Cumulative lifetime maternal stress and epigenome-wide placental DNA methylation in the PRISM cohort

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Abstract

Evolving evidence links maternal stress exposure to changes in placental DNA methylation of specific genes regulating placental function that may have implications for the programming of a host of chronic disorders. Few studies have implemented an epigenome-wide approach. Using the Infinium HumanMethylation450 BeadChip (450K), we investigated epigenome-wide placental DNA methylation in relation to maternal experiences of traumatic and non-traumatic stressors over her lifetime assessed using the Life Stressor Checklist-Revised (LSC-R) survey (N = 207). We found differential DNA methylation at epigenome-wide statistical significance (FDR = 0.05) for 112 CpGs. Additionally, we observed three clusters that exhibited differential methylation in response to high maternal lifetime stress. Enrichment analyses, conducted at an FDR=0.20, revealed lysine degradation to be the most significant pathway associated with maternal lifetimes stress exposure. Targeted enrichment analyses of the three largest clusters of probes, identified using the gap statistic, were enriched for genes associated with endocytosis (i.e., *SMAP1, ANKFY1*), tight junctions (i.e., *EPB41L4B*), and metabolic pathways (i.e., *INPP5E, EEF1B2*). These pathways, also identified in the top 10 KEGG pathways associated with maternal lifetime stress exposure, play important roles in multiple physiological functions necessary for proper fetal development. Further, two genes were identified to exhibit multiple probes associated with maternal lifetime stress (i.e., *ANKFY1*, *TM6SF1*). The methylation status of the probes belonging to each cluster and/or genes exhibiting multiple hits, may play a role in the pathogenesis of adverse health outcomes in children born to mothers with increased lifetime stress exposure.

Keywords: DNA methylation, maternal stress, placenta, PRISM cohort, metabolism, endocytosis

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Abbreviations: DNA, deoxyribonucleic acid; GC, glucocorticoid; BDNF, brain-derived neurotrophic factor; IGF2, insulin-like growth factor 2; GNASXL, guanine nucleotide-binding protein, alpha stimulating extra-large; HSD11B2, 11beta-hydroxysteroid dehydrogenase; CpG, cytosine-guanine site; SNP, single nucleotide polymorphism; ReFACTor, reference-free adjustment for cell type composition; FDR, false discovery rate; RNA, ribonucleic acid; U7, U7 small nuclear 24 pseudogene; GAM, generalized additive model; KEGG, Kyoto Encyclopedia of Genes and Genomes; SMAP1, small ArfGAP 1; INPP5E, inositol polyphosphate-5-phosphatase E; EPB41L4B, erythrocyte membrane protein band 4.1 like 4B; TM6SF1, transmembrane 6 superfamily member 1; ANKFY1, ankyrin repeat and FYVE domain containing 1;PRISM, PRogramming of Intergenerational Stress Mechanisms; BIDMC, Beth Israel Deaconess Medical Center; BWH, Brigham and Women's Hospital; LSC-R, Life Stressor Checklist Revised; LSCRwt, Life Stressor Checklist Revised weighted; PCA, principal components analysis; BMIQ, Beta Mixture Quantile dilation; EWAS, epigenome-wide association studies; GO, gene ontology

Introduction

Maternal psychosocial stress exposure occurring preconception and during gestation is associated with increased risk for a range of maladaptive outcomes in offspring, including lower birth weight, smaller head circumference, asthma, and altered stress-related hormone levels (e.g., cortisol).[1,2] These links are likely mediated by stress-induced physiological changes that affect the *in utero* environment. Cumulative lifetime stress, particularly exposure to traumatic events, is especially likely to lead to persistent psychophysiological alterations in the mother[3,4] that, in turn, influence perinatal outcomes, offspring neurobehavioral development, and other complex disorders.[5-7] Notably, trauma exposures are more highly prevalent among low-income and racial/ethnic minority populations.[8] Thus, consideration of lifetime exposures to traumatic events and other stressors in these groups may be particularly informative of health disparities. However, the mechanisms underlying these associations remain poorly defined.

Epigenetics incorporates the role of the environment as a regulator of gene expression and likely plays a key part in fetal programming.[9,10] Animal[9,11,12] and human[13,14] data show that epigenetic modifications of disease risk begin *in utero* and can undergo stable regulation that mediates persistent changes in biologic and behavioral phenotypes over the lifespan. DNA methylation is the most widely studied epigenetic mechanism. The demonstration of inter-individual variation in DNA methylation profiles in newborns as a result of maternal lifetime stress is fundamental to establishing a role for epigenetic variation in response to maternal stress in the programming of human diseases.

Studies examining associations between prenatal maternal stress or stress correlates (e.g., maternal psychological functioning, socioeconomic adversity) and DNA methylation have largely examined epigenetic changes in cord blood at birth or child blood DNA and have focused on a handful of candidate genes (e.g., the promoter region of the glucocorticoid [*GC*] receptor, brain-derived neurotrophic factor [BDNF], imprinted genes such as insulin-like growth factor 2 [*IGF2*], and guanine nucleotide-binding protein, alpha stimulating extra-large [*GNASXL*]).[15-18] Growing evidence suggests that the placenta may constitute a model organ to consider mechanisms linking maternal exposures to developmental programming of children's health.[19] The placenta is a significant regulator of maternal-fetal signaling throughout pregnancy influencing a range of physiological functions via release of cytokines, neurosteroids, and neurotransmitters into the fetal circulation.[20] Moreover, environmentally induced perturbations in the maternal milieu can result in changes in placental function and signaling that impact fetal development.[21] While a growing number of animal and human studies have explored associations between prenatal environmental exposures and DNA methylation changes in the placenta, including particulate air pollution, endocrine disrupting chemicals, tobacco smoke, and nutrition,[22-25] research considering prenatal maternal stress in this regard remains sparse. A study in rodents has linked maternal stress during pregnancy with gene-specific (*HSD11B2* gene promoter) changes in placental DNA methylation.[26] Further, two small human studies examined associations between prenatal maternal perceived stress (N = 61)[27] and chronic war-related stressors (N = 24)[28] and differences in placental DNA methylation in glucocorticoid pathway genes.

In this study, we examined associations between maternal cumulative lifetime exposures to traumatic and non-traumatic stressors and epigenome-wide methylation in the placenta among women enrolled in a longitudinal ethnically diverse pregnancy cohort.

Results

Sample characteristics: Maternal and child characteristics for the full sample (N = 238) and the analytic sample (N = 207) are presented in **Table 1**. The analytic sample is composed of those participants with complete stress exposure data and placental tissue. In the full sample, the average maternal age at enrollment was 30.2 years; the majority of the sample were racial/ethnic minorities (Black/Haitian, 40%; Hispanic, 19%; multi-racial, 8%); 26% reported having less than or at most a high school degree; 54% of the children were male, and nearly all children (91%) were born full term. There were no significant differences between the full sample and the analytic sample on maternal age, race/ethnicity, or education or child sex or gestational age.

Epigenome-wide analysis: **Figure 1** outlines the probe filtering steps taken prior to analysis. We tested the association between methylation levels of 365,193 CpGs and maternal stress exposures using semi-continuous linear regression models. In these regression models, population structures were accounted for through incorporating four principle components of CpGs near cis-SNPs[29]; cell type heterogeneity was accounted for by including Reference-Free Adjustment for Cell Type Composition (ReFACTor) components.[30] The resulting P values and their distribution on the genome are illustrated in **Figure 2**. The genome inflation factor of the resulting P-value distribution is 1.158 (**Supplemental Figure S1 and Table S1**), suggesting there is no or minimum inflation of type-I errors. Using FDR correction (Benjamini-Hochberg) for 365,193 tests, we observed epigenome-wide statistically significant (FDR = 0.05) associations between maternal lifetime traumatic and non-traumatic stressors and placental DNA methylation for 112 CpGs in covariate-adjusted analyses; 616 CpGs were differentially methylated at the FDR=0.20 level (**Supplement Table S2**). The 616 CpGs were enriched for enhancers (p-value < 0.0001) and CpG islands (p-value < 0.0001) but not shores, shelves, or promoters; enrichment for CpGs located in enhancers, islands, shores, shelves, or promoters was not observed at the FDR=0.05 level. Further stability selection and permutation analysis suggests the CpG probe, cg22065664, to be the most significantly associated with maternal lifetime stress (**Supplement Table S2**). This probe does not annotate to a gene but it is located in an enhancer near RNA, U7 small nuclear 24 pseudogene (*U7)*. Results from all models [i.e., semi-continuous, simple linear regression and generalized additive model (GAM)] can be found in the online data supplement (**Supplement Tables S1 and S2**).

Epigenome-wide pathway enrichment analysis using an FDR level of 0.20: The enrichment analysis based on 616 probes, which showed strong to moderate association with stress exposures (FDR=0.20) and correspond to 459 genes, revealed 43 enriched pathways (**Supplement Table S3**). The top 10 significant KEGG biological pathways are listed in **Table 2**. The most significant KEGG pathway was Lysine Degradation (FDR = 3.04E-08), which includes 56 genes; 9 of those genes correspond to our set of significant probes. Other significant KEGG pathways encompass multiple KEGG classes including cellular processes, environmental information processing, human diseases, metabolism, and organismal systems and development.

Associations at FDR level equal to 0.20 with heatmap: For the 616 CpGs significantly associated with maternal lifetime stress at the FDR level of 0.20 (**Supplement Table S2**), a heatmap was generated to visualize the placental DNA methylation pattern. The gap statistic was used to identify the optimal number of clusters (**Supplement Figure S2**). Based on initial clustering into 10 groups (**Supplement Figure S3**), we selected the top 3 largest clusters for further analysis. We observed distinct patterns of demethylation among 2 clusters of probes and one cluster showed increased methylation in association with increased maternal lifetime stress (**Figure 3**). Pathway enrichment analyses conducted among each cluster of probes and their corresponding genes (top cluster n = 112, middle cluster n = 111, bottom cluster n = 94) observed in the heatmap revealed further enrichment of 3 of our top 10 KEGG pathways: endocytosis (middle cluster), metabolic pathways (top cluster), and tight junction (bottom cluster) (**Figure 3**). The three most significant probes, mapping to genes associated with the three clusters, were cg13976799 [mapping to small ArfGAP 1 (*SMAP1*) as part of the endocytosis pathway], cg14024579 [mapping to inositol polyphosphate-5-phosphatase E (*INPP5E*) as part of the metabolic pathway], and cg13531977 [mapping to erythrocyte membrane protein band 4.1 like 4B (*EPB41L4B*) as part of the tight junction pathway]. All three probes were significant at the FDR=0.10 level; cg13531977 (*EPB41L4B*) was significant at the FDR=0.05 level. Generally speaking, CpG probes located in genes found in the endocytosis and metabolic pathways showed patterns of hypomethylation while probes associated with genes in the tight junction pathway were hypermethylated. Though there appeared to be additional regions of hyper- and hypomethylation among a small fraction of participants with low maternal lifetime stress, these regions were not associated with low maternal lifetime stress across all participants, nor were the patterns associated with covariates (i.e., maternal race/ethnicity, maternal age, child's sex).

Identification of probes with "multiple" hits: Of the 112 CpGs significant at the FDR=0.05 level, 3 probes mapped to two genes where at least one other neighboring probe with an FDR value < 0.05 was located. These include the following genes: transmembrane 6 superfamily member 1 (*TM6SF1*, probe cg03063639) and ankyrin repeat and FYVE domain containing 1 (*ANKFY1*, probes cg24084898 and 13303203). Locus-zoom-in plots of P-value distributions along the genome were generated for *TM6SF1* (**Figure 4**) and *ANKFY1* (**Figure 5**). Within these genes, we observed spikes of multiple probes showing associations with higher maternal lifetime stress; the top most significant hits are highlighted in red (FDR = 0.10). Scatterplots showing the relationship between maternal lifetime stress and the methylation status of the significant probes listed above can be found in the supplement (**Supplement Figure S4**).

Discussion

To our knowledge, this is the first human study to examine associations between cumulative lifetime maternal experiences of traumatic and non-traumatic stressors and epigenome-wide placental DNA methylation. We observed epigenome-wide statistically significant associations between increased maternal lifetime stress and placental methylation at 112 CpGs using a strict FDR of 0.05; a more liberal FDR of 0.20 resulted in an additional 504 significant CpGs, mapping to 459 genes.

Maternal lifetime exposure to stress was most significantly (FDR = 2.22E-6) associated with the placental methylation status of cg22065664. This finding turned out to be very robust as it remained the top hit following our permutation analysis. While cg22065664 is not located within a gene, there are some

characteristics which make it an interesting target for future studies. The probe is located in an intergenic enhancer and distal enhancers play a major role in the cell type-specific regulation of gene expression. While it is unclear whether the DNA methylation status of this upstream enhancer has an effect on gene expression, many intergenic enhancers have been associated with adverse birth outcomes (e.g., early-onset preeclampsia) and their methylation status has been shown to be retained in adult tissues.[31,32] Tissue-specific DNA methylation of intergenic enhancers are notably most variably in cancer cells[33] and global loss of DNA methylation in cancer cells has been shown to activate a large number of enhancer regions.[34] Whether maternal lifetime stress results in global hypomethylation, consequent activation of intergenic enhancers, and ultimately changes in gene expression in placenta requires further investigation. Further, the probe is located next to *U7*, a pseudogene. Although previously treated as "junk DNA", it has been shown that some pseudogenes have important gene regulatory roles and are capable of being transcribed into RNA. How the methylation status of cg22065664 impacts the expression and function of *U7* is unclear.[35,36]

Enrichment analyses revealed the KEGG lysine degradation as the most significant pathway. In humans, lysine is an essential amino acid, and thus must come from food. Lysine is incorporated into collagen, one of the most important components of connective tissue; therefore, its supply is required during embryonic development and early childhood.[37] The main catabolic pathway for lysine is the Saccharopine (e-N-[L-Glutaryl-2]-L-lysine) pathway; this is a mitochondrial pathway leading to the formation of Acetyl-CoA (Acetyl-Coenzyme-A), which is oxidized and used for energy production.[38] Research suggests decreases in lysine can lead to placental insufficiency, and reduced placental transport of lysine is observed in cases of intrauterine growth restriction.[39,40] Other significant KEGG pathways identified highlight the potential associations between maternal lifetime stress and cellular processes (tight junction and endocytosis), environmental information processing (PI3K-Akt signaling pathway, neuroactive ligand-receptor interaction, rap1 signaling pathway), human diseases (human papillomavirus infection, pathways in cancer), metabolism (metabolic pathways), and organismal systems and development (progesterone-mediated oocyte maturation). Interestingly, increased prenatal maternal perceived stress has been associated with multiple differentially methylated regions involved in the neuroactive ligand receptor interaction KEGG pathway in cord blood.[41] While the characterization of stress exposure and timing of exposure in the study by Trump and colleagues[41] differ from our study, these findings suggest some stressors may affect similar biological mechanisms (i.e., neuroactive ligand receptor interactions).

Targeted pathway enrichment analyses, conducted among the 3 largest clusters of probes observed in our heatmap, revealed further enrichment of three of our top 10 KEGG pathways: endocytosis, metabolic pathways, and tight junction. The most significant probe identified in the endocytosis cluster is located in the gene *SMAP1*. *SMAP1* encodes for a type II membrane glycoprotein which plays an important role in the erythropoietic stimulatory activity of stromal cells whose deficiency can lead to embryonic lethality.[42] While there are no studies to support our finding that maternal lifetime stress has an impact on *SMAP1* methylation and/or gene expression, other environmental exposures have been associated with *SMAP1* gene expression, specifically metal mixture exposures.[43] The expression of *INPP5E* and *EEF1B2*, where two of the most significant probes identified in the metabolic cluster of our

heatmap are mapped, has also been shown to be impacted by environmental exposures. *INPP5E* expression appears to be influenced by current smoking status[44], while *EEF1B2* expression correlates with maternal tobacco smoke exposure during pregnancy.[45] Despite the fact that maternal lifetime stress is not a chemical exposure, both tobacco smoke and psychosocial stress have been shown to induce similar biological responses, such as oxidative stress.[46-50] Maternal lifetime stress was also associated with increased methylation of a probe located in the gene *EPB41L4B*. This gene is part of the tight junction cluster and plays important roles in cytoskeleton changes associated with steroid-induced cell differentiation and development.[51,52] This is likely due to its involvement in the glucocorticoid receptor (GR) pathway, a main target for the stress-related steroid hormone cortisol, where it serves as a GR stimulatory gene.[53] While glucocorticoids are vital to the development and survival of the fetus, exposure to maternal stress and excess glucocorticoids *in utero* can be harmful for fetal development and growth.[54,55] Given our results and the findings highlighted above, it is plausible that *INPP5E, EEF1B2, and EPB41L4B* play roles in environmentally-induced (via both physical and psychosocial exposures) placental changes that may lead to adverse perinatal outcomes.

The gene targets outlined below exhibited multiple probes whose methylation status is associated with increased maternal lifetime stress (**Figures 4 and 5**). *TM6SF1* encodes a widely expressed lysosomal transmembrane protein which is vital for facilitating protein trafficking via organelle fusion.[56] Interestingly, lysosomal dysfunction has been associated with neonatal intestinal disorders preventing proper nutritional absorption.[57] Placental expression of TM6SF1 appears to be upregulated by environmental exposures such as prenatal alcohol consumption[58]. However, in some instances the regulation of TM6SF1, as a result of postnatal environmental insult (i.e., house dust mite exposure) doesn't appear to be associated with the methylation status of its promotor.[59] This raises the question of whether *TM6SF1* expression is regulated by DNA methylation and thus warrants further investigation. The other gene to exhibit differential methylation of multiple cpg probes associated with maternal lifetime stress is *ANKFY1,* also known as Rabakyrin-5*. ANKFY1* is widely expressed in adults and in fetal tissues during development and plays a major role in endocytosis.[60] Overexpression of *ANKFY1* has been shown to increase macropinocytosis, the non-selective uptake of solute molecules and nutrients, while knockdown of *ANKFY1* suppresses the process.[60] Macropinocytosis serves as a feeding mechanism and is particular relevant for nutrient-deprived environments[61] and nanoparticle uptake.[62] Nanoparticles can cross the placenta barrier[63] and cause fetotoxicity in offspring by disrupting placental function and structure.[64] Environmental exposure to nanoparticles can occur through many sources such as consumer spray products and cosmetics. [65,66] Thus, one could hypothesize that maternal lifetime stress exposure could result in increased uptake of nanoparticles at the maternal-fetal interface due to altered expression of *ANKFY1* and its subsequent increase in macropinocytosis. Together, these findings underscore the potential importance of *TM6SF1* and *ANKFY1* in fetal and infant development consequent to maternal stress exposure.

This study has notable strengths. The study is comprised of a largely racial/ethnic minority population of pregnant women at increased risk for stress exposure and consequent adverse health outcomes among their offspring. Further, because PRISM is a longitudinal cohort, we will be able to examine the impact of these epigenetic changes on child developmental outcomes. Moreover, in the data analysis, we

implemented a novel semi-continuous regression model to better detect associations with high stress exposures. When compared with traditional approaches, such as the ordinary linear regression and GAM (generalized additive model) on our data set, the semi-continuous regression model showed more favorable control of type-I errors and/or power (**Supplementary Table S1**).

There are also limitations worth noting. First, our study focused on maternal lifetime stress/trauma; it is possible that different stress domains exert differential effects on placental DNA methylation, and this possibility should be examined. Second, our analyses were limited to placental samples. The effects of maternal lifetime stress/trauma on other fetal tissues (e.g., umbilical cord blood) should be explored. Third, the sample size prevented us from examining differences among racial/ethnic groups; potential racial/ethnic differences should be tested among larger racially/ethnically-diverse cohorts as they might provide insight into racial inequalities in health. Lastly, it is important to replicate our findings; to date, we have been unable to locate a cohort suitable for replication due to differences in tissue type and the operationalization (e.g., psychopathology, natural disaster) and timing (e.g., current vs. lifetime) of stressors.

In summary, the placenta's role in nutrient transfer and organ development is important for the proper development of the fetus. Epigenetic modification of the placental epigenome may provide a possible connection between maternal stress exposure and alteration in gene expression that might set in motion fetal programming signatures that shape early childhood conditions. Our study found that maternal lifetime stress/trauma is associated with changes in the epigenome-wide methylation status of multiple loci in genes that have key roles in cellular processes and metabolism. Both of these processes broadly speaking, and more specifically the genes for which the significant loci were mapped, appear to be important for early embryo development and metabolism. These findings may provide insight into the biological mechanisms linking maternal stress exposure and adverse perinatal/child health outcomes.

Methods

Study Population

Participants included women enrolled in the PRogramming of Intergenerational Stress Mechanisms (PRISM) study, a prospective pregnancy cohort of mother-child pairs originally designed to examine how perinatal stress influences child development as well as examining the role of the placenta in environmental programming. In brief, N=238 women were recruited from prenatal clinics during the first or second trimester (27 ± 8 weeks of gestation) from the Beth Israel Deaconess Medical Center (BIDMC) from March 2011 to August 2012 and from the Icahn School of Medicine at Mount Sinai from November 2012 to August 2014. Eligibility criteria included: (i) English- or Spanish-speaking; (ii) age ≥18 years at enrollment; (iii) singleton pregnancy; and (iv) willingness to provide placenta samples at birth. Procedures were approved by the institutional review boards at the Brigham and Women's Hospital (BWH) and the Icahn School of Medicine at Mount Sinai. BIDMC relied on BWH for review and oversight of the protocol. Written informed consent was obtained from all participants in their primary language.

Maternal Lifetime Stress

Maternal lifetime exposure to stress and potentially traumatic events was assessed in the second trimester using the validated 30-item Life Stressor Checklist-Revised (LSC-R).[67] The LSC-R includes experiences particularly relevant to women (e.g., sexual assault, interpersonal violence) and questions reflecting the participant's assessment of the severity of the negative impact (ranging from 1 [not at all] to 5 [extremely]) of each endorsed event during the past 12 months. The LSC-R has established test-retest reliability and validity in diverse populations.[67,68] A weighted score of all endorsed stressful life events (traumatic and non-traumatic) that considered the self-reported negative impact of each event was computed (weighted Life Stressor Checklist Revised, LSCRwt); scores could range from 0-150 (range in present sample = 0-96), with higher scores indicating greater exposure to and impact of exposure to stressful and traumatic events.

Placenta Specimen Collection and Processing

Placentas were sampled per a published protocol[69] immediately after birth in 238 women. Each of four samples (~1 cm^3) was taken on the fetal side ~4 cm from the cord insertion site and ~1-1.5 cm below the fetal membrane to avoid membrane contamination. The deciduas and fetal membranes were removed, the sample was rinsed in a cold PBS bath, cut into smaller pieces (~0.1 cm^3), and placed into 1 ml of RNA*later*™ RNA Stabilization Reagent (Qiagen). Samples in RNA*later* were placed at -4°C for $\leq$24 h; excess RNA*later* was then removed and samples were placed at -80°C until DNA extraction.

DNA Extraction and Bisulfite Treatment

DNA was isolated using the Gentra Puregene kit (Qiagen, Germantown, MD) and quantified using an Implen Nanophotometer Pearl (Westlake Village, CA). The origin of placental tissue from the fetal side of the organ was confirmed by the near-perfect agreement of placenta and cord blood samples in 64 genotyping probes used for identity verification. Across these 64 genotyping probes, the range of the Pearson correlation coefficients between cord blood and placenta within each participant was 0.99 to 1.00.

500ng of DNA was bisulfite-treated using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA) analyzed by the Infinium Methylation Assay. Samples were arranged on chips and plates with a stratified randomization followed by statistical checks for balance on birthweight z-score, gestational age, sex, and city of collection.

DNA Methylation Profiling

Illumina Infinium HumanMethylation450 array: HumanMethylation450 BeadChips (Illumina Inc., San Diego, CA, USA) were used to interrogate 485,577 DNA methylation sites and to generate a measure of the methylation proportion at each site. Specifically, single-CpG-site methylation values were quantified after bisulfite conversion using fluorescence measures at site-specific probes, which was computed as the methylated intensity divided by the sum of both the methylated and unmethylated intensities. Methylation values ranged from zero (for a fully unmethylated CpG site) to one (for a fully methylated CpG site).

Quality Control, Preprocessing and Normalization: The presence of failed arrays or outliers was checked with detection P values (all samples passed with detection P values < 0.05 in >99% of probes) and through visualization of principal components analysis (PCA). PCA plots and the analysis of five pairs of technical replicates that were arranged across chips and plates were further used to assess potential batch effects. Sample identity was checked via imputed sex and agreement of genotype with paired tissues. Data were preprocessed using background correction,[70] dye bias, and probe type adjustment.[71] BMIQ (Beta Mixture Quantile dilation) intra-sample normalization was applied to all probes to adjust the methylation values of Infinium II probes into a statistical distribution characteristic of Infinium I probes. The *BMIQ* function in the R package *wateRmelon* was used.[72]

Probe Filtering: Prediction models were not performed on all 485,577 methylation probes. Probe filtering included the removal of 1217 probes with detection P value > 0.05 in >1% of the samples, 64 SNPs, 3089 CpH sites, 29127 cross-reactive probes, 74645 CpG sites with variants within 10 base pairs, which are common to Asian, American, African, and European populations with a frequency of >1%, and 9323 and 48 probes on chromosomes X and Y, respectively. An additional 2865 CpG sites with a multimodal distribution of methylation (Dip test's P value < 0.05) were excluded (**Figure 1**).

Statistical Analysis

Placenta tissue and LSC-R data were available for 238 participants.

ReFACTor analysis for cell heterogeneity adjustment: The cell heterogeneity adjustment was conducted using a Reference-Free Adjustment for Cell-Type composition (ReFACTor) analysis. ReFACTor is an unsupervised method for the correction of cell-type heterogeneity in epigenome-wide association studies (EWAS), which is based on a variant of principal component analysis (PCA).[30] The ReFACTor algorithm requires a CpG sites-by-samples matrix of beta-normalized methylation levels, a number of assumed cell types (k) and a number of CpG sites to use for computing the ReFACTor components (t). As suggested by the authors of this method (https://github.com/cozygene/refactor), before running ReFACTor we exclude problematic probes, as well as consistently methylated probes and consistently unmethylated probes. Specifically, filtered-out probes included 1,217 probes with a detection p-value >0.05 in >1% of the samples, 64 genotyping SNPs, 3,089 CpH sites, 29,127 cross-reactive probes [2], 74,645 CpG sites with variants within 10 base pairs that are common to Asian, American, African and European populations with a frequency of >1% [3], 9,371 probes on chromosome X or Y, 99,878 and 141,322 CpG sites with mean methylation value > 0.8 or < 0.2, respectively. The resulting dataset for the ReFACTor analysis included 126,864 probes and 238 unique samples. Guidelines for parameter selection guided us to set k and t equal to 3 and 500, respectively. Since the authors of this method observed that adjusting the methylation levels, before running ReFACTor, for genome-wide affecting factors, such as gender and global ancestry, improves the performance of ReFACTor, we decided to pass to ReFACTor also a samples-by-covariates matrix of covariates including gender and principal components for ancestry. The software output is a matrix with the first k ReFACTor components for each individual which can be included in downstream analyses as covariates. We noted from a scatterplot matrix that these three derived variables were uncorrelated with each other (as we would expect).

EPISTRUCTURE analysis for ancestry adjustment: The adjustment for population structure was conducted using principal components calculated on a subset of 4,905 CpG sites pre-screened to be highly correlated with local single nucleotide polymorphisms (cis-SNPs).[29] Scatter plots showed the first four principal components of the between-probe relations of these CpGs capturing genetic information separated samples by the maternal self-reported race/ethnicity and thus were included in further association testing to avoid confounding due to population stratification.

Epigenome-wide Association (EWAS) Analysis: Association testing was performed using CpG methylation β-values based on data from 207 participants, excluding 31 individuals with very high stress scores (LSCRwt > 80). For each probe, outliers were evaluated on the methylation M-value scale. Methylation β-values were first transformed into M-values through logit transformation. Any M-value above or below the interquartile range (defined as having values outside 3 times the interquartile range below Q1 or above Q3) was truncated to its nearest range value prior to association testing. Evidence supports the notion that low to moderate levels of stress are normative (i.e., commonly encountered in the population) and thus, when stress exposure is within this range it is likely to have limited impact on epigenetic processes; however, when stress exposure and/or the perceived impact of the stressor is high or more severe, there is more likely to be biological consequences at the cell level.[73,74] This hypothesis is supported by the empirical distributions of LSCRwt scores and M-values observed in our study. As the examples in the supplement illustrate (**Figure S4**), the scatterplots of LSCRwt vs. the M-value of individual CpG probes across all subjects consistently shows a mass of data points at the low LSCRwt end (i.e., weaker effect is observed between LSCRwt and methylation). This observation motivated us to introduce two variables ($LSCRwt_i^B, LSCRwt_i^T$) to better capture the information in the tail distribution of LSCRwt scores and at the same time to prevent the variability among small LSCRwt scores to dilute the signals in statistical tests. Specifically we define these variables as follows:

$$LSCRwt_i^B = \begin{cases} 1, & \text{if } LSCRwt_i \leq \alpha \\ 0, & \text{otherwise} \end{cases}$$

$$LSCRwt_i^T = \begin{cases} LSCRwt_i, & \text{if } LSCRwt_i \geq \alpha \\ 0, & \text{otherwise} \end{cases}$$

In the above definition, $LSCRwt_i^B$ is a binary variable indicating whether the participant was exposed to high or low stress; while $LSCRwt_i^T$ is a truncated continuous variable measuring the effect of extreme stress exposures among the high stress group.

We then used the following model to test for associations between methylation and stress, where y_{ij} represents the methylation M-value of individual i (1,…,207) for probe j (1,…,365193):

$$y_{ij} = \alpha + \beta_B LSCRwt_i^B + \beta_T LSCRwt_i^T + \sum_k \beta_k X_{ki}$$

where the covariates (X_{ik}) included principal components to adjust for population stratification (k=1, 2, 3, 4)[29] and cell heterogeneity (k=5, 6, 7)[30], child's sex (k=8), and maternal age at enrollment.

Statistical testing was conducted using the ANOVA F-statistic, comparing the model stated above to a null model containing neither stress related term. We compared results from our semi-continuous model with those based on a simple linear model and a generalized additive model based on fitting a smoothed spline to the data as implemented in R the "gam" package (**Supplement Table S1**). For the semi-continuous model we considered many values of α corresponding to percentile increments from the 80th to the 96st percentile of LSCRwt. For further investigations, we selected the semi-continuous model where α =31 corresponding to the 92nd percentile of LSCRwt. This model shows low genomic inflation (λ_{GC}=1.16, **Supplement Figure S1**), maintains an adequate number of samples above the threshold (n=15), and demonstrates enhanced power to detect associations as compared to either the simple linear or the generalized additive model. The –log10 (ANOVA P values) from the semi-continuous linear regression for 365,193 CpGs across the genome were plotted (**Figure 2**). Direction of association was based on the variable, $LSCRwt_i^B$ or $LSCRwt_i^T$, with the lowest model P value. To further substantiate our findings, we also conducted a stability selection[75] based permutation test to identify methylation probes significantly associated with LSCRwt scores (see **Supplement Text**).

Heatmap Visualization of Methylation Data: Methylation beta values for the 616 probes significant at FDR = 0.20 were first adjusted for genetic and cell background and then normalized before hierarchical clustering was performed (see **Supplement Text**). The number of clusters (K=10) was selected based on gap statistic (**Supplemental Figures S2 and S3**).[76] Visual inspection for patterns of methylation in association with maternal lifetime stress (LSCRwt) was conducted. **Figure 3** presents a heatmap based on the top three largest clusters (K=3).

Epigenome-wide Enrichment Analysis: We performed gene ontology (GO) and KEGG pathway analyses for the set of 616 probes significant at the FDR = 0.20 level (**Supplementary Table S2**) and for the each of the probe sets corresponding to the top 3 clusters shown in **Figure 3**. The epigenome-wide enrichment analyses for GO Terms and KEGG Pathways was conducted using the Bioconductor 'missMethyl' package for R.[38] Enrichment analyses were conducted based on a background probe set comprising the full set of 365,193 probes used in EWAS analysis. We utilized the modified hypergeometric test option of the 'gometh' function to account for selection bias due to the increased probability of finding an association for genes with a larger numbers of probes.[38,77] All significant KEGG biological pathways at an FDR=0.05 can be found in the **supplementary table S3**. **Figure 3** shows adjusted methylation values for the top 3 clusters, where cluster membership is shown on the left bar; the probes/genes belonging to each of the enriched pathways are highlighted in the bars to the right of the heatmap. We further performed pathway, CpG region, and promoter enrichment analysis using the two-sided doubling mid p-value hypergeometric test[78] for probes selected for significance at the FDR = 0.05 (n=112), FDR = 0.10 (n=267), and FDR = 0.20 (n=616) levels.

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Figure 1: Flowchart of Probe Filtering. The flow chart outlines the filtering steps used prior to analysis.

Figure 2: Epigenome-wide Association Results between Maternal Lifetime Stress and Methylation of 365193 CpGs Measured in Placenta for the Semi-Continuous Model. Each point represents the genomic location (x-axis) and the –log10 p-values based on the semi-continuous model for association test (y-axis) for a single probe. Horizontal lines depict the epigenome-wide significance level corresponding to FDR=0.05 (solid) and FDR=0.20 (dashed). The set of probes/genes highlighted in the text are indicated by red triangles.

Figure 3: Heatmap of M-values for 130 Gene-annotated Probes Significantly Associated with Maternal Lifetime Stress at the FDR = 0.10 Level. Heatmap shows adjusted and normalized methylation β -values with genetic background and cell heterogeneity factors removed, for the leading three clusters that consist of 317 probes of the 616 significant probes (FDR=0.20). For each gene/probe (rows), the cluster membership is shown in the bar to the left and the significance level based on the semi-continuous regression model is shown to the immediate right. Pathway enrichment testing was conducted for the probes belonging to each cluster; genes belonging to enriched KEGG pathways ($p<0.05$) are highlighted in the bars to the far right. For each sample (column), the LSCRwt is plotted (top) and the child's sex and maternal age is shown in the bars directly above the heatmap.

Figure 4: Zoomed-in plot featuring functional annotations for TM6SF1. Zoomed-in plot (top panel) shows the –log10 p-values for the semi-continuous model within a 10Kb neighbor of cg03063639/TM6SF1 on the genome. Functional annotations are derived from the ENCODE project and include regulatory, gene, CpG island, and GC content tracks. The lower panel shows pairwise correlations among the CpG sites selected. The plot was generated using the 'CoMet' packaged in R.

Figure 5: Zoomed-in plot featuring functional annotations for ANKFY1. Zoomed-in plot (top panel) shows the –log10 p-values for the semi-continuous model within a 16kb neighbor of cg13303203/ANKFY1 on the genome. Functional annotations are derived from the ENCODE project and include regulatory, gene, CpG island, and GC content tracks. The lower panel shows pairwise correlations among the CpG sites selected. The plot was generated using the 'CoMet' packaged in R.

Table 1. Sample Characteristics

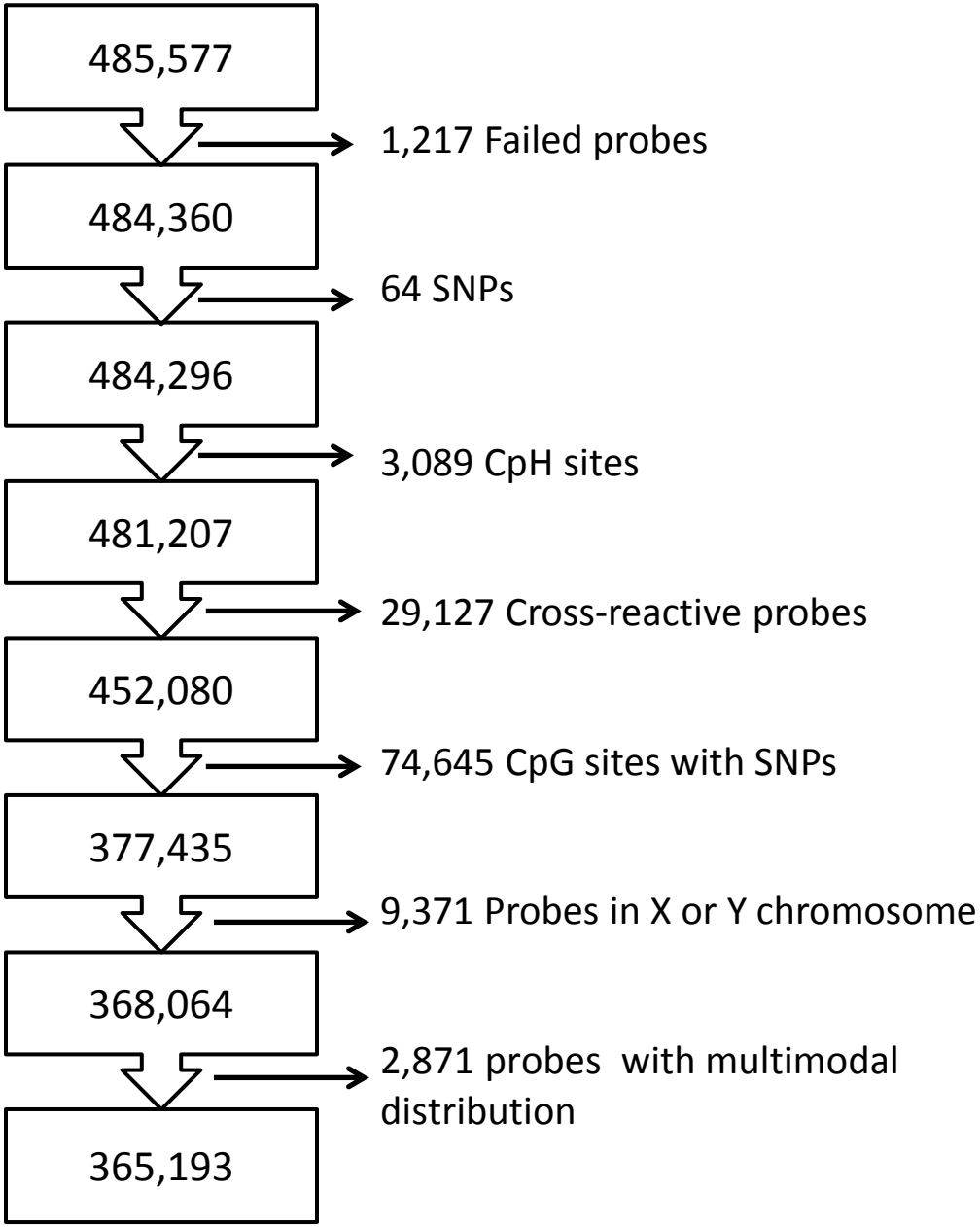
	Full Sample (N=238)	***Analytic Sample (N=207)***
Categorical Characteristics	***No. (%)***	***No. (%)***
Education		
<= High School degree	62 (26)	56 (27)
Missing	7 (2)	3 (1)
Race		
White	78 (33)	70 (34)
Black	94 (40)	90 (43)
Hispanic	46 (19)	35 (17)
Other/Mixed	20 (8)	13 (6)
Child sex		
Male	129 (54)	109 (52)
Child born full term		
Yes[a]	217 (91)	189 (91)
Continuous Characteristics	***mean ± SD[b]***	***mean ± SD***
Age at enrollment in years	30.2 ± 5.6	30.2 ± 5.7
Gestational Age in weeks	39.0 ± 1.8	39.0 ± 1.8
Life Stressor Checklist-Revised, weighted score (LSCRwt)	--	12.5 ± 12.3

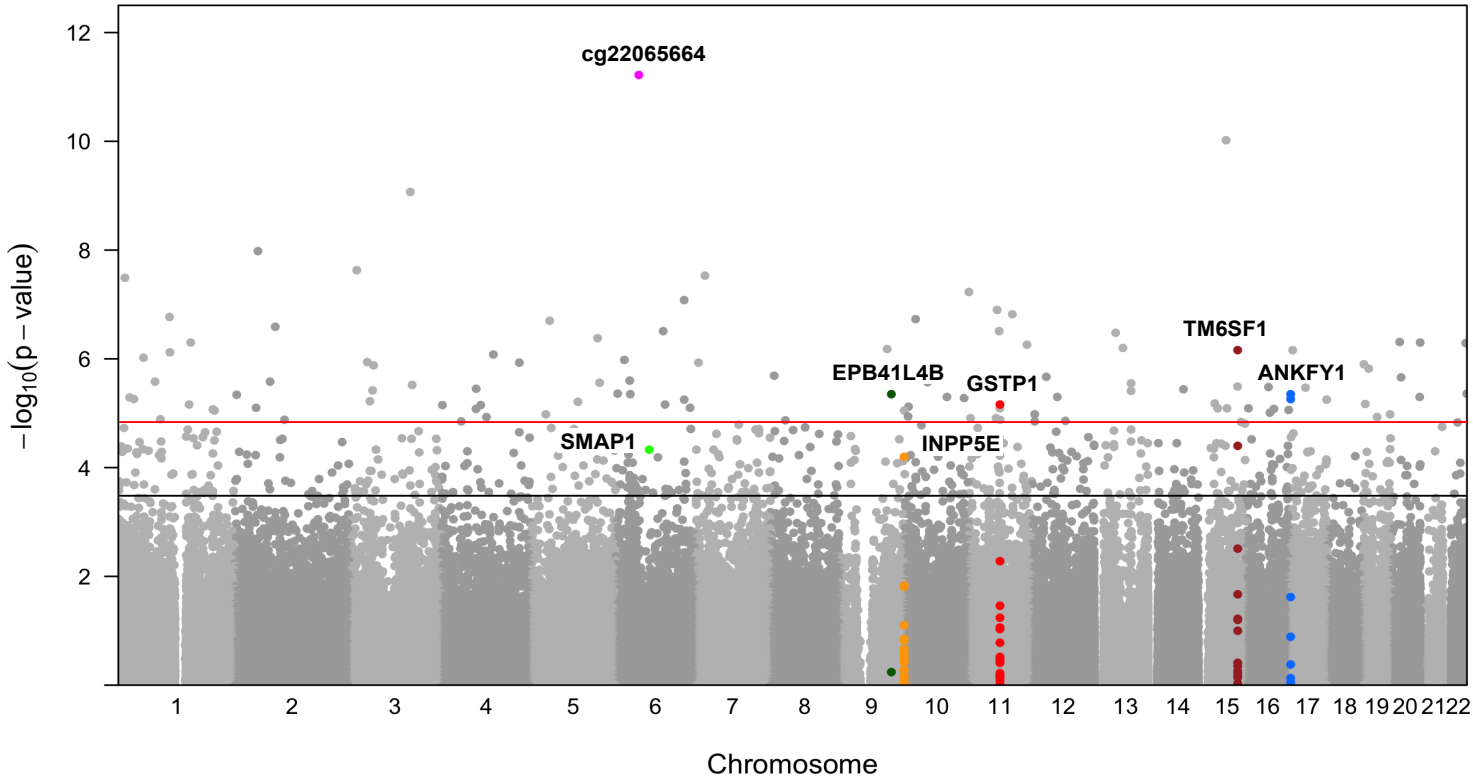
[a]Children born from 37 to 42 weeks of gestation; [b]SD, Standard Deviation

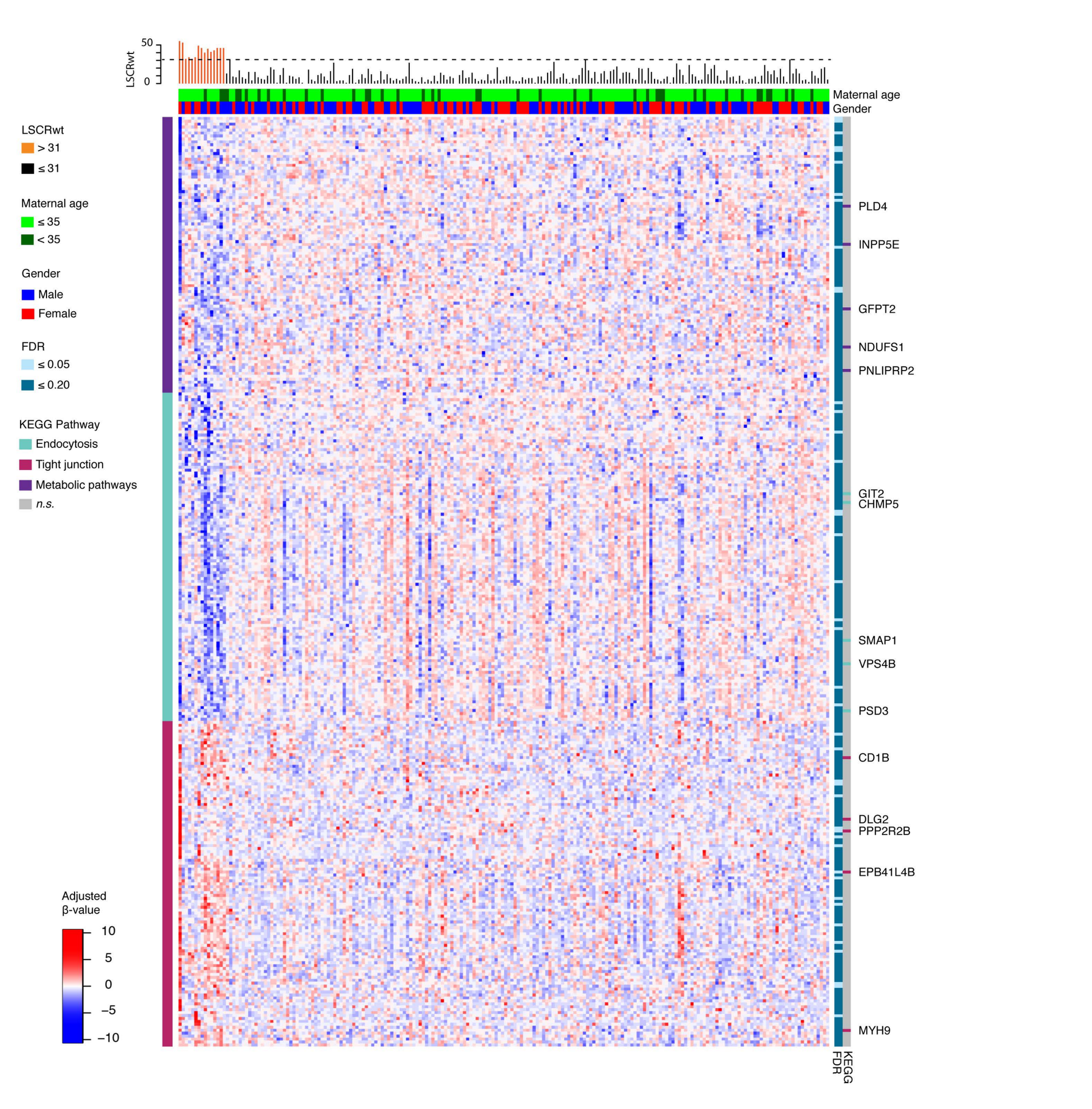
Table 2. Top 20 Significant KEGG Biological Pathways Affected by Maternal Lifetime Stress, Grouped by KEGG Class

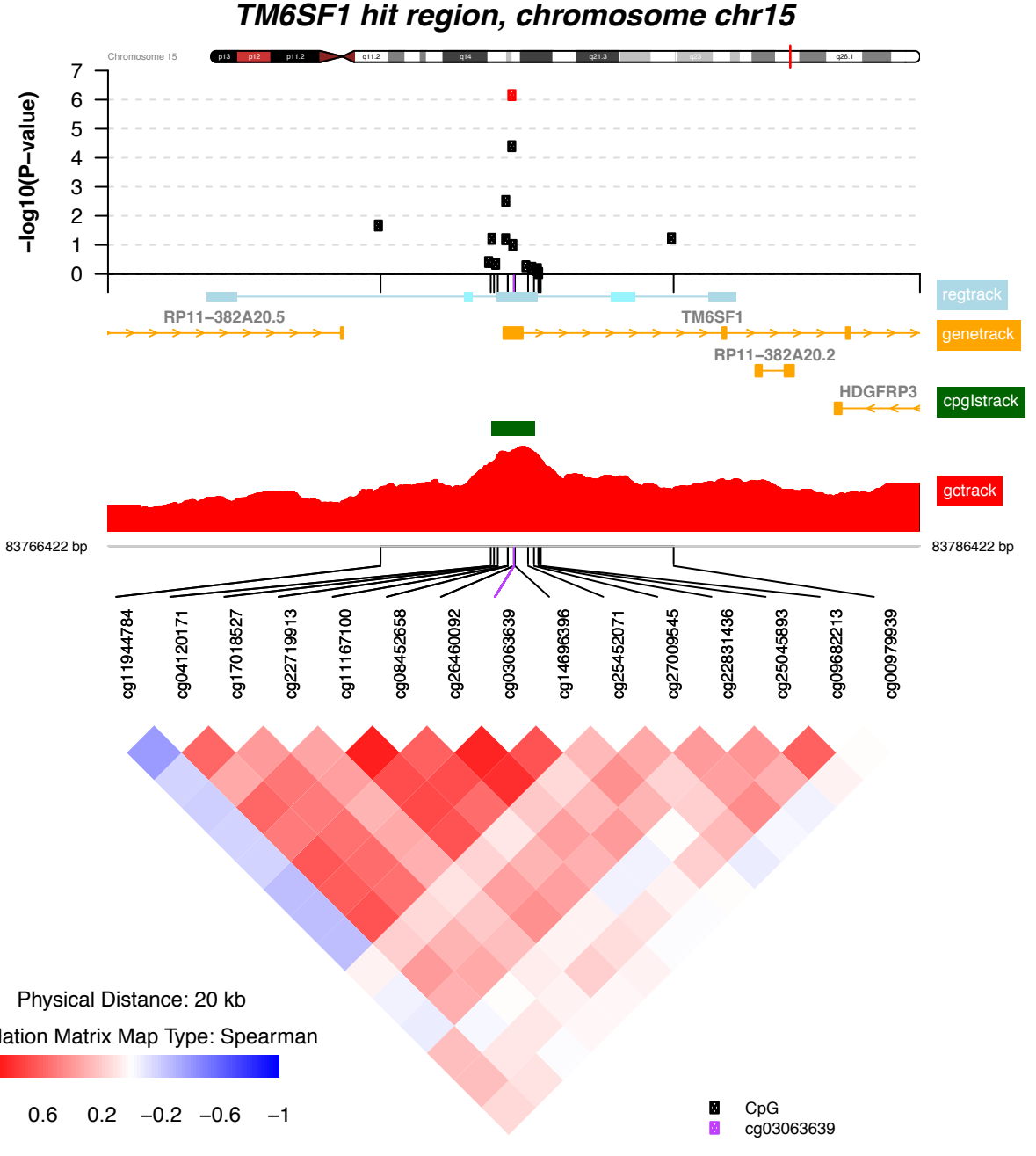
KEGG Class/Path ID	***KEGG Pathway Name***	***N***	***DE***	***FDR***
Cellular Processes				
hsa04530	Tight junction	162	9	2.11E-05
hsa04144	Endocytosis	252	13	1.00E-06
Environmental Information Processing				
hsa04151	PI3K-Akt signaling pathway	317	13	6.56E-06
hsa04080	Neuroactive ligand-receptor interaction	257	11	5.67E-06
Hsa04015	Rap1 signaling pathway	206	11	6.93E-06
Human Diseases				
hsa05165	Human papillomavirus infection	302	11	0.0002
hsa05200	Pathways in cancer	384	15	4.74E-06
Metabolism				
hsa00310	Lysine degradation	56	9	3.04E-08
hsa01100	Metabolic pathways	1190	20	6.41E-05
Organismal Systems/Development				
hsa04914	Progesterone-mediated oocyte maturation	86	7	4.42E-05

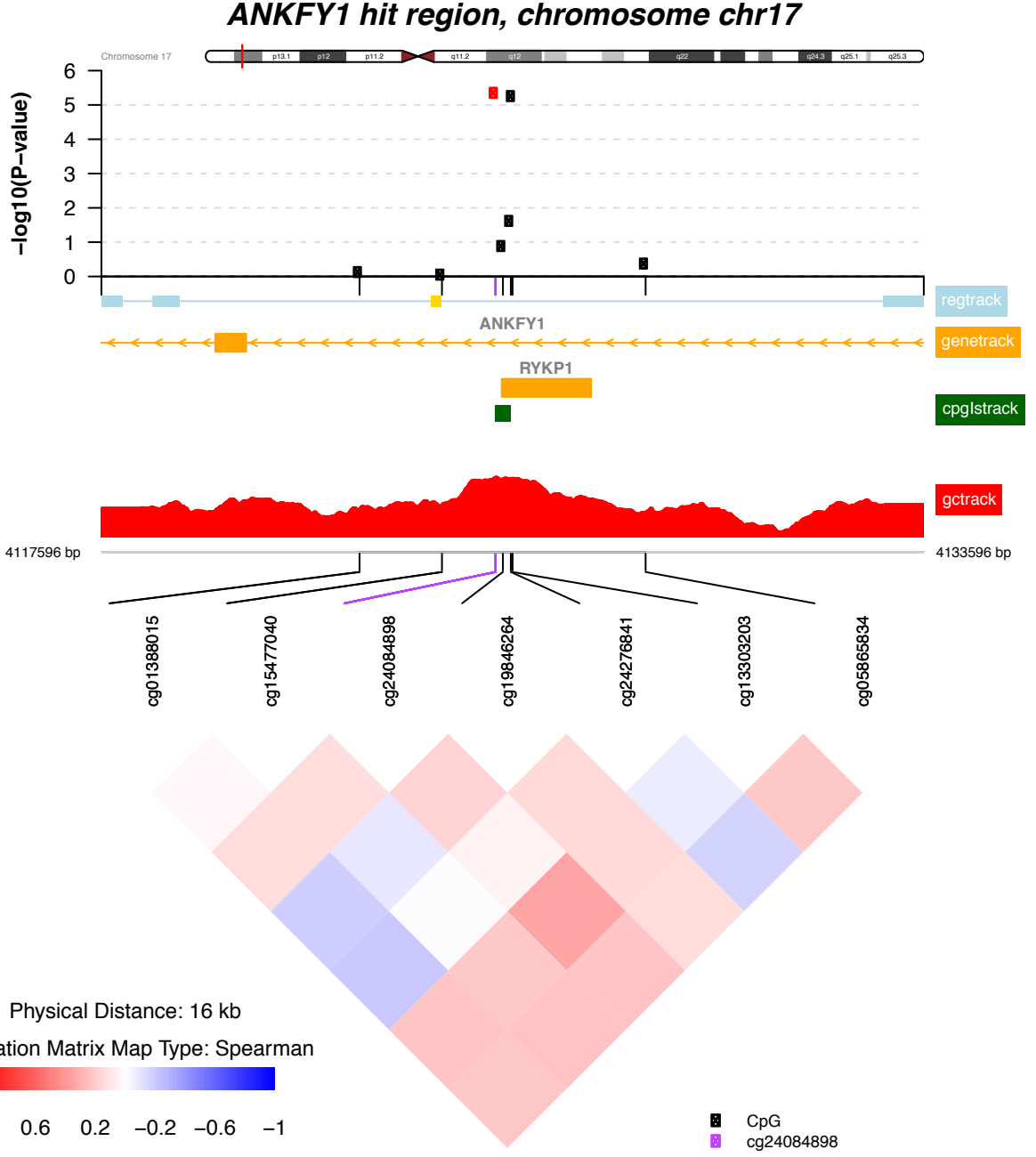
Based on 616 significant probes (FDR=0.20) using a background probe set based on the 365,193 probes used in association testing. Abbreviations: N, number of genes corresponding to the full set of probes used in our association test that belong to that pathway; DE, number of genes corresponding to our set of significant probes (FDR =0.20) that belong to the KEGG pathway in question; FDR, false discovery rate using Benjamini-Hochberg correction.


485,577
1,217 Failed probes
484,360
64 SNPs
484,296
3,089 CpH sites
481,207
29,127 Cross-reactive probes
452,080
74,645 CpG sites with SNPs
377,435
9,371 Probes in X or Y chromosome
368,064
2,871 probes with multimodal distribution
365,193


cg22065664
SMAP1
EPB41L4B
INPP5E
GSTP1
TM6SF1
ANKFY1
−log10(p−value)
Chromosome
12
10
8
6
4
2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22


LSCRwt
50
0
Maternal age
Gender
LSCRwt
> 31
≤ 31
Maternal age
≤ 35
< 35
Gender
Male
Female
FDR
≤ 0.05
≤ 0.20
KEGG Pathway
Endocytosis
Tight junction
Metabolic pathways
n.s.
Adjusted
β-value
10
5
0
−5
−10
PLD4
INPP5E
GFPT2
NDUFS1
PNLIPRP2
GIT2
CHMP5
SMAP1
VPS4B
PSD3
CD1B
DLG2
PPP2R2B
EPB41L4B
MYH9
KEGG
FDR


TM6SF1 hit region, chromosome chr15
Chromosome 15
p13
p12
p11.2
q11.2
q14
q21.3
q26.1
−log10(P−value)
7
6
5
4
3
2
1
0
regtrack
RP11−382A20.5
TM6SF1
genetrack
RP11−382A20.2
HDGFRP3
cpgIstrack
gctrack
83766422 bp
83786422 bp
cg11944784
cg04120171
cg17018527
cg22719913
cg11167100
cg08452658
cg26460092
cg03063639
cg14696396
cg25452071
cg27009545
cg22831436
cg25045893
cg09682213
cg00979939
Physical Distance: 20 kb
Correlation Matrix Map Type: Spearman
1
0.6
0.2
−0.2
−0.6
−1
CpG
cg03063639


ANKFY1 hit region, chromosome chr17
Chromosome 17
p13.1
p12
p11.2
q11.2
q12
q22
q24.3
q25.1
q25.3
−log10(P−value)
6
5
4
3
2
1
0
regtrack
ANKFY1
genetrack
RYKP1
cpgIstrack
gctrack
4117596 bp
4133596 bp
cg01388015
cg15477040
cg24084898
cg19846264
cg24276841
cg13303203
cg05865834
Physical Distance: 16 kb
Correlation Matrix Map Type: Spearman
1
0.6
0.2
−0.2
−0.6
−1
CpG
cg24084898

ONLINE DATA SUPPLEMENT

Cumulative lifetime maternal stress and epigenome-wide placental DNA methylation in the PRISM cohort

Kelly J. Brunst [a]*, Nicole Tignor [b]*, Allan Just [c], Zhonghua Liu [d], Xihong Lin [d], Michele R. Hacker [e], Michelle Bosquet Enlow [f], Robert O. Wright [c], Pei Wang [b], Andrea Baccarelli [g], Rosalind J. Wright [c, h]

*These authors contributed equally to this work

Objective

This study evaluates the relationship between maternal lifetime stress and DNA methylation in the placentas of 207 newborns from a multi-ethnic prenatal birth cohort.

Additional Methods/Results

Maternal lifetime exposure to stress and potentially traumatic events was assessed using the Life Stressor Checklist-Revised (LSC-R).[1] Placenta tissues were collected at birth and sampled from the fetal side. Placentas were stored at -80°C. DNA was isolated using Qiagen Tissue DNA extraction kits (Qiagen, Valencia CA) and quantified using an Implen Nanophotometer Pearl (Westlake Village, CA). 500ng of DNA was bisulfite-treated using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA) analyzed by the Infinium Methylation Assay. *Illumina Infinium HumanMethylation450 array:* HumanMethylation450 BeadChips (Illumina Inc., San Diego, CA, USA) were used to interrogate 485577 DNA methylation sites and to generate a measure of the methylation proportion at each site.

Association analysis was conducted using a semi-continuous model, linear regression, and a generalized additive model (GAM) (**see Supplement Table S1**). For the semi-continuous model, we considered many values of α corresponding to percentile increments from the 80th to the 96th percentile of LSCRwt. We selected the semi-continuous model where α =31 corresponding to the 92nd percentile of LSCRwt. This model shows low genomic inflation (λ_{GC}=1.158, **see Supplement Figure S1**), maintains an adequate number of samples above the threshold (n=15), and demonstrates enhanced power to detect associations as compared to the linear regression model. The result of GAM, on the other hand, showed a worse control of type-I errors than the semi-continuous regression model, giving a much higher genomic inflation factor of λ_{GC}=1.26 (**see Supplementary Table S1**).

To further substantiate our findings, we also conducted a combined stability selection and permutation test approach to identify methylation probes significantly associated with LSCRwt scores. First, we generated 100 data sets by subsampling two thirds of the participants in the original data (n_{sub}=138) and conducted the semi-continuous association test described above for all probes based on each subsampling data set. We recorded the selection frequencies, *k* (ranging from 0 to 100), of each probe as the number of times the probe was selected across 100 subsampling runs at a p-value cutoff of α1 = 0.00001. We then permuted the order of stress scores and repeated the above subsampling procedures. We derived the final FDR by comparing the distribution of selection frequencies, *k,* in the permuted runs

to that on the observed data. The results of all models tested can be found in s**upplementary Tables S1 and S2**.

Heatmaps were used to visualize patterns of methylation. First, methylation beta values of the 616 probes significant at FDR = 0.20 were adjusted for genetic background and cell type heterogeneity using the same set of principal component adjustments in the semi-continuous model, and were then normalized to have mean 0 and sd 1. Hierarchical clustering based on complete-linkage was performed based on adjusted methylation values of the 616 probes. The gap statistic[2], which provides a measure of error in terms of within cluster-dispersion, was calculated for cluster numbers 1 through 50 and plotted (**Supplemental Figure S2**). Based on values of gap statistic, we select number of clusters to be K=10. **Supplemental figure S3** shows a heatmap corresponding to cluster number of 10, where rows (probes) were ordered based on the hierarchical clustering results (left bar); probe sets corresponding to each of the top three clusters were boxed in yellow; a bar showing the category of statistical significance for each probe is shown to the left. Columns (participants) were organized by separately clustering methylation values for samples above and below the α=31 threshold. Samples above the threshold were clustered into three groups (left), while samples below were randomly ordered. Bars showing categories of LSCRwt, maternal age, and child's gender are shown for each participant (top).

Scatterplots were created for the significant hits at an FDR=0.05 level (**see Supplementary Figure S4**).

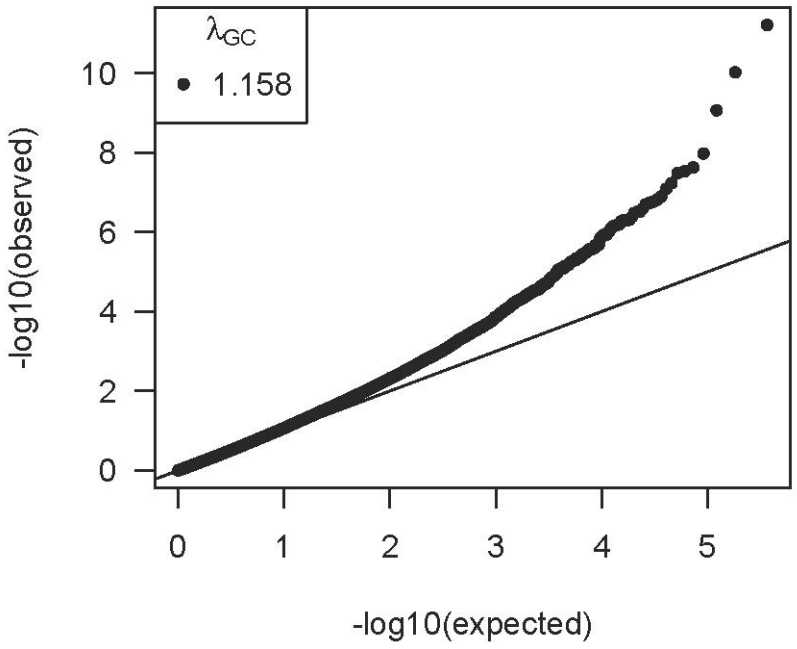


Figure S1. Quantile-quantile (Q-Q) plot for the pt92 semi-continuous model. The x-axis shows the expected –log10 p-value, and the y-axis shows the observed –log10 p-value based on the ANOVA F-test of association. The genomic inflation factor, the ratio of the medians of the observed and expected p-value distributions (chi-squared), is shown in the top left.

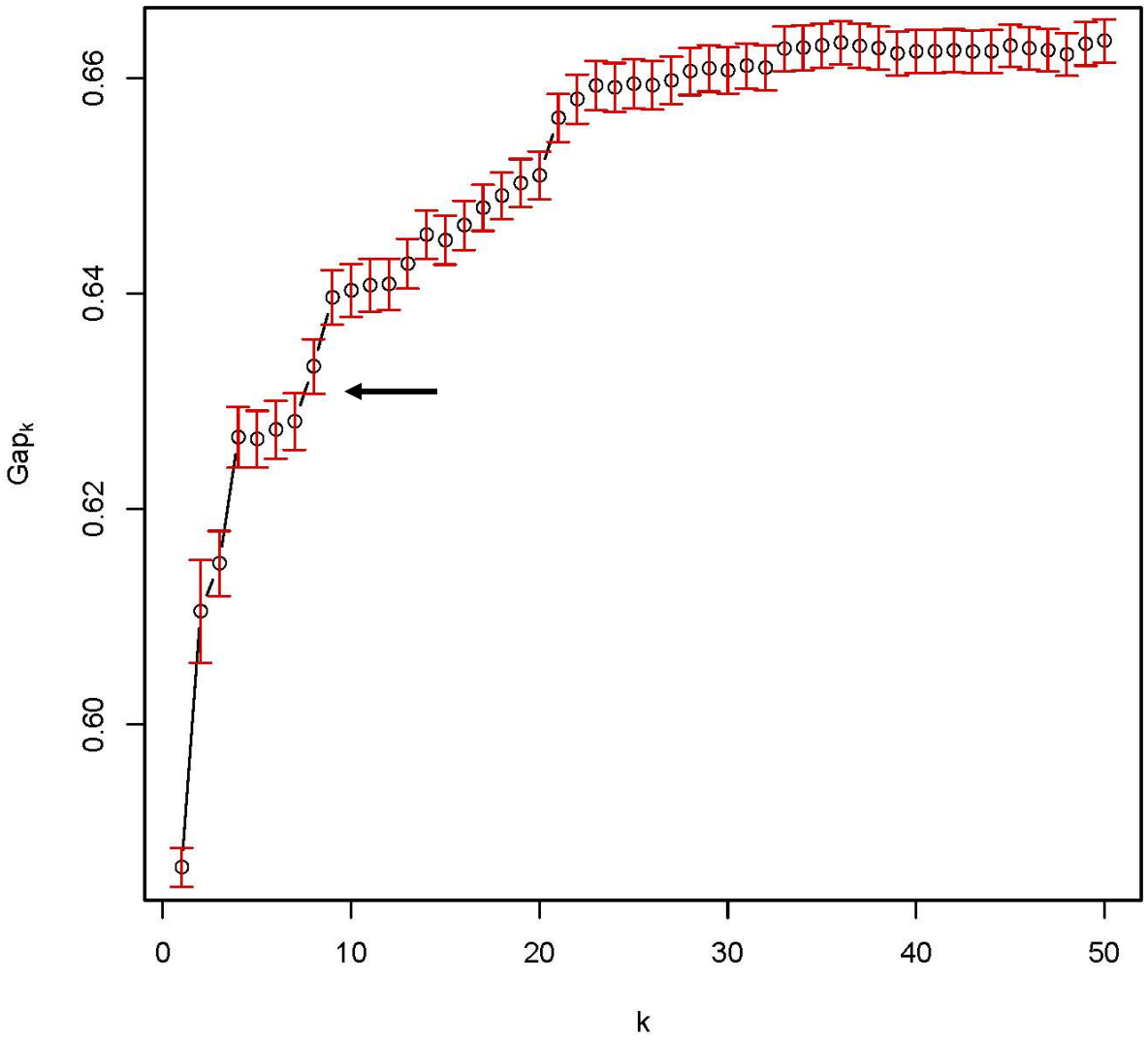


Figure S2. Gap statistic for different numbers of clusters. The gap statistic, which provides a measure of error in terms of within cluster-dispersion, was calculated for cluster numbers 1 through 50 (k) and plotted. The data point corresponding to the selected cluster size (K=10) is labelled by ←.

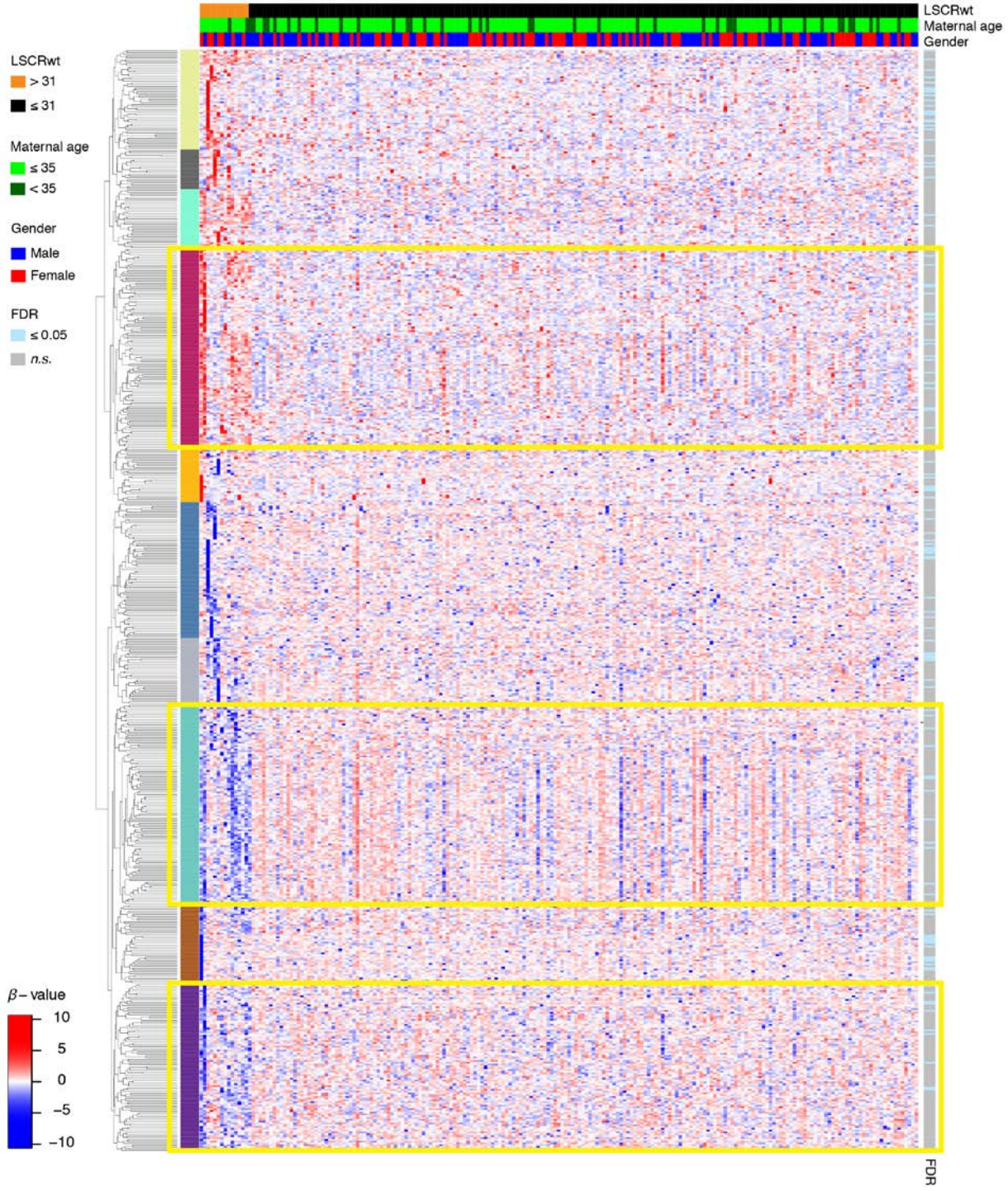


Figure S3. Heatmap of adjusted β -values. Heatmap is based on methylation beta values for 616 significant probes (FDR < 0.20) pre-adjusted for genetic and cell background. K-means clustering was performed on the adjusted methylation values and an optimal cluster size (K=10) was selected based on the gap statistic. Cluster assignments are shown in the bar to the left. KEGG pathway enrichment testing was performed for the collection of 616 genes.

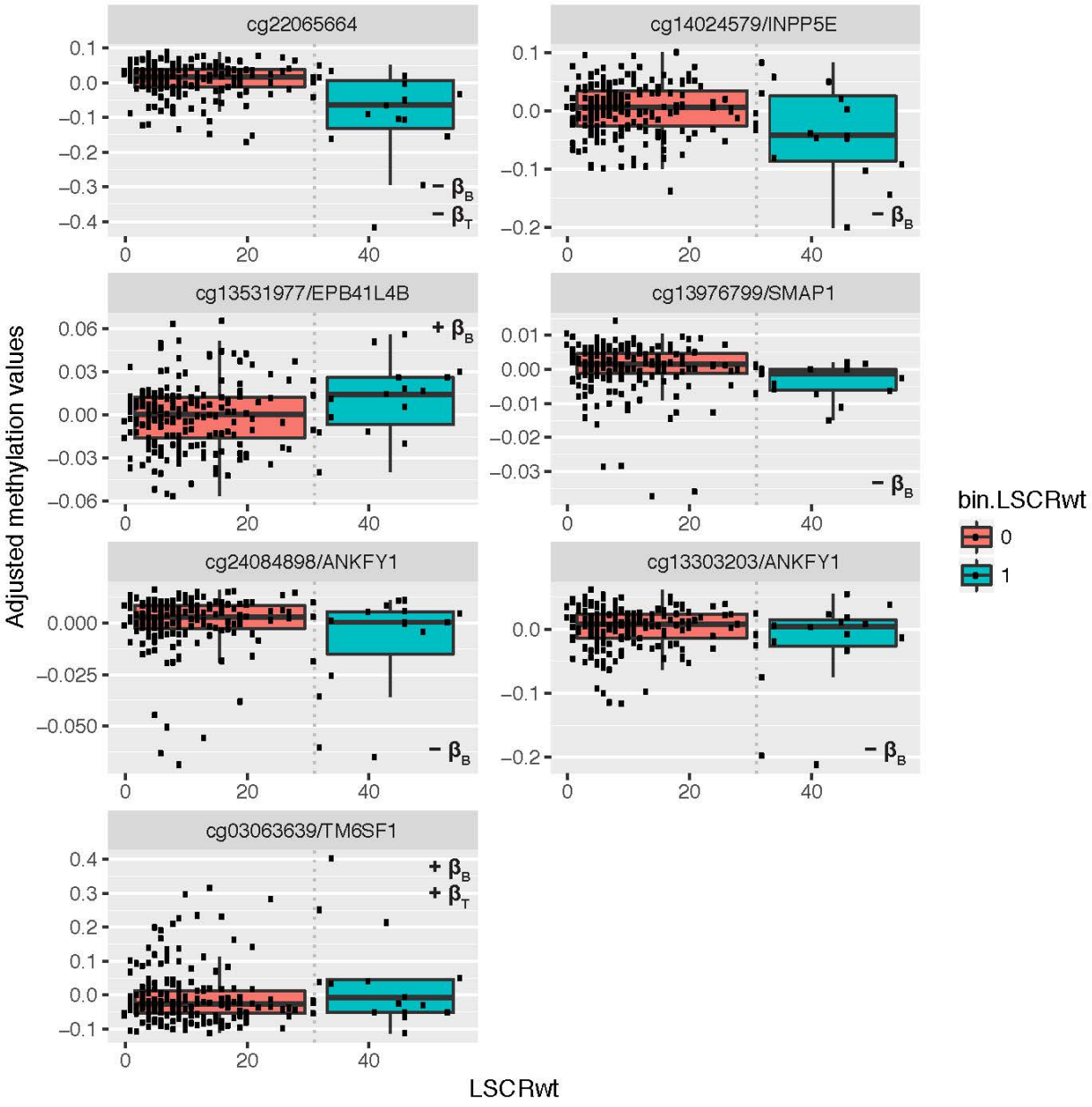


Figure S4: Boxplots and scatterplots for selected probes. For each probe, the corresponding scatterplot shows adjusted methylation values (y-axis) for each sample arranged on the x-axis by maternal stress score. The vertical dashed line shows the location of the alpha threshold (α=31) used to construct the binary and truncated variables used in our semi-continuous genome-wide tests of association. The direction of the estimated beta coefficients for each of these terms (β_T and β_B) are indicated when significant ($p<0.05$) and when the magnitude of beta is larger than 0.001.

References

1. Wolfe J, Kimerling R. *Gender issues in assessment of posttraumatic stress disorder.* New York: Guilford; 1997.
2. Tibshirani R, Walther G, Hastie T. Estimating the number of data clusters via the Gap statistic. *Journal of the Royal Statistical Society.* 2001;63:411-423.