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Archival Report

Sensitive Periods for the Effect of Childhood Adversity on DNA Methylation: Results From a Prospective, Longitudinal Study

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ABSTRACT

BACKGROUND: Exposure to early-life adversity is known to predict DNA methylation (DNAm) patterns that may be related to psychiatric risk. However, few studies have investigated whether adversity has time-dependent effects based on the age at exposure.

METHODS: Using a two-stage structured life course modeling approach, we tested the hypothesis that there are sensitive periods when adversity induces greater DNAm changes. We tested this hypothesis in relation to two alternatives: an accumulation hypothesis, in which the effect of adversity increases with the number of occasions exposed, regardless of timing; and a recency model, in which the effect of adversity is stronger for more proximal events. Data came from the Accessible Resource for Integrated Epigenomic Studies, a subsample of mother–child pairs from the Avon Longitudinal Study of Parents and Children (n = 691–774).

RESULTS: After covariate adjustment and multiple testing correction, we identified 38 CpG sites that were differentially methylated at 7 years of age following exposure to adversity. Most loci (n = 35) were predicted by the timing of adversity, namely exposures before 3 years of age. Neither the accumulation nor recency of the adversity explained considerable variability in DNAm. A standard epigenome-wide association study of lifetime exposure (vs. no exposure) failed to detect these associations.

CONCLUSIONS: The developmental timing of adversity explains more variability in DNAm than the accumulation or recency of exposure. Very early childhood appears to be a sensitive period when exposure to adversity predicts differential DNAm patterns. Classification of individuals as exposed versus unexposed to early-life adversity may dilute observed effects.

Keywords: Childhood adversity, Children, DNA methylation, Epigenetics, Longitudinal, Sensitive periods

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Exposure to childhood adversity, including poverty (1), abuse (2,3), family dysfunction (4,5), and other stressors (6,7), is a common and potent determinant of mental health across the lifespan, increasing risk of childhood- and adult-onset psychiatric disorders by at least twofold (8–10). Although the biological mechanisms explaining this relationship are poorly understood, accumulating evidence suggests that adversity may become programmed molecularly, leaving behind biological memories that persistently alter genome function and increase susceptibility to mental disorders. Indeed, dozens of candidate gene and epigenome-wide association studies (EWASs) in both animals and humans have shown that early-life adversity is associated with persistent alterations in the epigenome (11–15), including changes in DNA methylation (DNAm), which is the most studied epigenetic mechanism involving the addition of methyl groups to cytosines in the DNA sequence (16,17). These differential DNAm sites can alter gene expression, providing a mechanism by which gene by environment interactions affect biological responses (18).

Recent evidence, particularly from animal studies, suggests that epigenetic programming may be developmentally time sensitive and that there may be sensitive periods (19,20) when adversity exposure is more likely to induce DNAm changes. For instance, rodent experiments have demonstrated the existence of sensitive periods for different aspects of epigenetic regulation—from embryonic reprogramming to postnatal exposure—leading to differences in epigenetic outcomes and gene expression (21–25). Recent work in nonhuman primates also suggests that there are differential effects on DNAm based on whether adversity exposure, including maternal separation, occurred at birth or later in development (26). However, few human studies, whether candidate gene studies (16,27–29) or EWASs (30–32), have examined the time-dependent effects of psychosocial adversity on DNAm; nearly all human epigenetic studies have instead focused on the presence versus absence of exposure to early-life adversity. Thus, it is unknown whether there are age stages at which adversity differentially affects DNAm, children are therefore

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more vulnerable, and prevention efforts could be most efficacious.

This study aimed to address this limitation by using data from a prospective birth cohort of children to test the hypothesis that there are sensitive periods associated with DNAm alterations following adversity exposure. To test this hypothesis, we used a two-stage structured life course modeling approach (SLCMA) (33,34) to examine the effect of repeated exposure to seven types of childhood adversities across three developmental periods (in very early childhood, before 3 years of age; early childhood, 3–5 years of age; and middle childhood, 6–7 years of age) on DNAm profiles at 7 years of age. Recognizing that alternative conceptual models have been proposed to explain the effects of adversity, we also used the SLCMA to determine whether the sensitive period model explained more variability in DNAm relative to two other theoretical models described in the life course epidemiology literature (35–37): 1) an accumulation model (38–40), in which the effect of adversity on DNAm increases with the number of occasions exposed, regardless of timing; and 2) a recency model (41), in which the effect of adversity on DNAm is stronger for more proximal events. Finally, to evaluate the potential advantage of the SLCMA relative to the standard EWAS approach, which would ignore the timing or frequency of adversity, we examined the number of epigenome-wide significant loci identified by each approach and evaluated their degree of overlap.

METHODS AND MATERIALS

Sample and Procedures

Data came from the Avon Longitudinal Study of Parents and Children (ALSPAC), a population-based birth cohort (42–44). The ALSPAC generated blood-based DNAm profiles at birth and 7 years of age as part of the Accessible Resource for Integrated Epigenomic Studies (ARIES), a subsample of 1018 mother–child pairs from the ALSPAC (45). The ARIES mother–child pairs were randomly selected out of those with complete data across at least five waves of data collection (Supplement 1).

Measures

Exposure to Adversity. We examined the effect of seven adversities shown previously to associate with epigenetic marks (46–48): 1) caregiver physical or emotional abuse (49–52); 2) sexual or physical abuse (by anyone) (49–52); 3) maternal psychopathology (53,54); 4) one adult in the household (55); 5) family instability (29,56); 6) financial stress and/or poverty (57,58); and 7) neighborhood disadvantage and/or poverty (59). These adversities were chosen because they capture experiences that deviate from a child's expected social and physical environment (60). Each adversity was measured via maternal report on at least four occasions at or before 7 years of age either from a single item or from psychometrically validated standardized measures. Specific time periods of assessment varied across adversity type (Supplement 1). For each adversity type, we generated three sets of encoded variables (Supplement 1): 1) a set of variables indicating the presence of the adversity at a specific developmental stage versus absence of the adversity at that stage, to test the sensitive period hypothesis; 2) a single variable denoting the total number of time periods of exposure to a given adversity, to test the accumulation hypothesis; and 3) a single variable denoting the total number of developmental periods of exposure, with each exposure weighted by the age of the child during the measurement time period, to test the recency hypothesis; this variable upweighted more recent exposures, allowing us to determine whether more recent exposures were more impactful.

DNA Methylation. DNAm was measured at 485,000 CpG dinucleotide sites across the genome using the Illumina Infinium Human Methylation 450K BeadChip microarray (Illumina, San Diego, CA). DNA for this assay was obtained from cord blood at birth and peripheral blood leukocytes at 7 years of age. DNA was stored and extractions were completed at 5 to 8 years after collection of cord blood and within 3 weeks after collection of peripheral blood at age 7 (61). DNAm wet laboratory procedures, preprocessing analyses, and quality control were performed at the University of Bristol [Supplement 1 and Relton *et al.* (45)]. DNAm levels are expressed as a β value representing the proportion of cells methylated at each interrogated CpG site.

Prior to analysis, raw methylation β values, which are preferred over *M* values because of their interpretability (62), were normalized (63) to remove or minimize the effects of variation due to technical artifacts. To adjust for DNAm variation due to cell-type heterogeneity in peripheral and cord blood samples, we estimated cell counts from DNAm profiles (64) and regressed these estimates from the normalized β values. Additionally, to remove possible outliers, we winsorized the β values at each CpG site, setting the bottom 5% and top 95% of values to the 5th and 95th quantile, respectively (65).

Covariates. To adjust for baseline sociodemographic differences in the cohort, all analyses additionally controlled for the following variables, measured at birth (Supplement 1): child race and/or ethnicity; child birth weight; maternal age; number of previous pregnancies; sustained maternal smoking during pregnancy; and parent social class (66). Justification for the inclusion of parent social class as a covariate and alternative results from analyses that exclude social class as a covariate are presented in Supplement 1.

Data Analysis

Our primary analyses involved comparing the three theoretical models using the SLCMA, which was originally developed by Mishra *et al.* (34) and later extended by Smith *et al.* (33,67) to analyze repeated exposure data across the life course (Supplement 1). The major advantage of the SLCMA is that it provides an unbiased way to compare multiple competing theoretical models simultaneously and identify the most parsimonious explanation for the observed outcome variation. The SLCMA uses least angle regression (LARS) (68) and an associated covariance test (69) to identify the single theoretical model (or potentially more than one model working in combination) that explains the most outcome variation (R^2). Compared with other methods for structured life course analysis, LARS has greater statistical power (33) and does not overinflate effect size estimates (68) or bias hypothesis tests (69). The SLCMA has been used in several life course epidemiology studies (70,71), including studies of other birth cohorts (72,73). The LARS procedure functions under the same assumptions as multiple linear regression.

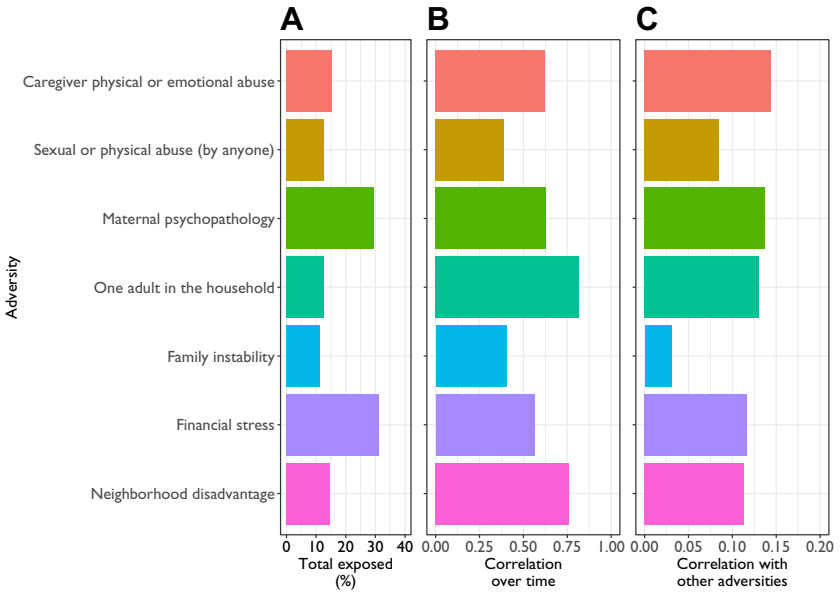

Figure 1. Exposure to adversity in the Accessible Resource for Integrated Epigenomic Studies data set. The figure displays the lifetime prevalence by 7 years of age of exposure to each adversity (labeled as "total exposed"), the average correlation between exposure to one type of adversity at one time point and exposure to that same adversity at a second time point (labeled as "correlation over time"), and the average correlation between exposure to one type of adversity and exposure to a second type of adversity (labeled as "correlation with other adversities"). **(A)** The lifetime prevalence of each adversity varied by type. The most commonly reported adversities were financial stress (31%) and maternal psychopathology (29%). The remaining adversities were less reported, but still common: caregiver physical or emotional abuse (15%), neighborhood disadvantage (15%), sexual or physical abuse (by anyone; 13%), one adult in the household (13%), and family instability (11%). **(B)** Among specific types of adversity, exposures tended to correlate over time, with neighboring time points being more related than distant time points. For instance, exposure to one adult in the household and neighborhood disadvantage were most strongly correlated over time (r = .54–.93 and r = .67–.89, respectively), whereas exposure to family instability (r = .11–.74) and sexual or physical abuse (r = .02–.69) were more weakly correlated across time. **(C)** The average correlation of having ever been exposed to the other adversities was modest across adversities, suggesting that we were capturing unique subtypes of adversity.

In the first stage, we entered the set of encoded variables described previously into the LARS variable selection procedure (68). LARS identified the variable with the strongest association with the outcome, thus identifying whether the sensitive period, accumulation, or recency model was most supported by the data. Therefore, for each CpG site, one unique LARS model was selected for each of the seven types of adversity. For each selected model, we performed a covariance test of the null hypothesis that the variable selected is unassociated with the outcome. With respect to multiple testing, the covariance test p values are adjusted for the number of variables included in the LARS procedure, controlling the type I error rate for each adversity type and CpG site. To adjust for confounding during the first stage, we regressed each encoded variable on the covariates and implemented LARS on the regression residuals (67).

In the second stage, the theoretical model shown in the first stage to best fit the observed data for a specific type of adversity was then carried forward to a multiple regression framework, where measures of effect were estimated. Positive effect estimates thus indicate elevated (hyper-) methylation, and negative effect estimates indicate decreased (hypo-) methylation. Only models with a covariance test p value $< 1 \times 10^{-7}$, the standard Bonferroni correction threshold for epigenome-wide statistical significance, were included in the second stage. The same covariates were also included in the second stage. We compared the distribution of theoretical models across the Bonferroni-significant CpG sites with an omnibus χ^2 test, which tested the null hypothesis that the theoretical models were likely to be represented among the significant results in proportion to the frequency in which they were tested.

To evaluate the loss or gain of information when using a simpler versus more complex analytic approach, we also performed seven EWASs (one for each type of adversity) to evaluate the association between lifetime exposure to adversity (coded as ever vs. never exposed) and DNAm across all

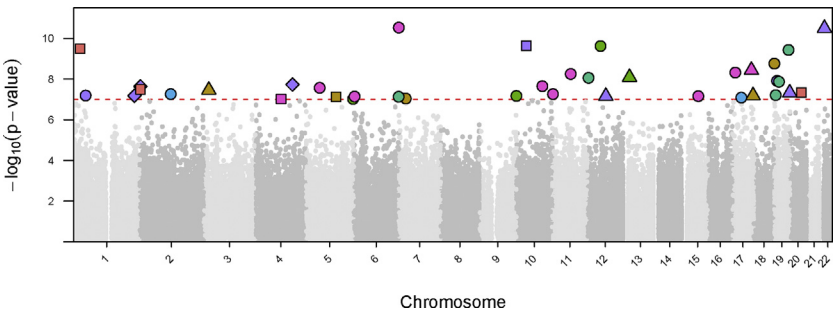

Figure 2. A Manhattan plot displays top CpG sites associated with exposure to adversity. In this Manhattan plot, the x-axis is the chromosomal position for each CpG site and the y-axis is the $-\log_{10} p$ value for the association between exposure to adversity and DNA methylation values at each CpG site. The dashed line shows the epigenome-wide significance level, with each CpG site above the line representing a statistically significant association ($p < 1 \times 10^{-7}$). The color of each CpG site refers to the type of adversity. The shape of each CpG site indicates the life course model tested. The sensitive period hypotheses were encoded as a circle for very early childhood, a triangle for early childhood, and a square for middle childhood. The recency and accumulation hypotheses were encoded as a diamond. As shown, CpG sites significantly affected by exposure adversity were distributed throughout the genome. There was no obvious genomic spatial pattern by adversity type or timing of exposure.

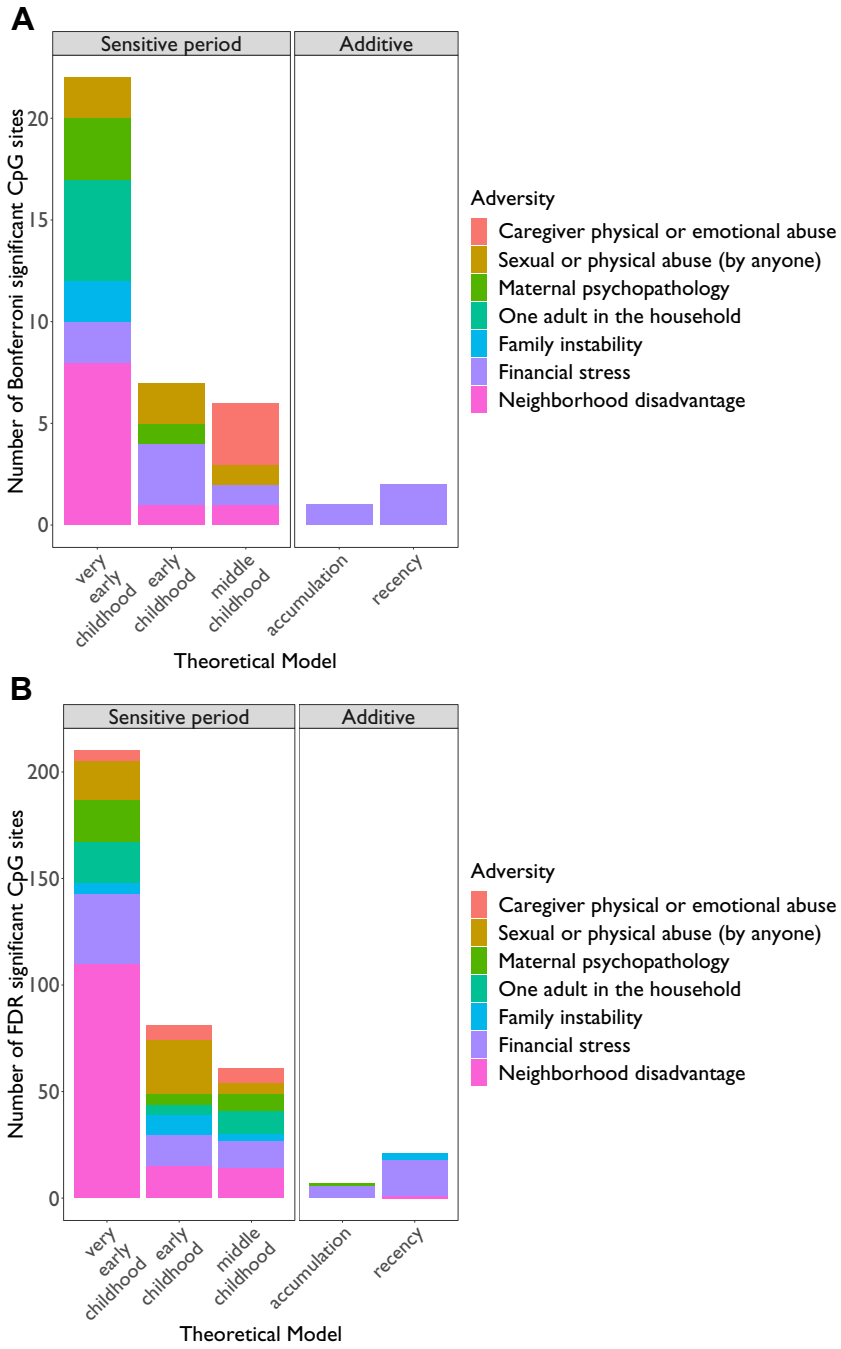

Figure 3. The figure illustrates the frequency at which each life course theoretical model was chosen for each type of adversity. Each plot displays the number of CpG sites for which adversity significantly predicted methylation, after controlling for covariates and correcting for multiple comparisons using **(A)** a Bonferroni threshold ($p < 1 \times 10^{-7}$, n = 38 sites) and **(B)** a false discovery rate (FDR) correction $q < .05$ (n = 380 sites). The distribution of theoretical models chosen first by the least angle regression procedure for top CpG sites was significantly different from what would be expected by chance, with exposure to adversity during sensitive periods, especially during very early childhood, more frequently predicting methylation.

CpG sites. The EWAS results were then compared with the SLCMA to determine whether the two approaches yielded similar or distinct conclusions regarding the number of significant loci detected.

We also performed sensitivity analyses to evaluate the fit of the LARS selection procedure, determine the degree of differential methylation present at birth, and control for genetic variation. We examined the biological significance of the findings by 1) examining the correlation in methylation between blood and brain tissue for the top CpG sites using an online database (74); 2) investigating enrichment of regulatory elements annotated to false discovery rate (FDR)–significant CpG sites; 3) performing a functional clustering analysis of all gene ontology terms for genes annotated to FDR-significant sites in DAVID 6.8 (75); and 4) assessing the selective constraint of these genes using the Exome Aggregation Consortium (76).

Table 1. Results of the SLCMA in ARIES, With Annotation to the Closest Gene, for the Bonferroni–Significant CpG Sites ($p < 1 \times 10^{-7}$)

Adversity	CpG Site	First Hypothesis Chosen by LARS Procedure	DNAm in Unexposed Group, β[a]	DNAm in Exposed Group, β[a]	Increases in R^2[b]	p Value[c]	Effect Estimate, β[d]	SE[d]	Lower 95% CI[d]	Upper 95% CI[d]	Chr[e]	Coordinate, bp[e]	Nearest Gene[f]	Distance to Nearest Gene, bp[f]
Caregiver physical or emotional abuse (n = 719)	cg10713431	Middle childhood (6 years of age)	.132	.139	.025	4.59×10^{-8}	.008	.0019	.004	.012	20	43933204	*MATN4*	0
	cg12023170[g]	Middle childhood (6 years of age)	.074	.086	.038	3.17×10^{-10}[i]	.013	.0022	.008	.017	1	23751761	*TCEA3*	499
	cg19825600[g,h]	Middle childhood (6 years of age)	.458	.384	.027	3.23×10^{-8}	−.072	.0158	−.103	−.041	2	3704501	*ALLC*	1283
Sexual or physical abuse (by anyone) (n = 703)	cg01370449	Very early childhood (2.5 years of age)	.244	.334	.030	8.87×10^{-8}	.083	.0168	.050	.116	7	27183369	*HOXA-AS3*	0
	cg06430102	Very early childhood (2.5 years of age)	.926	.862	.037	1.69×10^{-9}[i]	−.058	.0103	−.078	−.038	19	1151960	*SBNO2*	0
	cg19170021	Early childhood (4.75 years of age)	.734	.827	.028	6.41×10^{-8}	.092	.0209	.051	.134	17	79077169	*BAIAP2*	0
	cg05072819[g]	Early childhood (5.75 years of age)	.040	.053	.030	3.49×10^{-8}	.014	.0027	.009	.019	3	20081367	*KAT2B*	155
	cg05936516	Middle childhood (6.75 years of age)	.128	.153	.031	7.47×10^{-8}	.025	.0048	.016	.035	5	114507066	*TRIM36*	0
Maternal psychopathology (n = 691)	cg04583813	Very early childhood (8 mo of age)	.900	.878	.031	6.57×10^{-8}	−.023	.0046	−.032	−.014	10	560323	*DIP2C*	0
	cg08171937	Very early childhood (2.75 years of age)	.016	.017	.034	2.33×10^{-10}[i]	.001	.0003	.001	.002	12	49454761	*RHEBL1*	3705
	cg10666628	Very early childhood (2.75 years of age)	.020	.021	.029	9.29×10^{-8}	.002	.0004	.001	.003	5	179050666	*HNRNPH1*	0
	cg17806989	Early childhood (5 years of age)	.981	.975	.032	8.16×10^{-9}[i]	−.006	.0012	−.009	−.004	13	25338287	*RNF17*	12
One adult in the household (n = 710)	cg08337366[a]	Very early childhood (8 mo of age)	.934	.906	.029	6.07×10^{-8}	−.032	.0066	−.045	−.019	19	6371622	*ALKBH7*	820
	cg10192047	Very early childhood (8 mo of age)	.016	.019	.029	1.31×10^{-8}[i]	.003	.0007	.002	.005	19	18722754	*TMEM59L*	926
	cg26990406	Very early childhood (8 mo of age)	.868	.728	.027	7.22×10^{-8}	−.142	.0308	−.203	−.082	7	178829	*FAM20C*	14138
	cg24468070	Very early childhood (1.75 years of age)	.038	.058	.034	3.63×10^{-10}[i]	.023	.0044	.014	.031	19	54976501	*CDC42EP5*	0
	cg03397307	Very early childhood (2.75 years of age)	.025	.030	.030	8.46×10^{-9}[i]	.005	.0010	.003	.007	12	3862423	*CRACR2A*	56
Family instability (n = 703)	cg18311384	Very early childhood (2.5 years of age)	.019	.022	.027	7.97×10^{-8}	.002	.0005	.001	.003	17	34842312	*ZNHIT3*	159
	cg27637303	Very early childhood (2.5 years of age)	.345	.420	.028	5.32×10^{-8}	.078	.0168	.045	.111	2	118942893	*INSIG2*	75295
Financial stress (n = 774)	cg11631610	Very early childhood (8 mo of age)	.949	.923	.027	1.20×10^{-8}[i]	−.027	.0057	−.038	−.016	19	11322739	*DOCK6*	0
	cg06783003	Very early childhood (1.75 years of age)	.860	.893	.024	6.25×10^{-8}	.037	.0083	.021	.053	1	45116008	*RNF220*	0
	cg01050704[g]	Early childhood (5 years of age)	.017	.019	.025	4.68×10^{-8}	.002	.0005	.001	.003	19	59084995	*MZF1-AS1*	0
	cg02006977	Early childhood (5 years of age)	.015	.017	.024	6.87×10^{-8}	.002	.0005	.001	.003	12	69139955	*SLC35E3*	0
	cg21299458	Early childhood (5 years of age)	.110	.147	.035	3.19×10^{-11}[i]	.038	.0070	.024	.052	22	20779896	*SCARF2*	0
	cg19219503	Middle childhood (7 years of age)	.922	.889	.031	2.28×10^{-10}[i]	−.035	.0071	−.049	−.021	10	37414802	*ANKRD30A*	0
	cg11714846	Accumulation	.923	.915	.023	6.64×10^{-8}	−.005	.0011	−.007	−.003	1	230419534	*GALNT2*	1658
	cg21924472	Recency	.756	.770	.027	1.87×10^{-8}	.003	.0006	.002	.004	4	139600734	*LINC00499*	255235
	cg24996440	Recency	.566	.585	.026	2.28×10^{-8}	.004	.0009	.003	.006	2	3583570	*RNASEH1*	9119
Neighborhood disadvantage (n = 702)	cg00928478	Very early childhood (1.75 years of age)	.020	.018	.027	2.19×10^{-8}	−.002	.0005	−.003	−.001	10	99078824	*FRAT1*	196
	cg01954337	Very early childhood (1.75 years of age)	.050	.059	.028	5.32×10^{-8}	.008	.0018	.005	.012	11	3819010	*NUP98*	0
	cg04996689	Very early childhood (1.75 years of age)	.029	.035	.028	2.63×10^{-8}	.006	.0011	.003	.008	5	52285560	*ITGA2*	0
	cg12069925	Very early childhood (1.75 years of age)	.042	.048	.030	4.72×10^{-9}[i]	.007	.0014	.004	.009	17	11900858	*ZNF18*	72
	cg14522055	Very early childhood (1.75 years of age)	.030	.035	.028	6.77×10^{-8}	.005	.0011	.003	.007	15	64338757	*DAPK2*	235
	cg19157140	Very early childhood (1.75 years of age)	.014	.016	.037	2.87×10^{-11}[i]	.002	.0005	.001	.003	7	766323	*PRKAR1B*	0

Table 1. Continued

Adversity	CpG Site	First Hypothesis Chosen by LARS Procedure	DNAm in Unexposed Group, β[a]	DNAm in Exposed Group, β[a]	Increases in R^2[b]	*p* Value[c]	Effect Estimate, β[d]	SE[d]	Lower 95% CI[d]	Upper 95% CI[d]	Chr[e]	Coordinate, bp[e]	Nearest Gene[f]	Distance to Nearest Gene, bp[f]
	cg21740964	Very early childhood (1.75 years of age)	.160	.173	.025	7.13×10^{-8}	.014	.0028	.008	.019	6	3849331	*FAM50B*	299
	cg24826892[g]	Very early childhood (1.75 years of age)	.016	.018	.030	5.50×10^{-9}[i]	.003	.0006	.002	.004	11	71159390	*DHCR7*	0
	cg08546016	Early childhood (5 years of age)	.050	.056	.029	3.63×10^{-9}[i]	.006	.0012	.004	.009	17	72776238	*TMEM104*	0
	cg12412390	Middle childhood (7 years of age)	.038	.046	.030	9.59×10^{-8}	.008	.0016	.005	.011	4	96469286	*UNC5C*	0

ARIES, Accessible Resource for Integrated Epigenomics Studies; bp, base pair; Chr, chromosome; CI, confidence interval; DNAm, DNA methylation; LARS, least angle regression; SLCMA, structured life course modeling approach.

[a]In the DNAm column, values are unadjusted DNA methylation (β values) averaged within the group.

[b]In the Increases in R^2 column, values represent the increase in R^2 explained by the first hypothesis chosen after accounting for covariates.

[c]In the *p* column, each value gives the *p* value for the covariance test, which assesses the significance of the increase in R^2 explained.

[d]In the Effect Estimate, SE, Lower 95% CI, and Upper 95% CI columns, values are the parameter estimate, standard error, and lower and upper limits of the 95% confidence interval, respectively, of the regression coefficient of the first hypothesis chosen.

[e]The Chr and Coordinate columns, respectively, give the chromosome and position of the CpG site.

[f]The Nearest Gene and Distance to Nearest Gene columns give the gene symbol of and distance in bases to the nearest gene to the CpG site (as measured from the transcription start site), respectively.

[g]In list of potentially noisy probes compiled by Naeem *et al.* (86) (i.e., cross-reactive probes, probes with single nucleotide polymorphisms, insertions or deletions, and/or repeat regions, probes affected by unknown factors).

[h]In list of potentially noisy probes compiled by Chen *et al.* (87) (i.e., cross-reactive probes, probes with single nucleotide polymorphisms).

[i]Significant at $p < 1.43 \times 10^{-8}$, a more stringent *p* value threshold that accounted for the testing of seven types of adversity ($[1 \times 10-7]/7 = 1.43 \times 10^{-8}$).

RESULTS

Sample Characteristics and Distribution of Exposure to Adversity

Demographic characteristics of the ARIES analytic sample are shown in Table S1 in Supplement 1 for the total sample and for children exposed to any adversity ($n = 650$, 67%, experienced at least one adversity at some point in their lifetime). Details on the prevalence and correlations of exposure across time are also reported in Figure 1 and Figure S1 and Table S2 in Supplement 1. Of note, differences in the prevalence of exposure across time are unlikely to affect model selection, as all variables are automatically standardized by the LARS procedure.

Model Comparison and Effect Estimation

We identified 38 CpG sites ("top sites") that were differentially methylated at 7 years of age following exposure to adversity ($p < 1 \times 10^{-7}$) (Figure 2). Methylation at most sites ($n = 35$) was related to the developmental timing of exposure to adversity, especially adversity during very early childhood, meaning between birth and age 2 years (Figure 3A). In fact, exposure to adversity during very early childhood explained variability at more CpG sites (22 in total) than expected, while the accumulation and recency models were associated with fewer CpG sites than expected (one and two CpG sites, respectively; $\chi^2 = 7.40$, $p = .02$).

As shown in Table 1 and Figure 3A, neighborhood disadvantage was the type of adversity predicting the greatest number of genome-wide methylation differences (10 CpG sites), followed by financial stress (nine CpG sites), sexual or physical abuse (by anyone) (five CpG sites), and one adult in the household (five CpG sites). Maternal psychopathology, caregiver physical or emotional abuse, and family instability were associated with differences at four, three, and two CpG sites, respectively.

Across all 38 top sites, exposure to adversity was typically associated with hypermethylation (73.7% positive beta coefficients; $\chi^2 = 8.53$, $p = .004$) (Table 1). On average, exposure to adversity during a sensitive period was associated with a 2.5% difference in methylation level (β) after controlling for all covariates (range 0.1%–14.2%). For the two CpG sites associated with recency of exposure to financial stress, one additional adverse event was associated with a 0.3% to 0.4% increase in methylation per year of age at the event. For the single site associated with accumulation of exposure, one additional adverse event was associated with a 0.5% decrease in methylation. Of these 38 CpG sites, 14 remained statistically significant after we imposed a more stringent *p* value threshold that accounted for the testing of seven types of adversity ($p = [1 \times 10^{-7}]/7 = 1.43 \times 10^{-8}$) (Table 1).

After relaxing the multiple testing correction threshold to an FDR $q < .05$, there were 380 CpG sites affected by exposure to adversity (Figure 3B, and Table S3 in Supplement 2). As with the top 38 Bonferroni-significant sites, methylation at 352 of the 380 FDR-significant sites was best explained by sensitive period models (Figure 3B, and Table S3 in Supplement 2). Exposure in very early childhood explained methylation variation at more CpG sites than expected from the background for neighborhood

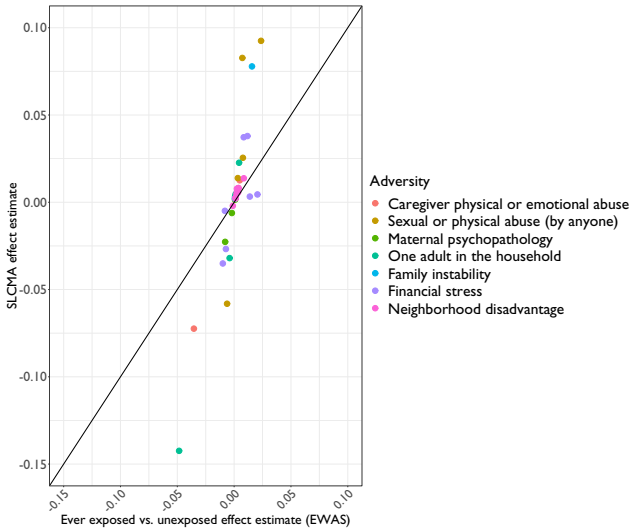

Figure 4. A scatterplot displays increased power in the structured life course modeling approach (SLCMA) shown by the comparison of beta estimates from the epigenome-wide association study (EWAS) vs. SLCMA approaches. In this scatterplot, the y-axis represents the β estimates associated with the 38 top CpG sites derived for the SLCMA; the x-axis represents the β estimates associated with the same 38 CpG sites obtained from EWASs. Different types of adversity are indicated by colors. The black straight line denotes the 1:1 correspondence between the two sets of β values. Substantial positive deviation from the line suggests increased power in the SLCMA. For most CpG sites, the magnitudes of effect were larger for the SLCMA compared with the EWAS results.

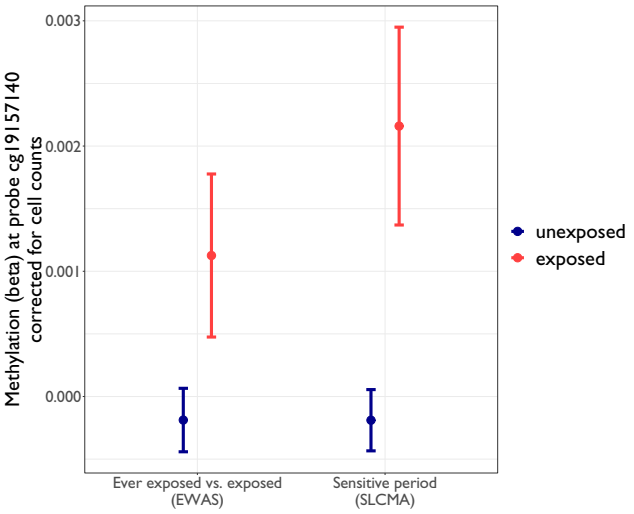

Figure 5. Comparison of epigenome-wide association study (EWAS) vs. structured life course modeling approach (SLCMA) estimates for the top CpG site identified in the SLCMA, cg19157140. The effect estimates and the confidence intervals obtained from the EWAS approach comparing ever exposed to never exposed to neighborhood disadvantage for cg19157140 are presented on the left. The stage 2 effect estimates and confidence intervals obtained from the SLCMA comparing being exposed to neighborhood disadvantage at 1.75 years of age with being unexposed at 1.75 years of age for the same CpG site are displayed on the right. The top CpG site in the SLCMA, which suggested a sensitive period at 1.75 years of age associated with the effects of neighborhood disadvantage, was nonsignificant after correction for multiple testing (p = .0002) in the epigenome-wide association study of neighborhood disadvantage.

disadvantage (Figure S2 in Supplement 1). The effects of adversity type and timing on methylation were distributed throughout the genome (Figure S3 in Supplement 1).

Exposed Versus Unexposed Analysis

Across the seven EWASs, which separately evaluated the effect of ever versus never exposed to each type of adversity on CpG site DNAm, only one statistically significant result emerged (Figure S4 in Supplement 1); this was for cg02431672, a locus located on chromosome 1 79 kb away from the gene *FAM183A*, and it was associated with exposure to abuse ($\beta = -.005$; $p = 1.77 \times 10^{-8}$).

Overall, there was very little overlap in identified CpG sites across the top SLCMA and EWAS results. Most of the top 38 sites had effect estimates that were larger in the SLCMA compared with the EWAS (Figure 4). There was also little overlap in findings across specific CpG sites. For example, the cg02431672 locus, which was the top hit in the EWAS of abuse, did not emerge as a top hit in the SLCMA of abuse, failing to appear in the list of FDR-significant loci (p = .0138). Similarly, the top CpG site in the SLCMA (cg19157140), which suggested a sensitive period at 1.75 years of age associated with the effects of neighborhood disadvantage, was nonsignificant in the corresponding EWAS (β = .001; p = .0002) (Figure 5). These results suggest that the SLCMA allowed us to more effectively identify methylation differences among children with and without a history of exposure to adversity.

Sensitivity Analyses

Evaluation of the LARS Selection Procedure. There was no evidence in support of compound theoretical models, whereby more than one theoretical model explained the most outcome variability. For each of the top 38 CpG sites, the marginal increase in variance of methylation explained by additional steps of the LARS procedure was not significant (each $p > .05$) (Figure S5 in Supplement 1), suggesting that methylation was best explained by a single theoretical model.

Evaluation of Methylation at Birth for Top CpG Sites. Adversity-associated methylation differences occurred during very early childhood for most top CpG sites. To assess whether the observed differences in DNAm existed at birth, we examined the effect of the selected exposure on DNAm in cord blood for the top 38 sites. We found that DNAm differences at birth were significant for only one of the 38 sites ($p < .05/38$, or .00132), suggesting that the differences in DNAm at 7 years of age mainly occurred after birth, as a result of exposure to postnatal stressors (Table S4 in Supplement 2). Similar results were obtained when examining the 380 FDR-significant loci, where significant differences at birth were detected at only six of the 380 probes (Table S4 in Supplement 2). An example of a site differentially methylated at birth and an example of a site nondifferentially methylated at birth are shown in Figure S6 in Supplement 1.

Correction for Genetic Variation. Genetic variation did not appear to influence observed DNAm differences at the top CpG sites. Using a database of methylation quantitative trait loci of the ARIES cohort (77), there were 658 single nucleotide polymorphisms associated with DNAm at 17 of the top 38 sites. After controlling for genetic variation at methylation quantitative trait loci linked to these 17 sites, the effect of exposure to adversity remained significant (each FDR $q < .05$; Table S5 in Supplement 1), suggesting that adversity could have caused these methylation differences distinct from genetic sequence variation.

Exploring the Biological Significance of Findings

Correlation Between Blood and Brain Tissue. On average, methylation in blood at the top 38 sites was slightly positively correlated with methylation in four brain regions (prefrontal cortex: $r_{avg} = .10$, entorhinal cortex: $r_{avg} = .11$, superior temporal gyrus: $r_{avg} = .11$, cerebellum: $r_{avg} = .06$) (Table S6 in Supplement 1). CpG sites with methylation that is highly correlated between blood and brain tissue may be indicative of important interindividual covariation (i.e., due to adversity) or a strong genetic influence on methylation, while those that are uncorrelated may still be biomarkers of a response to adversity.

Enrichment of Regulatory Elements. As compared with all autosomal loci tested, FDR-significant loci were more likely to be located in gene promoters ($\chi^2 = 9.92$, $p = .002$) and less likely to be in gene enhancers ($\chi^2 = 3.86$, $p = .049$; Figure S7A in Supplement 1). Furthermore, the location of FDR-significant loci differed from all other loci tested relative to CpG islands ($\chi^2 = 42.92$, $p < .0001$) (Figure S7B in Supplement 1). With eFORGE 1.2 (78), we also tested whether FDR-significant loci colocalize with markers of transcriptional activity. FDR-significant loci were not enriched for DNase I hypersensitivity sites or histone marks in any tissue or cell type after correction for multiple comparisons (each $q > .05$). The strongest trend for enrichment was detected in the analysis of all histone marks in fetal thymus cells (uncorrected $p = .0007$). Annotations at each FDR-significant site are presented in Table S3 in Supplement 2.

Biological Processes Potentially Affected by Adversity. Genes near the FDR-significant sites ($n = 365$ genes) corresponded to 158 clusters of gene ontology biological process terms (75). The top 11 gene ontology term clusters, including positive regulation of developmental growth, axon development, and neuron apoptotic process, were more likely to be represented than by chance (average enrichment $p < .05$) (Figure S8 in Supplement 1).

Additionally, we uncovered evidence of functional constraint for these genes. Genes annotated to FDR-significant sites were more highly constrained, as measured by the probability of intolerance to loss-of-function variation from the Exome Aggregation Consortium (76), than the rest of the autosomal genes tested (permutation $p = .0001$) (Figure S9 in Supplement 1). This indicates a greater importance of these genes, on average, to survival and reproduction over human evolution.

DISCUSSION

This prospective study used data from a large population-based sample of children to test three competing life course theoretical models describing the association between exposure to childhood adversity, measured repeatedly across the first 7 years of life, and DNAm at 7 years of age. By comparing these theoretical models to each other, we could evaluate which one explained the most variation in DNAm. To our knowledge, this is the first use of the SLCMA in an epigenome-wide context.

The main finding of this study is that the effect of adversity on DNAm depends primarily on the developmental timing of exposure. In our Bonferroni-corrected analysis, we identified 38 CpG sites that were differently methylated following exposure to adversity, with more than half of these loci showing associations based on adversity occurring during very early childhood, meaning before 3 years of age. Exposure in very early childhood was associated with DNAm differences for nearly all adversity types. In contrast, the effects of exposure in middle childhood were largely detected only for arguably the most severe forms of adversity exposure (e.g., sexual or physical abuse). These results are consistent with those of at least one human longitudinal study (16) and multiple animal studies (21,22,24,25) in emphasizing the existence of sensitive periods (19,20)—particularly occurring shortly after birth—when epigenetic programming is maximally dynamic in response to parental care disruptions and other environmental inputs. The lack of detectable sensitive periods in one recent study (32) may be due to focusing only on adversities occurring at or after 5 years of age. Interestingly, neither the accumulation nor recency of the adversity explained considerable variability in DNAm. The observed DNAm differences were absent at birth, identified for a range of adversities, and unrelated to genetic variation. The absence of support for an accumulation model is surprising, given previous research linking cumulative time spent in institutional care to DNAm status in stress-related genes (29).

Perhaps more importantly, our results suggest that broad classifications of individuals as exposed versus unexposed to "early life" adversity—although commonly used—may dilute observed effects and fail to detect DNAm differences among people exposed to adversity during specific life stages. These findings support the value of more detailed phenotyping, which is meaningful given the trend in psychiatric genetics toward minimizing phenotypic precision in the service of maximizing sample size. The lack of overlap in identified loci across the SLCMA and EWAS suggest that refinement of the environmental phenotype—by treating each time point of exposure as unique—may better capture underlying signal. Indeed, results of a power analysis suggest that the EWAS of exposed versus unexposed will be underpowered when the true underlying relationship between exposure and outcome depends on the timing or amount of exposure (Supplement 1). Thus, more precise phenotyping could preserve study power and provide more mechanistic insights to guide targeted interventions.

These findings also raise important questions regarding why exposure to adversity in the first 3 years of life may be particularly salient in influencing DNAm patterns. When adversity occurs early in life, it coincides with the initial and foundational sculpting

of brain architecture. Experiences of childhood adversity, which represent deviations from expected cognitive, social, and sensory inputs (60), may be more likely to be wired into neural circuitry during this especially vulnerable stage in brain development. Relatedly, DNAm patterns are known to be dynamic across the life course. It may be that very early exposure to adversity produces more stable DNAm changes that persist across the life course, in contrast with later exposure to adversity. With more longitudinal studies of DNAm, the field of psychiatric epigenetics will be better positioned to determine not only when are the most vulnerable life stages for DNAm changes to occur, but also the extent to which these adversity-induced DNAm patterns persist over time.

Although these findings emphasize the importance of exposure timing, greater insights are needed regarding the age stages when adversity may be most harmful, as mixed results have emerged among the small number of studies comparing the effects of early versus later adversity. Some retrospective studies have shown that adolescent DNAm patterns are more strongly associated with life stress during adolescence than earlier periods (27). However, other studies have found potentially persistent effects of childhood adversity into adolescence (31) and adulthood (11), even after accounting for subsequent stress exposure. A recent study also found that the effects of adversity timing may be gene specific (29). As epigenetic patterns appear to vary over the life course (26,79), longitudinal studies are needed to study the developmental trajectories of DNAm and evaluate the extent to which these adversity-induced DNAm differences persist or attenuate over time, and operate independently of or in interaction with subsequent experience to ultimately predict mental health outcomes. Ideally, these longitudinal studies would include repeated measures of prenatal and postnatal adversity exposure and investigate whether any adversity-associated DNAm signatures predict psychopathology. If our findings about the importance of sensitive periods do replicate, these results would emphasize the need to prioritize policies and interventions toward children exposed to adversity within the first 3 years of life, when the biological effects of adversity may be most profound.

Several limitations are noted. First, some adversity measures were drawn from single items. Parents may have also underreported exposure to stigmatizing experiences (80,81), especially if they were implicated in the exposure (82). However, the prevalence of several adversities, including those capturing possible experiences of abuse, were similar to and even greater than those reported from some nationally representative samples (9,83). Second, as with any longitudinal study, there was attrition over time, which could result in bias owing to loss of follow-up. However, ARIES children were sampled from among those with the most complete longitudinal data. Within the field of epigenetics, efforts are now underway to understand the consequences of attrition and how potential biases arising from attrition could be mitigated through multiple imputation or other strategies. Third, we were unable to examine the impact of experiencing multiple adversities simultaneously, because each adversity was measured at a slightly different time point. Fourth, the DNAm samples were obtained from peripheral tissue and not the brain; multiple data sets, however, are starting to identify limited though important shared DNAm patterns across central nervous system and peripheral tissue (84). Fifth, we were unable to directly examine whether DNAm at the identified loci influenced gene expression of the nearest genes. Future work using a sample with both methylation and expression data is needed to clarify the functional consequences of significant CpG sites. Finally, the *p* values derived from the covariance tests could be potentially inflated, as the test relies on asymptotic theories and therefore does not theoretically guarantee the control of type I error rate in a finite sample (69). However, the covariance test might be a more sensitive method to detect signals compared with other postselection significance tests that make fewer assumptions (85). As the relative statistical power of the available tests remains unclear, simulation studies are underway to identify the best inference tools in different settings and the statistical power of the SLCMA with varying effect sizes.

In summary, this study lends further support to the evidence base showing that DNAm patterns are responsive to experience. However, these results reveal that DNAm patterns may be most influenced by exposures during sensitive periods in development. Efforts may therefore be needed to move beyond crude comparisons of those exposed versus unexposed to early-life adversity.

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