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ARTICLE

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

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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 5×10^6 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.

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 [2] (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.

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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 personality 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, IHG). 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 (Mawi 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 epigenetic 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 51, the reads were mapped to human RefSeq genome hg19.

QC for the data was performed on the FASTQ data using FastQC1 (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 Bismark3 as well. The output SAM files were converted to BAM files and sorted using SAMtools4 (version 1.3.1).

CpG methylation calling for was carried out using function processBismarkAln() in R (version 3.2.2) package methylKit5 (version 0.99.0). 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 re-calculated 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 women met the criteria for depression at the 6-month postpartum timepoint (Table 1).

Number of results

We identified many thousands of regions of DNA that were alternatively methylated in infants in association with each of the maternal characteristics (Table 1). The majority of the hits were in noncoding regions, with promoter sites being the next most common after intronic and intergenic regions. See Supplementary Table 1 for the full gene list identified.

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 1. Sample demographics.

Behavioral sample		Epigenetic sample		
			Mean	SD
Mean	SD			
Mother's age	31.39	5.26	33.21	4.73
Mother's parity	0.90	0.98	1.24	0.94
Maternal depression				
EPDS in pregnancy	7.90	6.34	6.98	5.38
EPDS at 6 months postpartum	7.06	5.38	6.60	5.12
	N	%	N	%
Mother's race				
White Non-Hispanic	16	51.61	76	51.01
Hispanic	5	25.81	20	13.42
Black or Af-Am	1	3.23	4	2.68
Asian	8	25.81	40	26.85
Pacific Islander	0	0	3	2.01
Other	1	3.23	6	4.03
Child's race				
White Non-Hispanic	15	53.57	71	48.63
Hispanic	5	17.86	25	17.12
Black or Af-Am	1	3.57	3	2.05
Asian	5	17.86	28	19.18
Pacific Islander	0	0	1	0.68
Other	2	7.14	18	12.33
Child sex				
Male	14	45.16	71	47.97
Female	17	54.84	77	52.03
Mother's employment				
Homemaker	7	22.58	27	18.12
Employed for wages	18	58.07	87	58.39
Self-employed	2	6.45	13	8.72
Unemployed	2	6.45	14	9.40
Other	2	6.45	8	5.37
Mother's level of education				
Some HS	1	3.23	1	0.67
HS degree	0	0	0	0
Some college	3	9.68	11	7.38
Associate's/technical	3	9.68	7	4.70
Bachelor's	8	25.81	47	31.54
Graduate	16	51.61	83	55.70
Mother's history of depression				
Yes	16	51.61	48	37.5
No	15	48.39	80	62.5
Mother depressed at 6-month follow-up				
Yes	2	6.45	17	11.81
No	29	93.54	127	88.19

| Mother's characteristic	Alternatively methylated regions in infant DNA
Maternal history of maltreatment (CTQ)	16,185
Maternal attachment insecurity (ASQ)	11,176
Pregnancy depressive symptoms (EPDS)	19,348
Postpartum depressive symptoms (EPDS)	16,150

Total Ns are 31 for the epigenetic sample and 156 for the behavioral sample. Columns do not all sum to overall N because of individual-level missing data in some categories.

Overlap among the pathways associated with each maternal characteristic was high, mirroring the generally highly significant associations among each of these variables that are observed clinically [45, 46]. Of 160 pathways identified in infants (see

Table 2. Top ten biological pathways with components alternatively methylated in infant DNA in association with each maternal clinical variable.

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%	Wnt signaling pathway	127	2.0%	5.0%	Wnt signaling pathway	131	2.2%	5.9%
Gonadotropin-releasing hormone receptor pathway	98	1.6%	4.3%	Gonadotropin-releasing hormone receptor pathway	76	1.6%	4.2%	Gonadotropin-releasing hormone receptor pathway	110	1.7%	4.3%	Gonadotropin-releasing hormone receptor pathway	91	1.5%	4.1%
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%	Integrin signalling pathway	97	1.5%	3.8%	Integrin signalling pathway	90	1.5%	4.0%
Integrin signalling pathway	79	1.3%	3.5%	Integrin signalling pathway	56	1.2%	3.1%	Inflammation mediated by chemokine and cytokine signaling pathway	92	1.4%	3.6%	Inflammation mediated by chemokine and cytokine signaling pathway	87	1.4%	3.9%
Cadherin signaling pathway	71	1.2%	3.1%	Angiogenesis	54	1.1%	3.0%	Angiogenesis	79	1.2%	3.1%	Angiogenesis	72	1.2%	3.2%
Angiogenesis	68	1.1%	3.0%	Heterotrimeric G-protein signaling-Gi alpha and Gs alpha mediated pathway	52	1.1%	2.9%	CCKR signaling map	73	1.1%	2.9%	CCKR signaling map	67	1.1%	3.0%
CCKR signaling map	65	1.1%	2.9%	Cadherin signaling pathway	49	1.0%	2.7%	Huntington disease	66	1.0%	2.6%	Cadherin signaling pathway	67	1.1%	3.0%
Huntington disease	63	1.0%	2.8%	Huntington disease	48	1.0%	2.7%	EGF receptor signaling pathway	64	1.0%	2.5%	Huntington disease	60	1.0%	2.7%
Heterotrimeric G-protein signaling-Gi alpha and Gs alpha mediated pathway	58	1.0%	2.5%	CCKR signaling map	46	1.0%	2.6%	PDGF signaling pathway	63	1.0%	2.5%	Alzheimer disease-presenilin pathway	57	0.9%	2.5%
Alzheimer disease-presenilin pathway	48	0.8%	2.1%	EGF receptor signaling pathway	40	0.8%	2.2%	Cadherin signaling pathway	61	0.9%	2.4%	PDGF signaling pathway	55	0.9%	2.5%
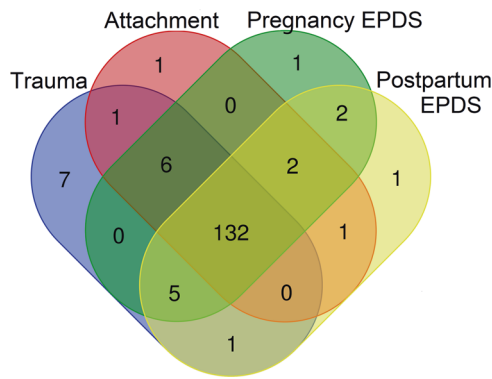
Fig. 1 Overlap among biological pathways with components alternatively methylated in infants in association with each maternal variable. The vast majority of pathways are affected in all four conditions. Maternal childhood maltreatment (CTQ) has the most unique pathways not associated with the other 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
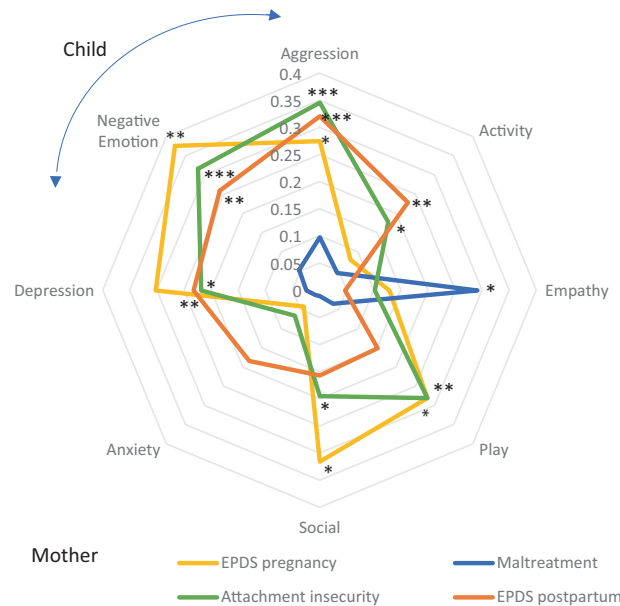
Fig. 2 Radar plot of maternal variable: child outcomes. ITSEA (18 months age, $N = 156$). Maternal attachment insecurity and depressive symptoms postpartum are associated with toddler negative emotion and aggression. Effect magnitudes shown as absolute values for easier comparison. Signs of the associations between maternal attachment insecurity and pregnancy depression with toddler social and play were negative; all others were positive. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

were correlated with the measure of toddler empathy ($p \leq 0.05$). Five of the 34 NFATC1 regions were correlated with toddler aggression, negative emotion, or both ($p \leq 0.05$). 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

Table 3. Correlations of mean methylation density within selected genetic regions from the result set with selected infant behavioral outcomes.

		Mean GNG8 region 1	Mean GNG8 region 2	Mean GNG8 region 3	Mean NFATC1 region 1	Mean NFATC1 region 10	Mean NFATC1 region 19	Mean NFATC1 region 25
ITSEA empathy	Pearson correlation	0.512**	0.354	−0.416*	−0.270	0.048	0.114	−0.109
	p value	0.005	0.065	0.028	0.191	0.814	0.572	0.588
ITSEA aggression	Pearson correlation	−0.194	−0.198	0.111	0.393*	−0.374*	−0.281	0.406*
	p value	0.314	0.304	0.566	0.047	0.050	0.147	0.032
ITSEA negative emotion	Pearson correlation	−0.177	−0.234	0.332	0.502**	−0.331	−0.414*	0.394*
	p value	0.359	0.221	0.079	0.009	0.086	0.029	0.038

Results passing correction for multiple hypotheses are underlined.

ITSEA Infant-Toddler Social Emotional Assessment.

Uncorrected results are starred (*p ≤ 0.05; **p ≤ 0.01).

catenin signaling [61], gonadotropin-gonadal steroid pathways [63], and immune function [62].

Some of these pathways may be of direct functional consequence in the sampled tissue—buccal epithelial cells [64]—particularly those pathways involved with immunological and inflammatory responses and with cell-cell communication. Buccal epithelial cells have barrier and moistening functions as well as roles in the recognition of pathogenic invaders and production of cytokines [65], all functions that are heavily modulated in response to external stressors. Thus, pathways involving pathogen detection and immune response have clear relevance to cellular functions in the sampled tissue.

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 neurotransmission, 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 behavioral 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 result 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.

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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 KLH 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. IHG is PI of the parent study, provided guidance for study design, data acquisition and analysis, and reviewed and edited the manuscript for publication.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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