative stress model that were below significance of $p < 5.0 \times 10^{-5}$ to determine possible genetic differences that may lead to population-level variations and functional consequences of DNA methylation changes. This was carried out by querying the catalog of *cis*- and *trans*- mQTLs from the Genetics of DNA

Methylation Consortium (GoDMC) of >30,000 participants of European ancestry[56]. (http://mqtldb.godmc.org.uk/index.php)

Expression Quantitative Trait Methylation (eQTM) Analysis

We utilized eQTM analysis to explore whether there was any association between DNA methylation of particular CpG sites and gene expression. A catalogue of 39,749 eQTMs from children's blood compiled by the Exposome-omics-Wide Association Study (ExWAS) conducted in the Human Early Life Exposome (HELIX) project was used to test whether the five significantly methylated CpG sites from the primary analysis had any functional correlation to specific genes. The catalogue was downloaded from http://www.helixomics.isglobal.org/.

Code Availability

The code used for this EWAS meta-analysis is available from the corresponding authors upon reasonable request.

Results

Cohort Summaries

The twelve cohorts in this meta-analysis comprise 5,496 total mother-child dyads. All twelve cohorts participated in the primary analysis of cumulative stress measures. Descriptive details are presented in Table 1. Most cohorts were relatively close to 50% male offspring with the exception of three cohorts (DCHS-450K, DCHS-EPIC, MARBLES) comprised of the majority being

male. Mean maternal age ranged from 27.0 to 34.5 years. Categorization and description of certain covariates between the cohorts may have differed slightly, but this is noted within Table 1 where applicable. Details describing the cumulative stress model score for each cohort, as well as the distribution of participants for each stressor specific domain can be found in Table 2.

Maternal Cumulative Stress During Pregnancy and DNA Methylation (Primary Meta-Analysis)

Using the Saffari cutoff ($p < 2.4x10^{-7}$)[50], increases in cumulative stress were significantly associated with differential DNA methylation of cg26579032 (Figure 2a). Cohort-level data suggest increases in cumulative stress are associated with lower DNA methylation of the locus for all but 1 cohort, PRISM (Table 3, Figure 2a, 2b). This CpG site is located in chromosome 11 on a North Shore CpG region nearest to the Alpha-Ketoglutarate Dependent Dioxygenase (*ALKBH3*) gene. The genomic inflation factor (λ) of the resulting *p*-value distribution is 1.0 (Table 3, Figure 2b). The association remained significant after further adjustment for maternal smoking during pregnancy, birth weight, and gestational age at birth. There was no appreciable heterogeneity across cohorts (I^2=0) for this loci. Comparison of effect magnitude and direction for each cohort compared to the total meta-analysis effect can be seen graphically in Figure 3.

Stress-Specific Domains and DNA Methylation (Secondary Meta-Analyses)

Next, the relationships between prenatal stressor specific domains and DNA methylation in cord blood were examined. Experiencing conflict with friends and family, abuse, and death of a loved one were associated with differential DNA methylation at three different loci at $p < 2.4x10^{-7}$. Supplementary Table 1 summarizes effect sizes of each of the significant CpG sites

associated with maternal stressful events from the primary (cumulative stress) and secondary analyses (stress-specific domains) and how they compare in each stress-specific domain.

Conflict with family or friends (Figure 2c, 2d) was associated with differential methylation at cg14228885 located in a CpG South Shore region of chromosome 9, which annotates nearest to the Aprataxin (*APTX*) gene (λ =1.3); the directionality of this association was positive for all 6 cohorts. This association did suggest possible heterogeneity across studies (I^2=54.5).

Abuse during pregnancy (Figure 2e, 2f) was associated with differential DNA methylation at cg02829783. This CpG loci is located in a CpG Island region of chromosome 7 that annotates to the intragenic region of Myeloid Differentiation Primary 88 (*MyD88*) gene (λ = 1.2). The association between abuse and DNA methylation of cg02829783 was negative for all 7 cohorts (Table 3). There was no heterogeneity across studies (I^2=24.2).

Death of a close friend or relative (Figure 2g, 2h) (λ = 1.2) was associated with differential DNA methylation at cg11714793 located in an Open Sea of chromosome 1 that annotates near the Serologically Defined Colon Cancer Antigen 8 (*SDCCAG8*) gene ; an additional site (cg23933606) with significant association to experiencing the death of a close friend or relative is located in the CpG North Shore region of chromosome 19 which annotates closest to the Ubiquitin Like With PHD And Ring Finger Domains 1 (*UHRF1*) gene region (λ = 1.2). The directionality of the association between death of a close friend or relative and DNA methylation of cg11714793 varied; the directionality was positive for 6 out of 9 participating cohorts. The second CpG site with a significant association, cg23933606, exhibited a positive effect in 11 of 12 cohorts. There

was no hetergoenity across studies demonstrated for either of the significant hits in this stressor specific domain (I^2=3.0 for cg11714793, I^2=27.7 for cg23933606).

Conflict with a partner (Supplementary Figure 1a, 1b) and financial stress (Supplementary Figure 1c, 1d) were not associated with DNA methylation in cord blood.

Sensitivity Analyses

The results of the sensitivity analyses are summarized in Supplementary Figure 2 and Supplementary Table 2. Specifically, Supplemental Figure 2a provides the results of the sensitivity analysis after removal of non-European cohorts. Supplemental Figure 2b provides the results of the sensitivity analysis after removal of cohorts using the EPIC Beadchip.

European-only Cohorts

In order to confirm that the results of the primary and secondary meta-analyses were not driven by ancestry, we conducted a sensitivity analysis using cohorts of primarily European descent (Supplementary Figure 2a). This excluded 3 cohorts: DCHS-450k, DCHS-EPIC, and PRISM for a total of n=5,082 remaining in the analysis. We observed a similar association for the cumulative stress model with all nine participating cohorts demonstrating a negative association between cumulative stress and DNA methylation of cg26579032 (Supplementary Table 2a). Similarly, death of a close friend or relative was significantly associated with DNA methylation of cg23933606 and directionality was mainly positive as detailed in the domain-specific secondary meta-analysis. Although signficant findings in conflict with family and friends and abuse models did not remain statistically signficant ($p < 2.4\text{x}10^{-7}$) after restricting to European-only cohorts, directionality and magnitude of association remained similar to the

primary meta-analysis (Supplementary Table 2a and Supplementary Figure 2a). Heterogenity also changed minimally from the primary analysis, ranging from I^2=0.0 – 62.3.

450k Only Cohorts

When removing all cohorts using the EPIC BeadChip, which includes DCHS-EPIC, ITU, and MARBLES for a total of n=4,882 remaining in the analysis, direction of effect for associations remained the same, however 4 of 5 CpG sites were no longer statistically significant (Figure 3c). In particular, DNA methylation of cg142288885 remained positively associated and statistically significant with conflict with family or friends in all 6 participating cohorts ($p < 1.16x10^{-8}$) (Supplemetary Table 2b and Supplementary Figure 2b). Additionally, heterogeneity for this loci was unaffected compared to the primary analysis and remained moderate (I^2=54.5).

When comparing the primary meta-analysis (Figure 3) with the sensitivity analyses (Supplementary Figure 2a, 2b), each cohort except for PRISM indicates a negative association with cg26579032, the CpG associated with cumulative stress. Directionality of association is also maintained for the significant sites identified in the secondary meta-analysis (Figure 3), when compared with the sensitivity analyses (Supplementary Figure 2a, 2b).

Further Adjusted Models

The stressor specific domain models were further adjusted for maternal smoking during pregnancy, mean birth weight, and gestational age at birth. All significant associations from the primary analysis remained significant, with only one from the model of death of close friend/relative (cg23933606) falling just above the significance cutoff with $p < 2.94x10^{-7}$ (Supplementary Table 2c).

In all sensitivity analyses, heterogenity for each of the five significant CpG sites from the primary analysis remained similar, ranging from I^2=0.0–62.3 (primary analysis range I^2=0.0-54.5). Please see Supplementary Table 2 for detailed information on heterogeneity (I^2) for each of most significant associations resulting from the primary analysis, and how they compared in secondary and sensitivity analyses.

Follow-Up Analyses (DMR, BECon, mQTL, eQTM)

A DMR analysis was performed to look for any associations between cumulative prenatal maternal stressful life events and DNA methylated regions in cord blood. We identified one statistically significant (FDR < 0.05) DMR located on chromosome 20 at position 36149185-36149271 that included 5 CpGs within the genomic region of the Bladder Cancer-Associated Protein (*BLCAP)* gene (Supplementary Table 3). Increases in prenatal cumulative maternal stressful life events were associated with decreased DNA methylation in this region. Supplementary Figure 4 is a visualization of the effect size of methylation for each cohort (prior to meta-analysis) at each of the 5 sites resulting from the DMR analysis.

For an analysis of blood and brain tissue correlation, we included CpGs with $p < 5.00 \times 10^{-5}$ [52] from the cumulative maternal stressful life events model in BECon[57], which resulted in the inclusion of 21 CpG sites for this analysis (Supplementary Table 4). Two CpG sites, cg03601372 and cg05213896, are highly correlated with all three areas of the brain analyzed, including frontal, parietal, and temporal cortex (Supplementary Figure 3). They also have high interindividual variability, which make them potentially informative CpGs enabling biological interpretation of blood-based human DNA methylation results [57]. The CpG site from the

primary meta-analysis that was significantly associated with DNA methylation, cg26579032, demonstrated negative correlation with the areas of the brain.

None of the cohort hits from the primary or secondary meta-analyses demonstrated any potential link with genomic influence in the GoDMC database with the exception of cg142288885 (death of close friend/relative) (Supplementary Table 5). This CpG loci also demonstrated increased heterogeneity in the primary, secondary, and sensitivity analyses as suggested by mostly moderate-level I^2 values.

We did conduct an analysis of functionality using the Human Early Life Exposome expression quantitative trait (eQTM) loci database[58] however, none of the five CpG sites from the primary or secondary meta-analyses were identified as an eQTM. Further functional analyses are necessary to better define the relationship between DNA methylation of our significant loci and gene expression.

Discussion

This meta-analysis across twelve cohorts from ten independent longitudinal studies revealed that prenatal maternal stressful life events are associated with cord blood DNA methylation in offspring. Increased cumulative stress during pregnancy was associated with altered DNA methylation of the offspring at cg26579032, located in the *ALKBH3* gene.

Interestingly, differential DNA methylation at other loci were also observed for the following stressors: conflict with family/friends (cg14228885), abuse (cg02829783), and death of a close friend/relative, which was associated with two CpG sites (cg23933606 and cg11714793). These loci are located within gene regions of *APTX*, *MyD88*, and both *UHRF1* and *SDCCAG8*, respectively. All five CpG sites mapped to genes that are implicated in various functions including neurodevelopment/neurodegeneration[59, 60] , immune and cellular function[61], regulation of global DNA methylation levels[62], metabolism[63], and schizophrenia risk[64-66]. Taken together, these data suggest that cumulative stress, as well as specific stressors, are associated with the DNA methylation status of some loci in offspring. While further studies and validation experiments are needed, these loci may identify interesting candidate loci for future investigation into mechanisms underlying the effects of prenatal maternal stress on offspring (neuro)development.

Mechanisms underlying how stress impacts our health are mostly unexplained. To our knowledge, this is the first study that utilizes an epigenome-wide approach, as well as several pregnancy cohorts in a meta-analysis to examine the association of maternal stressful life events during pregnancy and DNA methylation in cord blood.

Differential DNA methylation associated with cumulative maternal stressful life events during pregnancy was observed at cg26579032. This site mapped to *ALKBH3*, which was recently identified as a potential candidate gene in a schizophrenia transcriptome study[64], in addition to its many metabolic and cellular functions[63]. Similarly, the CpG site associated with death of a close friend/relative, cg11714793, mapped to *SDCCAG8,* a gene involved in schizophrenia risk, as well as cognition[65, 66]. Interestingly, the relationship between maternal

stressful life events during pregnancy have previously been associated with risk of schizophrenia. In one study, prenatal stress exposure (death of a relative including father, mother, sibling, child, or spouse) specifically in the first trimester raised risk of schizophrenia in the offspring (fully-adjusted relative risk 1.67) compared to pregnant mothers not experiencing death of a relative 6 months prior to or any time during pregnancy.[27] Similarly, other studies suggest that the timing and severity/magnitude of the stressful life event during gestation are important criteria when considering risk of schizophrenia in offspring[67]. Although we do not have information on timing of exposures for each cohort, it is interesting to note that there is an association with death of a close friend/relative in two genes, one of which has previously been linked to schizophrenia risk. A second CpG site associated with death of a close friend/relative, cg23933606, is within the *UHRF1* gene which is responsible for global methylation regulation of the epigenome in humans[62]. Animal studies of this gene have demonstrated its impact on neurogenesis and neurodevelopment, as well as its key role in methylation during fetal development[59].

Our analysis also revealed an association between abuse during pregnancy and differential methylation at cg0229783 within the *MyD88* gene. In humans, this gene encodes a protein involved in immune system function, specifically a toll-like receptor family member adaptor molecule[68]. In mice, it has been shown to be critical for central nervous system development in both structural and behavorial aspects, such as neocortical thickness, cortical neuron density, locomotion, and anxiety-like behaviors[60]. Additionally, several studies in Drosophila have mapped its function to brain plasticity[61].

There was a strong association (p = 1.16 x 10^{-8}) of differential DNA methylation at cg14228885 with conflict with family/friends, although we did not observe a similar association with conflict with a partner. This CpG maps to *APTX*, which is a DNA repair gene involved in several functions related to correcting aberrant DNA strand break mistakes. Mutations in this gene have major neurological consequences including severe motor and cerebellar ataxias, neuropathy, and ocular apraxia[69]. In our analyses, this was the only CpG site with strong genomic influence in the GoDMC database of mQTLs (all of which were *cis*-mQTLs), as well as some heterogeneity across studies in the primary meta-analysis, suggesting that the changes in methylation of this loci may be due to genomic effects and not prenatal maternal stressful life events alone. However, the genetic effects of cis-mQTLs between brain and blood have been shown to be highly correlated[70], and these data may suggest there could be both genetic and environmental (phenotypic) factors contributing to gene functionality.

Our DMR analysis identified a hypomethylated region of five methylation sites within the gene *BLCAP* that codes for a cell cycle regulator protein and associated with apoptosis. Li et al. provided evidence indicating the methylation of *BLCAP* was significantly reduced in women with preeclampsia and the gene was highly expressed in placenta[61]. Of additional interest, *BLCAP* also has a transcript variant within its 8.5kbp intron that encodes the Neuronatin (*NNAT)* gene. This gene is involved in central nervous system and brain development[71]. Together with our findings, these data underscore the importance of methylation at the maternal-fetal interface and its contribution to neurodevelopmental programming mechanisms.

It is important to note some limitations of this study. Previous studies have established that timing of adversity exposure in early development is key to predicting changes in DNA

methylation later in childhood and adolescence[72, 73]. However, given the constraints of the meta-analysis study design and harmonization of the stress domains across twelve cohorts, we do not have information on the precise timing and/or duration of the stress exposures for every cohort and each domain which could help to tailor intervention strategies aimed at reducing stress-related risk. Additonally, since this study is focused on cord blood methylation, we do not analyze longitudinal epigenomic data on the offspring. Thus, future studies which can incorporate timing of exposure during pregnancy and/or longitudinal epigenomic data in children where available, might help predict post-natal changes in DNA methylation. Furthermore, harmonization of covariates such as, maternal socioeconomic status, maternal education, and gestational age is also a limitation as these variables are difficult to standardize between different studies within the consortium for practical, societal, and cultural reasons. Second, methods of methylation data normalization in each cohort were varied and may contribute to differences in EWAS results[74], however, a majority of the quality control process was homogenous (batch variables, exclusion of outlying beta values, no transformation of beta values). Previous studies conducting meta-analysis with PACE cohorts have utilized a similar study pipeline and show support that differences in normalization protocols do not affect meta-analysis results[75, 76]. Additionally, we conducted a sensitivity analysis excluding the EPIC BeadChip data in order to minimize any variance of technical effects between cohorts. Results of this sensitivity analysis support the primary analysis results that any significant association of cord blood DNA methylation and maternal stressful life events were not dependent on normalization protocols. Third, our meta-analysis only has representation from two non-European cohorts (PRISM and DCHS), so generalizing our findings to other multi-ethnic

populations may be limited. This was confirmed when analyses were restricted to European-only ancestry cohorts, and the results of the primary meta-analysis remained unchanged. Fourth, we cannot rule out reporting bias of stressful life events or residual confounding by unmeasured variables known to impact the epigenome and neurodevelopment (e.g., environmental exposures such as air pollution, heavy metal exposure, etc.)[77-79]. Fifth, resilience was not evaluated in this study, and it can play a role in mitigating stress and coping[80]. It is also important to note that reporting bias and resilience are some of the reasons experienced stress can be a very individual-centric experience, and thus quanitifying with a cumulative stress score may not capture all the contributing factors involved. These limitations notwithstanding, this study has several notable strengths including the largest sample size to date, utilizing cord blood to measure DNA methylation, comprehensive analyses using robust statistical methods, and the exploration of multiple stress domains (including a cumulative stress index) in relation to the infant epigenome in the largest published study so far. While our sample size is large, a caveat to this advantage is the necessity to harmonize the exposure data in a way that it is comparable across cohorts. It is possible that this approach has introduced additional variability in our results likely biasing our results toward the null (i.e., minimizing the strength of associations between stressful life events and cord blood methylation).

In summary, each of the five CpG sites associated with different stressor specific domains map to intragenic regions of genes that have varying degrees of involvement in neurodevelopment and neurologic function. As a hypothesis-free meta-analysis of twelve cohorts from ten independent longitudinal studies, our results identify interesting candidates for future research, which will need to be followed up to establish their potential utility as

biomarkers of prenatal stress exposure, as well as potential functional relevance for child neurodevelopmental outcomes. Future studies should also consider the dynamic/static nature of these markers as the child ages and their association with structural and functional changes to the brain through the application of neuroimaging to further elucidate their links to both prenatal stress and child neurodevelopmental outcomes.

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Supplementary Materials

Supplementary information is available at *Molecular Psychiatry's* website.

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Figure and Table Legends

Figure 1. Geography of participating cohorts. This map describes the geographic location of the participants from each cohort in the PACE consortium that participated in this meta-analysis.

Figure 2: Manhattan plots (a, c, e, g) and related quantile-quantile plots (b, d, f, h) showing sites of DNA methylation in cord blood associated with cumulative prenatal maternal stressful life events and stress specific domains. Blue line indicates genome-wide significance based on Saffari recommended cutoff for 450k-array-based EWASes, ($p < 2.40x10\text{-}7$)[49]. Methylation of CpG sites that are significantly associated with MSLE are depicted by dots above the red line. Panels a and b: Cumulative stress, (λ=1.0). Panels c and d: Conflict with family or friends, (λ = 1.3). Panels e and f: Abuse, (λ=1.2). Panels g and h: Death of a close friend or relative, (λ=1.2).

Figure 3: Forest plot from primary and secondary meta-analyses results. CpG sites are the top five most significant associations from the primary and secondary meta-analyses results. For each CpG site, cohorts are arranged by largest to smallest sample size from top to bottom, with total meta-analysis effect ("Meta") on the bottom. Cohort sample sizes in figure legend refer to primary meta-analysis sample size.

Table 1: Characteristics and Demographics of Participating Cohorts. *MARBLES: medium and low socioeconomic status (SES) are combined; †Smoking is categorized into "none" and "any"; **MOBA2: medium and low SES are combined; ***PRISM: medium and high SES are combined ††smoking categorized into "no smoking" and "smoking".

Table 2: Characteristics of Stressor-Specific Domains For Participating Cohorts. *Cohort did not have sufficient data to contribute to this analysis.

Table 3: Primary and Secondary Meta-Analysis: Significant Associations of Prenatal Maternal Stressful Life Events and DNA Methylation in Cord Blood. *Direction of methylation for each cohort in the meta-analysis (+=increase, -=decrease, ?=CpG site unknown); cohorts in the following order: ALSPAC, DCHS-450K, DCHS-EPIC, GENR, ITU, LINA, MOBA1, MOBA2, MARBLES, POSEIDON, PREDO, PRISM.