Maternal attachment insecurity, maltreatment history, and depressive symptoms are associated with broad DNA methylation signatures in infants

Thalia K. Robakis1✉, Marissa C. Roth2, Lucy S. King3, Kathryn L. Humphreys4, Marcus Ho4, Xianglong Zhang4, Yuhao Chen5, Tongbin Li5, Natalie L. Rasgon3, Kathleen T. Watson4, Alexander E. Urban4 and Ian H. Gotlib3

© The Author(s), under exclusive licence to Springer Nature Limited 2022

The early environment, including maternal characteristics, provides many cues to young organisms that shape their long-term physical and mental health. Identifying the earliest molecular events that precede observable developmental outcomes could help identify children in need of support prior to the onset of physical and mental health difficulties. In this study, we examined whether mothers’ attachment insecurity, maltreatment history, and depressive symptoms were associated with alterations in DNA methylation patterns in their infants, and whether these correlates in the infant epigenome were associated with socioemotional and behavioral functioning in toddlerhood. We recruited 156 women oversampled for histories of depression, who completed psychiatric interviews and depression screening during pregnancy, then provided follow-up behavioral data on their children at 18 months. Buccal cell DNA was obtained from 32 of their infants for a large-scale analysis of methylation patterns across 5 x 10⁶ individual CpG dinucleotides, using clustering-based significance criteria to control for multiple comparisons. We found that tens of thousands of individual infant CpGs were alternatively methylated in association with maternal attachment insecurity, maltreatment in childhood, and antenatal and postpartum depressive symptoms, including genes implicated in developmental patterning, cell-cell communication, hormonal regulation, immune function/inflammatory response, and neurotransmission. Density of DNA methylation at selected genes from the result set was also significantly associated with toddler socioemotional and behavioral problems. This is the first report to identify novel regions of the human infant genome at which DNA methylation patterns are associated longitudinally both with maternal characteristics and with offspring socioemotional and behavioral problems in toddlerhood.

Molecular Psychiatry (2022) 27:3306–3315; https://doi.org/10.1038/s41380-022-01592-w

INTRODUCTION

Developing organisms receive cues from their early environment that guide their growth and development. These cues lead them to optimize their health and reproductive capacity for the environments they can expect to encounter as adults [1]. The ability to adapt behavior patterns flexibly to environmental cues enables the organism to thrive in a wide range of potential environments. Humans, in particular, are masters of behavioral adaptation to environmental variability.

The hypothesis of Developmental Origins of Health and Disease (DOHaD) posits that the early environment shapes the organism in ways that will affect tendencies toward health and disease in later life. This hypothesis, first articulated by David Barker in the context of fetal origins of cardiovascular disease [3], has also been extensively applied to psychiatric and behavioral adaptations [4].

In high-stress environments in which long reproductive life-spans are less likely, early reproductive success may be privileged over, for example, longevity or health in midlife and later [5–8]. Characteristics that precede early parenthood are also associated with worse mental and physical functioning in later life [9–11]. Thus, in order to mitigate mid- and late-life health risks that occur as byproducts of early life adaptation to particular environments, it is important to understand the complex physiological and behavioral effects that are set in motion by environmental exposures in early life [12].

Maternal characteristics are well established as major influences on infant development [13–15]. Even ordinary variations in maternal behavior have been shown to have lasting and detectable influences on child development [16]. Maternal characteristics may affect infants’ environments by influencing parenting behavior [17], the intrauterine milieu [18], the availability of resources, and exposure to adversity postnatally [19]. In turn, variation in the environment associated with maternal characteristics may alter the infant epigenome and, ultimately, child socioemotional and behavioral functioning. A growing literature is examining the varied physiological mechanisms that mediate adaptations to early life experience.
Methylation of cytosine-guanine residues in nuclear DNA is one important mechanism through which aspects of the early environment can exert lasting effects on transcription, translation, and protein expression and, therefore, on individual phenotype and behavior [20]. In the mid-2000s, researchers documented that DNA methylation of the glucocorticoid receptor in mice was both necessary and sufficient for the effects of maternal care in early life on adult behavioral patterns [21, 22]. This proof of principle provided the impetus for a broad array of investigations examining the role of epigenetic modifications to DNA during development in shaping health and behavioral outcomes by aspects of the early environment, including maternal behavior [23–29].

Maternal attachment style, maltreatment history, and depressive symptoms may combine to affect maternal behavior in ways that have the potential to shape offspring outcomes. Understanding the molecular processes that precede clinically observable offspring outcomes could facilitate the early identification of children at risk for adverse behavioral and physical development. Elucidating the relevant molecular events that mediate the association between the early environment and clinically observable outcomes could also guide the development of new approaches to early intervention.

We and others have shown that maternal attachment style, maltreatment history, and depressive symptoms are associated with detectable differences in preschooler behaviors, including increased frequency of both internalizing and externalizing behaviors [30–34]. An important next step is to investigate whether there are molecular events that are detectable in infancy that could prefigure the development of risk for socioemotional and behavioral problems in young children.

In the present study, we use next-generation sequencing along with clustering-based significance criteria to control for multiple comparisons to investigate the associations of infant patterns of DNA methylation across five million CpGs with three selected maternal characteristics: attachment style, maltreatment history, and depressive symptoms. We sought to identify molecular pathways in infants that are epigenetically modulated in association with each of these maternal characteristics and to explore the overlap and divergence among these pathways. We hypothesized that genetic regions in which patterns of methylation are systematically associated with maternal attachment style, maltreatment history, and depressive symptoms could be identified in infants. We hypothesized further that DNA methylation patterns in these regions would also be associated with subsequent socioemotional and behavioral problems in toddlerhood.

**METHODS**

**Participants**

This study was approved by the Stanford University IRB for Human Subjects Research, and all study participants completed informed consent prior to study participation. Participants were a subset of mother-infant dyads who participated in a larger observational study (PI, IMG). The cohort was oversampled for mothers with histories of depression and maltreatment history. Inclusion criteria were that mothers were age at least 18 years, had uncomplicated singleton pregnancies, and were fluent in English. Exclusion criteria were multiple or highly medically complicated pregnancy or insufficient English to participate. One hundred fifty-six women provided clinical and behavioral data (behavioral cohort), and 32 infants provided buccal swabs (epigenetic subcohort). One mother who provided behavioral data, but not an epigenetic swab from her infant, reported taking an antidepressant medication in pregnancy (bupropion). Information on psychotherapeutic treatment was not obtained.

**Measures**

When they were between 16–35 weeks gestation, women participated in an in-person visit that included administration of the depression modules (i.e., past and current major depressive disorder) from the Structured Clinical Interview for DSM-5 Disorders [35] and assessment of antenatal depressive symptoms (EPDS) [36]. At 6 months postpartum, mothers again completed the EPDS, a retrospective report of their history of childhood maltreatment using the Childhood Trauma Questionnaire (CTQ) [37], and attachment insecurity was assessed via the Attachment Style Questionnaire (ASQ) [38]. In addition, at this visit, a buccal swab (Mawu DNA Technologies) was collected from 32 of the infants for DNA analysis. When offspring were age 18 months, mothers reported on their socioemotional and behavioral problems using the Infant-Toddler Social-Emotional Assessment (ITSEA) [39].

The EPDS is a specialized self-report measure of depressive symptoms that is used for both screening and severity assessment in perinatal women and has been validated cross-culturally [40]. (Cronbach’s alpha = 0.88.) The ASQ is a self-report measure of attachment style [41, 42] that has been used previously in pregnant women [43] and in the study of postpartum depression [44]. The ASQ (alpha = 0.93) has five subscales. For this analysis, we computed an adjusted total attachment insecurity score in which the Confidence score was subtracted from the sum of the scores of the four other subscales, consistent with our previous work [45, 46]. The CTQ [37] is a self-report measure that yields maltreatment severity scores across five categories of childhood maltreatment. For this analysis, we combined scores across each category of maltreatment to yield an index of total severity of childhood maltreatment for each participant. Alpha across all items was 0.96. The ITSEA [39] is a broadband measure that yields information about social-emotional and behavioral competencies and difficulties in young children between the ages of 12 and 36 months. In this study we analyzed eight subscales: aggression/defiance (alpha = 0.73), activity/impulsivity (alpha = 0.57), depression/withdrawal (alpha = 0.71), general anxiety (alpha = 0.56), negative emotionality (alpha = 0.83), empathy (alpha = 0.73), social relatedness (alpha = 0.60), and imitation/play (alpha = 0.65). Data were managed using REDCap electronic data capture tools hosted at Stanford University [47, 48].

**DNA methylation analysis**

Genomic DNA was extracted from buccal swabs using the Blood & Tissue Culture DNA mini kit (Qiagen). Library construction was performed using SeqCap Epi Enrichment System (Roche), as previously described [46]. Buccal cells were chosen due to their better cell type purity and less divergent global epigenic profile versus other commonly available peripheral tissues such as blood or saliva [49, 50]. Bisulfite converted libraries captured by SeqCap Epi CpGiant Probes kit (the size of target regions is 80.5 Mb with >5.5 million CpGs) of all the samples were sequenced at the Functional Genomics Facility (Stanford University) on Illumina HiSeq 4000 platform by 2×150 paired-end sequencing with an average of 70 million reads generated for each sample. After trimming the adapters and low-quality ends by Cutadapt 5.1, the reads were mapped to human RefSeq genome hg19. QC for the data was performed on the FASTQ data using FastQC version 0.11.5. Low-quality bases were trimmed using Trimmomatic2 version 0.33.

Trimmed reads were aligned to the human reference genome (hg19) using Bismark3 (version 0.14.5), and PCR duplicates were then removed using Bismark as well. The output PERL files were converted to BAM files and sorted using SAMtools4 (version 1.3.1). CpG methylation calling for was carried out using function processBismarkAlignin R (version 3.2.2) package methylKit5 (version 0.99.2). Then, methylation densities at variant CpG loci (CpGs) for all 29 infants were merged together. CpGs located at ChrX and ChrY were removed from downstream analysis due to known association with child sex.

**Statistical analysis**

We developed a sliding-window based statistical analysis approach similar to the swDMR method previously described [51], which we have detailed previously [46]. The key difference is that instead of identifying regions (DMRs) differentially methylated between two groups of subjects, we identified methylation regions that are significantly associated with a continuous variable. Briefly, starting from each CpG locus, a window is defined if more than five CpGs are identified within a 1000 bp span. For each window identified, mean methylation density is calculated for each subject. Association analysis between each maternal characteristic and DNA methylation densities of infants is performed using linear regression, with adjustments for eight covariates. The multivariate regression model assumes a linear relationship between each of the independent variables.
(four maternal characteristics and eight covariates) and the dependent variables (methylation levels for infants). In statistical terms, the model is described as follows: methylation level ~ maternal clinical variable + age of child at time of swab + annual household income bracket + mother’s level of education + gestational age at delivery + child sex + mother’s age + mother’s smoking status + child race. The Benjamini–Hochberg [52] method is then applied to calculate false discovery rates (FDRs) based on the p values, with cutoff FDR = 0.05. Significant regions are extended by merging overlapping CpGs among nearby windows. The p values and FDRs are then recalculated to select new windows. This process is repeated iteratively until no neighboring significant regions within a 100 bp distance can be merged.

Molecular function classification
The online molecular function classification tool Panther [53] was used to explore the molecular function classifications for the obtained results.

Association of maternal characteristics with toddler behavioral outcomes
We computed Pearson correlations between each of the four maternal characteristics (childhood maltreatment, attachment insecurity, and antenatal and postpartum depressive symptoms) and each of the eight subscales of the ITSEA (aggression/defiance, activity/impulsivity, depression/withdrawal, general anxiety, negative emotionality, empathy, social relatedness, and imitation/play). We applied Bonferroni correction to the 32 tests.

Association of methylation density in selected candidate genes with toddler behavioral outcomes
In an additional exploratory analysis, two candidate genes were selected as putative intermediary variables to determine whether the infant DNA methylation signatures that were associated with maternal characteristics are also related to toddler behavior at 18 months. The Wnt pathway was selected for attention because it had the largest number of associated results for all of the tested maternal characteristics. Of all results matched to this pathway, two individual genes, GNG8 and NFATC1, contained regions alternatively methylated in association with all tested maternal characteristics. Therefore, these two genes were selected as candidate intermediary variables and assessed for associations with toddler behavioral outcomes using Pearson correlations. The three subscales of the ITSEA that were associated most strongly with each of the three tested maternal characteristics were selected as the dependent variables. Statistical correction by the method of Cheverud [54] was conducted using SPSS Statistics 27.

RESULTS
Sample demographics
The demographics of the sample reflect those of the San Francisco Bay Area’s Peninsula region, with an overrepresentation of highly educated individuals and persons of Asian descent and an underrepresentation of persons of African descent compared to the U.S. as a whole. The majority of mothers were employed. Half of the sample had histories of depressive disorders, but only two of the Bay Area’s Peninsula region, with an overrepresentation of highly educated individuals and persons of Asian descent and an underrepresentation of persons of African descent compared to the U.S. as a whole. The majority of mothers were employed. Half of the sample had histories of depressive disorders, but only two of the

| Variable                  | Epigenetic sample | Mean | SD  |
|---------------------------|-------------------|------|-----|
| Mother’s age              |                   | 31.39| 5.26|
| Mother’s parity           |                   | 0.90 | 0.98|
| Maternal depression       |                   |      |     |
| EPDS in pregnancy         |                   | 7.90 | 6.34|
| EPDS at 6 months postpartum|               | 7.06 | 5.38|
| Child’s race              |                   |      |     |
| White Non-Hispanic        |                   | 16   | 51.61|
| Hispanic                  |                   | 5    | 25.81|
| Black or Af-Am            |                   | 1    | 3.23 |
| Asian                     |                   | 8    | 25.81|
| Pacific Islander          |                   | 0    | 0    |
| Other                     |                   | 1    | 3.23 |
| Child sex                 |                   |      |     |
| Male                      |                   | 14   | 45.16|
| Female                    |                   | 17   | 54.84|
| Mother’s employment       |                   |      |     |
| Homemaker                 |                   | 7    | 22.58|
| Employed for wages        |                   | 18   | 58.07|
| Self-employed             |                   | 2    | 6.45 |
| Unemployed                |                   | 2    | 6.45 |
| Other                     |                   | 2    | 6.45 |
| Mother’s level of education|                |      |     |
| Some HS                   |                   | 1    | 3.23 |
| HS degree                 |                   | 0    | 0    |
| Some college              |                   | 3    | 9.68 |
| Associate’s/technical     |                   | 3    | 9.68 |
| Bachelor’s                |                   | 8    | 25.81|
| Graduate                  |                   | 16   | 51.61|
| Mother’s history of depression|          |      |     |
| Yes                       |                   | 16   | 51.61|
| No                        |                   | 15   | 48.39|
| Mother depressed at 6-month follow-up| |      |     |
| Yes                       |                   | 2    | 6.45 |
| No                        |                   | 29   | 93.54|

Molecular pathways identified
We used Panther [53] to identify molecular pathways represented by the coding regions we identified. Pathways with functions in neurodevelopment, signaling, cell-cell communication, and inflammation were prominently represented (Table 2).
### Table 2. Top ten biological pathways with components alternatively methylated in infant DNA in association with each maternal clinical variable.

| Pathway                                                                 | CTQ 152 total pathways | Genes % | Genes pathway hits | ASQ 143 total pathways | Genes % | Genes pathway hits | Preg EPDS 148 total pathways | Genes % | Genes pathway hits | Postpart EPDS 144 total pathways | Genes % | Genes pathway hits |
|------------------------------------------------------------------------|------------------------|---------|--------------------|------------------------|---------|--------------------|-------------------------------|---------|--------------------|-------------------------------|---------|--------------------|
| Wnt signaling pathway                                                  | 127                    | 2.1%    | 5.6%               | Wnt signaling pathway  | 89      | 1.9%               | 5.0%                         | 127     | 2.0%               | 5.0%                         | 131     | 2.2%               |
| Gonadotropin-releasing hormone receptor pathway                        | 98                     | 1.6%    | 4.3%               | Gonadotropin-releasing hormone receptor pathway | 76      | 1.6%               | 4.2%                         | 110     | 1.7%               | 4.3%                         | 91      | 1.5%               |
| Inflammation mediated by chemokine and cytokine signaling pathway      | 87                     | 1.4%    | 3.8%               | Inflammation mediated by chemokine and cytokine signaling pathway | 68      | 1.4%               | 3.8%                         | 97      | 1.5%               | 3.8%                         | 90      | 1.5%               |
| Integulin signalling pathway                                           | 79                     | 1.3%    | 3.5%               | Integulin signalling pathway | 56      | 1.2%               | 3.1%                         | 92      | 1.4%               | 3.6%                         | 87      | 1.4%               |
| Cadherin signalling pathway                                            | 71                     | 1.2%    | 3.1%               | Angiogenesis            | 54      | 1.1%               | 3.0%                         | 79      | 1.2%               | 3.1%                         | 72      | 1.2%               |
| Angiogenesis                                                           | 68                     | 1.1%    | 3.0%               | CCXR signalling map     | 52      | 1.1%               | 2.9%                         | 73      | 1.1%               | 2.9%                         | 67      | 1.1%               |
| CCXR signalling map                                                    | 65                     | 1.1%    | 2.9%               | Cadherin signalling pathway | 49      | 1.0%               | 2.7%                         | 66      | 1.0%               | 2.6%                         | 67      | 1.1%               |
| Huntington disease                                                     | 63                     | 1.0%    | 2.8%               | Huntington disease      | 48      | 1.0%               | 2.7%                         | 64      | 1.0%               | 2.5%                         | 60      | 1.0%               |
| Heterotrimeric G-protein signaling-G alpha and Gs alpha mediated pathway | 58                     | 1.0%    | 2.5%               | CCXR signalling map     | 46      | 1.0%               | 2.6%                         | 63      | 1.0%               | 2.5%                         | 57      | 0.9%               |
| Alzheimer disease-presenilin pathway                                   | 48                     | 0.8%    | 2.1%               | EGF receptor signalling pathway | 40      | 0.8%               | 2.2%                         | 61      | 0.9%               | 2.4%                         | 55      | 0.9%               |
Fig. 1 Overlap among biological pathways with components alternatively methylated in infants in association with each maternal variable. The vast majority of pathways are affected in all four conditions. Maternal childhood maltreatment (CTQ) has the most unique pathways not associated with the other variables (7). Pathways altered in association with maternal attachment insecurity (ASQ) and depressive symptoms (EPDS in pregnancy or postpartum) are more overlapping (2 pathways in ASQ but not EPDS, 4 pathways in EPDS but not ASQ). http://bioinformatics.psb.ugent.be/webtools/Venn/

Supplementary Table 2 for full pathway list), 132 contained components that were alternatively methylated in association with all three maternal characteristics (Fig. 1).

Toddler behavioral outcomes

Offspring socioemotional and behavioral problems in toddlerhood were associated with maternal attachment insecurity, maltreatment history, and depressive symptoms in pregnancy and postpartum (Fig. 2). Five of eight ITSEA subscales were associated with maternal postnatal depressive symptoms; six were associated with maternal attachment insecurity; and one was associated with maternal childhood maltreatment history (p < 0.05). Associations of maternal postpartum depressive symptoms with one ITSEA subscale (aggression) and of maternal attachment insecurity with three ITSEA subscales (aggression, negative emotion, and play) survived Bonferroni correction (p < 0.0016).

Maternal attachment insecurity and maternal postnatal depressive symptoms were associated with similar child socioemotional and behavioral problems in toddlers: both maternal characteristics demonstrated strong positive associations with child aggression and negative emotionality. In contrast, maternal history of childhood maltreatment showed only a weak positive association with toddler empathy that did not survive Bonferroni correction. Our results reflect those of many previous investigators in this area [55–60] but provided guidance for our candidate-gene investigation below.

Association of methylation density in selected candidate genes with toddler behavioral outcomes

We conducted an exploratory candidate-gene analysis to determine whether the infant DNA methylation signatures that were associated with maternal characteristics were also associated with toddler behavior at 18 months. Results associated with the Wnt pathway were chosen as a source of the candidate genes. Two individual genes, GNG8 and NFATC1, were selected as candidate intermediary variables (see Methods) and were assessed for associations with toddler behavioral outcomes. The three subscales of the ITSEA that were associated most strongly with each of the three tested maternal characteristics (Fig. 2) were selected as the dependent variables.

GNG8 included three regions that showed associations of methylation density with one or more maternal characteristics, and NFATC1 included 42 such regions, 8 of which were discarded for insufficient sequencing coverage. Two of the three GNG8 regions were correlated with the measure of toddler empathy (p ≤ 0.05). Five of the 34 NFATC1 regions were correlated with toddler aggression, negative emotion, or both (p ≤ 0.05). Multiple hypothesis correction was carried out following the method of Cheverud [52]. After correction, one of the GNG8 regions and one of the NFATC1 regions remained significant (Table 3). We did not conduct mediation tests as we did not expect that two selected genes out of thousands of potentially relevant candidates would carry statistically meaningful mediation.

DISCUSSION

Maternal attachment style, maltreatment history, and depressive symptoms all have the potential to affect the environment and, thus, the behavioral and physiological development of offspring. The molecular pathways by which these effects are transduced are not yet fully understood. Alteration of patterns of gene expression by environmental influences may be carried out in part by methylation of relevant regions of the genome; thus, identifying regions of the genome in which DNA methylation is associated with the factors of interest may yield clues to the biological pathways involved.

In this study we show that maternal attachment style, maltreatment history, and depressive symptoms are each associated with broad and overlapping peripheral DNA methylation signatures in women’s 6-month-old infants, as well as with socioemotional and behavioral outcomes in toddlerhood. Consistent with the fact that these maternal characteristics are associated with overlapping socioemotional and behavioral outcomes in offspring, they affect epigenetic regulation of many of the same genes and pathways in infants. Most prominently represented among the results were pathways involved with cell-cell communication, developmental patterning, growth, immune function/inflammatory response, and neurotransmission. Several of the most highly represented pathways have been identified in other studies of DNA methylation and early care experiences, including Wnt-β
Correlations of mean methylation density within selected genetic regions from the result set with selected infant behavioral outcomes.

| Region | ITSEA empathy Pearson correlation | p value | ITSEA aggression Pearson correlation | p value | ITSEA negative emotion Pearson correlation | p value | Results passing correction for multiple hypotheses are underlined. |
|--------|-----------------------------------|---------|------------------------------------|---------|------------------------------------------|---------|-----------------------------|
| region 1 | 0.114                            | 0.080   | 0.512**                           | 0.005   | 0.332                                    | 0.099   | Results passing correction for multiple hypotheses are underlined. |
| region 2 | 0.014                            | 0.027   | 0.410*                            | 0.048   | 0.234                                    | 0.079   | Results passing correction for multiple hypotheses are underlined. |
| region 3 | 0.023                            | 0.017   | 0.177                             | 0.032   | 0.079                                    | 0.005   | Results passing correction for multiple hypotheses are underlined. |
| region 19 | 0.054                            | 0.050   | 0.114*                            | 0.032   | 0.079                                    | 0.005   | Results passing correction for multiple hypotheses are underlined. |
| region 25 | 0.023                            | 0.017   | 0.177                             | 0.032   | 0.079                                    | 0.005   | Results passing correction for multiple hypotheses are underlined. |

Results passing correction for multiple hypotheses are underlined.

**Markers of cellular aging such as telomere length and electrokinetic index** [66] in buccal cells are also associated with physiological stress of various types, reflecting a body-wide burden of allostatic load. Autonomic and adrenocortical reactivity in children has been associated with shorter length of buccal cell telomeres [67]. Buccal epithelial cells are responsive to glucocorticoids [68], and methylation of the glucocorticoid receptor gene in buccal cells has been correlated with HPA-axis reactivity in response to social stress [69]. Thus, pathways relating to HPA-axis functions and stress response also have relevance to functions in this tissue type.

The results related to neurodevelopment and neurotransmission are more difficult to interpret. Multiple hits were retrieved that were related to axon guidance by netrins, Slit/Robo, and semaphorins, and to 5-HT and GABA signaling (Supplementary Table 1). Although several investigators have reported concordance in DNA methylation patterns between brain tissue and samples from the oral epithelium [70–73], the functional significance of this remains unclear. It is possible that there are functional implications related to environmental guidance of peripheral nerve cell axons, or to receipt of GABAergic and serotonergic signals from peripheral neurons. Alternatively, methylation of regions of the genome that are not normally expressed in buccal epithelium may occur as a byproduct, in parallel with functionally important methylation of those regions in other tissues, but without specific functional implications in the oral epithelium. A third possibility is that methylation in distal regions exerts regulatory effects elsewhere in the genome [74].

Regarding the direction of observed associations, both hypomethylation and hypermethylation were observed. Classically, hypermethylation in the promoter region blocks access of transcription machinery and reduces gene transcription [75], while hypermethylation in gene bodies typically directs the production of alternative transcripts [76] as well as the regulation of splicing [77].

We were not able to address gestational timing of exposures in the present study; however, we note that the infant global epigenetic profiles associated with antenatal and postnatal maternal depression were almost entirely overlapping (see Table 2 and Fig. 1), suggesting that more fine-grained dissections of the temporal course of depressive symptoms during pregnancy at an epigenomic level are unlikely to be illuminating for the question under study. This is in accordance with our previous work [78], where we compared leukocyte DNA methylation patterns across trimesters and postpartum. Consistent with the present study, we found that methylation in most regions of the genome was quite stable across pregnancy, with a few select regions showing unusual variability across trimesters. Based on these results, we propose that future epigenetic work on fetal vulnerability windows should focus in on the specific genomic regions where such temporal variability is observed. Epigenomic-level work, such as the current study, is not likely to be illuminating in this respect.

**Offspring outcomes associated with each maternal characteristic**

The results obtained in association with each maternal characteristic examined were highly coextensive (see Table 2 and Fig. 1), suggesting that these maternal characteristics have similar
developmental correlates. However, the areas of divergence are instructive.

Maternal depression in pregnancy was associated with the largest number of hits. More regions of infant DNA were alternatively methylated in association with antenatal maternal depressive symptoms than with maternal depressive symptoms measured concurrently with the infant swab. This suggests that the maternal depressive symptoms have a relatively greater impact on the infant epigenome antenatally than postnatally, a formulation consistent with the DoHAD hypothesis of prenatal programming [4, 79, 80].

Intriguingly, maternal childhood maltreatment severity had limited association with socioemotional and behavioral outcomes in toddlers, in contrast to maternal depression and attachment style, which were associated with several toddler socioemotional and behavioral outcomes (Fig. 2). It is possible that behavioral effects of maternal history of childhood maltreatment appear in dimensions that were not measured in this study, or that they might emerge at a later point in child development. We have elsewhere reported, and others have found, important associations of maternal history of childhood maltreatment with child behavioral outcomes at ages beyond 18 months [30, 31, 33].

Despite the divergence in their associations with the infant socioemotional and behavioral outcomes measured in this study, the result set for altered DNA methylation in association with maternal history of childhood maltreatment was equivalent in size to the result set for maternal depression. This suggests that even in the absence of an observable behavioral phenotype in toddlers, there are already patterns of events occurring on the molecular level that may prefigure later, clinically observable outcomes. Alternatively, it is possible that maternal history of childhood maltreatment is associated with behavioral or physiological outcomes in toddlers that were not captured by the ITSEA.

These results, while suggestive and not confirmatory, provide evidence that epigenetic modification of factors involved in behavioral responses can also be observed in peripheral tissues. In addition, they further support and extend our previous reports of powerful associations between maternal attachment security and maternal depressive symptoms specifically in the perinatal period [45, 46], demonstrating highly overlapping epigenetic and behavioral outcomes in offspring in association with maternal depression and insecure attachment, whereas a slightly different set of offspring outcomes was observed in association with maternal history of maltreatment.

Epigenetic signatures of maternal characteristics in infant genome are far broader than epigenetic signatures of maternal characteristics in the maternal genome

We previously reported on within-subject associations of psychological variables and DNA methylation signatures in pregnant women [46]. In comparison, the number of associations found between maternal psychological variables and infant DNA methylation signatures was larger by several orders of magnitude. This parallels an earlier study showing that maternal depressive symptoms were associated with altered DNA methylation in infant cord blood T cells, but not in T cells from the mothers themselves [81]. Together with other related work [82–84], these findings support the hypothesis that DNA methylation signatures are related most directly to environmental influences (in this case, environmental factors related to the mother’s experiences and behavior) and less directly to measurable characteristics of the individual.

Methodological differences from previous work

We note that there have been several previous array-based investigations of associations between maternal antenatal depression and infant DNA methylation [85–88]. All were performed on neonatal cord blood using the Illumina Infinium 450 K Beadchip. All reported either zero or a small, single- or double-digit number of associated DMRs, despite larger sample sizes than in our study in two cases.

The reasons for these discrepant results are not obvious, but we note several major methodological differences from previous work that may be relevant. First, we used next-generation sequencing, which yields precise, digital, sequence-based, strand-specific data, rather than the array approach, which is prone to errors introduced by probe cross-reactivity as well as stochastic variation in background fluorescence signal. Second, we obtained high-quality clinical data, including structured psychiatric interviews and assessments of childhood trauma and attachment insecurity on all mothers. Third, we used buccal cells, which have >95% cell type purity, rather than cord blood, which contains mixed blood cell lineages. Fourth, our DNA was obtained from infants at 6 months rather than at delivery. Fifth, our analytical strategy capitalized on the fact that transcriptional regulation by DNA methylation is often generated by clusters of methyl groups. We thus incorporated clustering information into our algorithm as a signal of biological significance, while analyses of array data more typically designate predetermined genomic regions as significantly differentially methylated or not, relying on the strength of the difference in methylation density signal to indicate significance.

We speculate that the tissue type purity and sliding-window based analysis may be the most relevant factors contributing to the return of our large number of positive results. Given the variability in epigenetic profiles among different tissues [49, 50], the use of a sample that contains mixed cell types runs the risk of diluting any signal beyond the possibility of discovery. For the analysis, relying on signal strength differences alone to indicate significance, as with the array data, means that a very large signal strength difference is required to overcome the statistical correction for large numbers of hypothesis tests, thus potentially missing many true positives. Our approach enabled us to avoid both these pitfalls.

We also note that a major advance in our study is that we were able to obtain confirmatory correlations of infant epigenetic markings with behavioral phenotypes at a later developmental timepoint, thus providing initial evidence to support the validity of the results obtained.

Association between infant DNA methylation patterns and toddler behavioral outcomes

Our working hypothesis was that alternative methylation patterns of infant genes would be a mediator of the causal pathway from maternal characteristics to offspring development. Having identified associations between maternal variables and infant DNA methylation patterns, a next step would be to test the DNA methylation results for mediation. However, it is not feasible to conduct mediation analyses in which the hypothesized mediator is a group of tens of thousands of individual results. Instead, we chose candidate genes and determined whether there was an association between the observed methylation patterns and behavioral outcomes.

While this result is not conclusive, it does demonstrate associations between DNA methylation patterns and offspring behavioral outcomes that are consistent with mediation of the association between maternal characteristics and offspring behavioral outcomes by alterations in patterns of DNA methylation.

We chose two candidate genes, GNG8 and NFATC1, based both on their inclusion within the most frequently identified molecular signaling pathway, the Wnt pathway, and on the fact that each of these genes contained regions that were significantly associated with each of the three maternal characteristics we examined. GNG8, which codes for a subunit of a guanine nucleotide binding protein, acts as a modulator or transducer in various
G-coupled transmembrane signaling systems. NFATC1, a nuclear factor of activated T cells, codes for a DNA-binding transcription complex playing a central role in inducible gene transcription during immune response. Both demonstrated uncorrected associations of infant DNA methylation density with one or more of the selected toddler behavioral outcomes, in addition to their association with the maternal characteristics, although only one region within GNG8 survived multiple testing correction. This suggests that in principle, these factors, acting in concert with the many other factors identified in this work, could play a causal role in the processes by which maternal psychiatric, personality, and life history characteristics shape early development.

Strengths and limitations

We should note four limitations of this study. First, the tissue we examined is, by necessity, peripheral, and may not reflect processes occurring in the central nervous system. Second, the study design is observational; thus, we can only describe associations, and cannot draw causal inferences. Third, the sample size is small, and although it is racially and socioeconomically reflective of the San Francisco Bay Area’s Peninsula region, it is not typical of the general U.S. population. Fourth, information on psychotherapeutic treatment was not obtained. Finally, we relied on maternal reports of child behavior without corroborating data from other observers. Thus, these data may be influenced by the maternal characteristics under study (although existing work supports the clinical validity of the ITSEA despite the potential for maternal mental health status to affect reporting [89–91]).

Despite these limitations, strengths of the study include behavioral data from offspring, the use of next-generation sequencing rather than less precise array-based methods, broad epigenomic coverage, and the novel statistical and theoretical approach.

CONCLUSIONS

In this study we show that methylation patterns in the buccal cell DNA of 6-month-old infants are associated with several key maternal characteristics: attachment style, maltreatment history, and depressive symptoms. Genes whose methylation patterns are altered in association with these characteristics include those related to cell-cell communication, developmental patterning, growth, immune function/inflammatory response, and neuro-transmission, suggesting that these are important molecular pathways that mediate the physiological effects of infant exposure to the specified maternal characteristics. We also studied offspring behavioral outcomes in toddlerhood associated with each of the three selected maternal characteristics and found that maternal attachment insecurity and depressive symptoms were similarly associated with offspring aggression and negative emotion at 18 months, while maternal history of childhood maltreatment was not strongly associated with most of the specified outcomes measured in the offspring.

Finally, in a proof-of-concept investigation of two candidate genes from the result set, we identified regions of the human infant genome at which DNA methylation patterns are associated both with maternal characteristics and prospectively with toddler behavior. Demonstration of associations of DNA methylation density with toddler socioemotional and behavioral outcomes provides additional evidence for functional significance of the obtained results, and suggests that our current set is a source of candidate molecular factors that could mediate the effects of maternal personality and life history on offspring development.

DATA AVAILABILITY

Data are accessible at https://figsharE.com/articles/dataset/1010i-CGATGT_S1_L001_R1_001_fastq.gz/17027099.
3314

27. Labonte B, Suderman M, Maussion G, Navarro L, Yerko V, Mahfaz L et al. Genome-wide epigenetic regulation by early-life trauma. Arch Gen Psychiatry. 2012;69:722–31.

28. Essex MJ, Thomas Boyce W, Hertzman C, Lam LL, Armstrong JM, Neumann S et al. Epigenetic vestiges of early developmental adversity: childhood stress exposure and DNA methylation in adolescence. Child Dev. 2013;84:58–75.

29. Provenzi L, Brambilla M, Scotto di Minico G, Montoriroso R, Bongarti R. Maternal caregiving and DNA methylation in human infants and children: systematic review. Genes, Brain Behav. 2020;19:e12616.

30. Roth MC, Humphreys KL, King LS, Mondal S, Gotlib IH, Robakis TK et al. Attachment security in pregnancy mediates the association between maternal childhood maltreatment and emotional and behavioral problems in offspring. Child Psychiatry Hum Dev. 2021;52:966–77.

31. Cooke JE, Racine N, Plamondon A, Tough S, Madigan S. Maternal adverse childhood experiences, attachment style, and mental health: pathways of transmission to child behavior problems. Child Abus Negl. 2019;93:27–37.

32. Goodman SH, Rouse MH, Connell AM, Broth MR, Hall CM, Heyward D. Maternal depression and child psychopathology: a meta-analytic review. Clin Child Fam Psychol Rev. 2011;14:1–27.

33. Hetherington E, Racine N, Madigan S, McDonald S, Tough S. Relative contribution of maternal adverse childhood experiences to understanding children’s externalizing and internalizing behaviours at age 5: findings from the All Our Families cohort. CMAJ Open. 2020;8:E352.

34. Thomas-Arngriou JC, Letourneau N, Dewey D, Campbell TS, Giesbrecht GF, APOEN Study Team. The role of HPA-axis function during pregnancy in the inter-generational transmission of maternal adverse childhood experiences to child behavior problems. Dev Psychopathol. 2021;33:284–300.

35. First MB. Structured clinical interview for the DSM. In: Cautin RL, editor. DSM-IV-TR. New York: Guilford Press; 1994. p. 128.

36. Bernstein DP, Stein JA, Newcomb MD, Walker E, Pogge D, Ahluvalia T et al. Development and validation of a brief screening version of the Childhood Trauma Questionnaire. Child Abuse Negl. 2003;27:169–90.

37. Feeden JA, Noller P, Hannah M. Assessing adult attachment. In: Sprengel MB, Berman WH, editors. Attachment in adults: clinical and developmental perspectives. New York: Guilford Press; 1994. p. 128–52.

38. Carer AS, Briggs-Gowan MJ, Jones SM, Little TD. The infant–toddler social and emotional assessment (ITSEA): Factor structure, reliability, and validity. J Abnorm Child Psychol. 2003;31:495–514.

39. Zubaran C, Schumacher M, Rixo MR, Foresti K. Screening tools for postpartum depression: validity and cultural dimensions. Afr J Psychiatry. 2010;13:357–65.

40. Alexander R, Feeden J, Hohaus L, Noller P. Attachment style and coping resources as predictors of coping strategies in the transition to parenthood. Pers Relatsh. 2016;6:302.

41. Karabekiroglu K, Rodopman-Arman A. Parental attachment style and severity of emotional/behavioral problems in toddlerhood. Arch Neuropsychiatry/Noropsikiatr Arisi. 2011;48:14–75.

42. Neuwald MF, Agranonik M, Portella AK, Fleming A, Wazana A, Steiner M, MAVAN Study Team. Transgenerational effects of maternal care interact with fetal growth and influence attention skills at 18 months of age. Early Hum Dev. 2016;92:493–8.

43. Whiffen VE, Kerr MA, Kallos L. Maternal depression, adult attachment, and children’s emotional distress. Fam Process. 2005;44:93–103.

44. Cao-Lei L, Elgbeili G, Massart R, Laplante DP, Szyf M, King S. Pregnant women’s cognitive appraisal of a natural disaster affects DNA methylation in their children 13 years later: Project Ice Storm. Transl Psychiatry. 2015;5:e01351. https://doi.org/10.1038/tp.2015.13.

45. Garg E, Chen Li, Nguyen TT, Pokhvisneva I, Chen LM, Unternaehrer E, MAVAN Study Team. The early care environment and DNA methyome variation in childhood. Dev Psychopathol. 2018;30:891–903.

46. Moore SR, McEwen LM, Quir J, Morin A, Mah SM, Barr RG et al. Epigenetic correlates of neonatal contact in humans. Dev Psychopathol. 2017;29:1517–38.

47. Theda C, Hwang SH, Czajko A, Loke YJ, Leong P, Craig JM. Quantification of the cellular content of saliva and buccal swab samples. Sci Rep. 2018;8:6944. https://doi.org/10.1038/s41598-018-25311-0.

48. Groeger S, Meyle J. Oral mucosal epithelial cells. Front Immunol. 2019;10. https://doi.org/10.3389/fimmu.2019.00208.

49. Kyrilenko I, Fajda O, Drach O, Popel’ S, Popel’ R, Zukow V. Relationships between Electrokinetic Index of buccal epithelium and some functional and metabolic parameters at men with chronic pyelonephritis. J Educ Health Sport. 2016;6:302–14. http://www.ios.ukw.edu.pl/index.php/johs/article/view/3369/4685.

50. Kroenke CH, Epel E, Adler N, Bush NR, Obradovic J, Lin J et al. Autonomic and adrenocortical reactivity and buccal cell telomere length in kindergarten children. Psychosom Med. 2011;73:533–40. https://doi.org/10.1016/j.psymed.2010.12.012.

51. Fowkes RC, Moradi-Bidhendi N, Branceloonee V, Zarivala MG, Brady J, Jessop DS et al. Annexin-A1 protein and its relationship to cortisol in human saliva. Psychoneuroendocrinology. 2013;38:722–7.

52. Edelmann S, Shalev I, Uzefosky F, Israel S, Knafal A, Kremer I et al. Epigenetic and genetic factors predict women’s salivary cortisol following a threat to the social self. PLoS ONE. 2012;7:e48597.

53. Smith AK, Kilaru V, Deppel T, Mercer KB, Bradley B, Conneely KN et al. DNA extracted from saliva for methylation studies of psychiatric traits: evidence tissue specificity and relatedness to brain. Am J Med Genet Part B: Neuropsychiatr Genet. 2015;168:36–44.

54. Lowe R, Gemma C, Bevan H, Hawa MI, Bazeos A, Leslie RD et al. Buccals are likely to be a more informative surrogate tissue than blood for epigenome-wide association studies. Epigenetics. 2013;6:445–54.

55. Thompson TM, Sharfi D, Lee M, Yrigollen CM, Naumova OY, Grigorenko EL. Comparison of whole-genome DNA methylation patterns in whole blood, saliva, and lymphoblastoid cell lines. Behav Genet. 2013;43:168–76.

56. Braun PR, Han S, Hing B, Nagahama Y, Gaul LN, Heinzman JT et al. Genome-wide DNA methylation comparison between live human brain and peripheral tissues with epigenome scans. Transl Psychiatry. 2013;3:E1–10.

57. Stadler MB, Murr R, Burger L, Ivanek R, Lienert F, Schöler A et al. DNA-binding factors shape the mouse methyome at distal regulatory regions. Nature. 2011;480:490–5.

58. Bayes J, Bird A. Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. EMBO J. 1992;11:327–37.
76. Maunakea AK, Nagarajan RP, Bilenyk M, Ballinger TJ, D’Souza C, Fouse SD, et al. Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature. 2010;466:253–7.

77. Galitz LM, Ahuvi Y, Gil A. The alternative role of DNA methylation in splicing regulation. Trends Genet. 2015;31:274–80.

78. Robakis TK, Lee S, Werner E, Liu G, Miller M, Wylie D, et al. DNA methylation patterns in T lymphocytes are generally stable in human pregnancies but CD3 methylation is associated with perinatal psychiatric symptoms. Brain, Behav. Immun-health. 2020;3:100044.

79. Monk C, Lugo-Candelas C, Trumpf P. Prenatal developmental origins of future psychopathology: mechanisms and pathways. Annu Rev Clin Psychol. 2019;15:317–44.

80. Kim DR, Bale TL, Epperson CN. Prenatal programming of mental illness: current understanding of relationship and mechanisms. Curr Psychiatry Rep. 2015;17:5.

81. Nemoda Z, Massart R, Suderman M, Hallett M, Li T, Coote M, et al. Maternal depression is associated with DNA methylation changes in cord blood T lymphocytes and adult hippocampi. Transl Psychiatry. 2015;5:e545.

82. Yehuda R, Daskalakis NP, Desarnaud F, Makotkine I, Lehrner A, Koch E, et al. Epigenetic biomarkers as predictors and correlates of symptom improvement following psychotherapy in combat veterans with PTSD. Front Psychiatry. 2013;4:118.

83. Perroud N, Salzmann A, Prada P, Nicastro R, Hoeppli ME, Furrer S, et al. Association between maternal depression during pregnancy and newborn DNA methylation. Transl Psychiatry. 2021;11:572. https://doi.org/10.1038/s41398-021-01697-w.

84. Lopez JP, Mamdani F, Labonte B, Beaulieu MM, Yang JP, Berlim MT, et al. Epigenetic regulation of BDNF expression according to antidepressant response. Mol Psychiatry. 2013;18:398–9.

85. Drzymalla E, Gladish N, Koen N, Epstein MP, Kobor MS, Zar HJ, et al. Association between maternal depression during pregnancy and newborn DNA methylation. Transl Psychiatry. 2021;11:572. https://doi.org/10.1038/s41398-021-01697-w.

86. Viuff AC, Sharp GC, Rai D, Henriksen TB, Pedersen LH, Kyng K, et al. Maternal depression during pregnancy and cord blood DNA methylation: findings from the Avon Longitudinal Study of Parents and Children. Transl Psychiatry. 2018;8:1–10.

87. Non AL, Binder AM, Kuzbarsky LD, Michels KB. Genome-wide DNA methylation in neonates exposed to maternal depression, anxiety, or SSRI medication during pregnancy. Epigenetics. 2014;9:964–72.

88. Schroeder JW, Smith AK, Brennan PA, Conneely KN, Kilariu V, Knight BT, et al. DNA methylation in neonates born to women receiving psychiatric care. Epigenetics. 2012;7:409–14.

89. Carter AS, Little C, Briggs-Gowan MJ, Kogan N. The infant–toddler social and emotional assessment (ITSEA): comparing parent ratings to laboratory observations of task mastery, emotion regulation, coping behaviors, and attachment status. Infant Ment Health J. 1999;20:375–92.

90. Van der Toorn SL, Huizink AC, Utens EM, Verhulst FC, Oormel J, Ferdinand RF. Maternal depressive symptoms, and not anxiety symptoms, are associated with positive mother–child reporting discrepancies of internalizing problems in children: a report on the TRAILS Study. Eur Child Adolesc Psychiatry. 2010;19:379–88.

91. Briggs-Gowan MJ, Carter AS. Social-emotional screening status in early childhood predicts elementary school outcomes. Pediatrics. 2008;121:957–62.

ACKNOWLEDGEMENTS

This work was supported by a Stanford Precision Health and Integrated Diagnostics Seed Funding grant to TKR, and by NIH Grants R21-HD090493 and R21-MH111978 to IHHG. AEU is a Tashia and John Morgridge Faculty Fellow of the Stanford Child Health Research Institute, is a Project-PI of the Stanford Center of Excellence in Genomics—Center for Personal Dynamic Regulomes (NIH HG007735-5, Center PI Changi), and acknowledges funding from and helpful discussions with Bruce Blackie. The sequencing data were generated on an Illumina HiSeq 4000 that was purchased with funds from NIH under award number S10OD018220. TL and YC are affiliated with Accura Science, LLC, and were financially recompensed for their expert biostatistical analysis. Code availability is at the discretion of Accura Science, by correspondence with TL. The REDCap platform services at Stanford are subsidized by (a) Stanford School of Medicine Research Office and (b) the National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health, through grant UL1 TR001085.

AUTHOR CONTRIBUTIONS

TKR conceived of the study, obtained targeted funding for the project, and wrote the paper. MCR managed data collection and contributed to data analysis, writing and editing. LSK and KKH contributed to study design, data collection, data analysis, interpretation, writing, and editing. MH performed the DNA processing and sequencing. XZ contributed to the analysis plan and to data acquisition and processing. YC and TL performed the bioinformatic analysis. NLR contributed to study design and reviewed and edited the final manuscript. KTW assisted with interpretation of results and reviewed and edited the final manuscript. AEU contributed to study design, oversaw acquisition and analysis of epigenetic data, and reviewed and edited the final manuscript. Code availability is at the discretion of Accura Science, by correspondence with TL. The REDCap platform services at Stanford are subsidized by (a) Stanford School of Medicine Research Office and (b) the National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health, through grant UL1 TR001085.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41398-022-01592-w.

Correspondence and requests for materials should be addressed to Thalia K. Robakis.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.