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**Examining the epigenetic mechanisms of childhood adversity and sensitive periods:
a gene set-based approach**

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Key Words: childhood adversity, sensitive periods, ALSPAC, plasticity, epigenetics

Abstract

Background: Sensitive periods are developmental stages of heightened plasticity when life experiences, including exposure to childhood adversity, have the potential to exert more lasting impacts. Epigenetic mechanisms, including DNA methylation (DNAm), may provide a pathway through which adversity induces long-term biological changes. DNAm shifts may be more likely to occur during sensitive periods, especially within genes that regulate the timing of sensitive periods. Here, we investigated the possibility that childhood adversity during specific life stages is associated with DNAm changes in genes known to regulate the timing and duration of sensitive periods.

Methods: Genome-wide DNAm profiles came from the Avon Longitudinal Study of Parents and Children (n=785). We first used principal components analysis (PCA) to summarize DNAm

variation across 530 CpG sites mapped to the promoters of 58 genes previously-identified as regulating sensitive periods. Gene-level DNAm summaries were calculated for genes regulating sensitive period *opening* ($n_{\text{genes}}=15$), *closing* ($n_{\text{genes}}=36$), and *expression* ($n_{\text{genes}}=8$). We then performed linear discriminant analysis (LDA) to test associations between seven types of parent-reported, time-varying measures of exposure to childhood adversity and DNAm principal components. To our knowledge, this is the first time LDA has been applied to analyze functionally grouped DNAm data to characterize associations between an environmental exposure and epigenetic differences.

Results: Suggestive evidence emerged for associations between sexual or physical abuse as well as financial hardship during middle childhood, and DNAm of genetic pathways regulating sensitive period opening and expression. However, no statistically significant associations were identified after multiple testing correction.

Conclusions: Our gene set-based method combining PCA and LDA complements epigenome-wide approaches. Although our results were largely null, these findings provide a proof-of-concept for studying time-varying exposures and gene- or pathway-level epigenetic modifications.

1. Introduction

Childhood adversity is commonly defined as social, emotional, and physical experiences that deviate from expected environmental inputs during development, including the presence of aversive stimuli or absence of an adaptive learning environment (Gilbert et al., 2009; McLaughlin et al., 2017). Exposure to childhood adversity can impact development, leading to changes in myriad neurological, behavioral, and health outcomes (Berens et al., 2017). These consequences are thought to be more impactful when adversity occurs during *sensitive periods* in

development, meaning periods of increased plasticity (meaning, both sensitivity and vulnerability) when individuals are more susceptible to life experiences, including exposure to childhood adversity (Knudsen, 2004). To that end, and consistent with sensitive period theories, recent studies are beginning to reveal that the effects of childhood adversity might be time-varying, with adversity exposures during early childhood possibly being more detrimental than exposures occurring earlier or later in development (Dunn et al., 2020).

Genetic processes play a key role in regulating the timing of sensitive periods. Both animal and human studies have identified more than 50 genes involved in shaping the *timing* of sensitive periods across brain development (Takesian and Hensch, 2013). Specifically, these genes are involved the opening, closing, and maintenance (or expression) of sensitive periods. *Opening* genes regulate when sensitive periods *begin*, accelerating or delaying the onset of sensitive period plasticity by modifying parvalbumin cell maturation or excitatory-inhibitory circuitry balance (Anomal et al., 2013; Takesian and Hensch, 2013). *Closing* genes regulate when sensitive periods terminate; these genes play important roles in the formation of perineuronal nets, which emerge with sensitive period closure and limit plasticity (Lee et al., 2017). Finally, *expression* genes maintain the duration of how long sensitive periods stay open, orchestrating structural changes that lead to circuit consolidation over time (Fagiolini et al., 2003).

The functions of these sensitive period genes have been predominantly identified in the primary sensory cortex using animal models (Hensch, 2004; Hooks and Chen, 2007; Sharma et al., 2015). However, evidence for similar plasticity regulating mechanisms in the prefrontal cortex and subcortical structures is emerging (Gogolla et al., 2009; Guirado et al., 2020). For example, we recently applied a gene set approach to examine how genetic pathways involved in

sensitive period functioning are associated with risk for depression in humans (Zhu et al., 2022). Our results suggest that genetic variation in *opening* genes is implicated in depression risk in the general population. We also found that individuals with high genetic risk for depression (as conferred by *opening*-related genes) and who were exposed to caregiver abuse during an empirically identified sensitive period (from ages 1 to 5) had the highest levels of depression in adolescence, compared to individuals who did not have sensitive-period related genetic susceptibility or exposure to childhood adversity. Together, these findings highlight the role of sensitive period-regulating pathways in shaping psychiatric vulnerabilities during development.

Evidence is lacking on how adversity *timing* shapes epigenetic modifications of these sensitive period pathways in humans. One major type of epigenetic modification, DNA methylation (DNAm), has been increasingly studied as a potential biological signature of exposure to childhood adversity. Emerging evidence suggests DNAm patterns may be most strongly influenced by adversity that occurs during the first five years of life, compared to adversity at other ages or the accumulation of adversity across time (Dunn et al., 2019; Lussier et al., 2022). However, existing research on epigenetic profiles linked to childhood adversity is limited. Candidate gene studies have narrow scope, cannot generate novel hypotheses, and lack replicability and sometimes generalizability (Parade et al., 2021). Results from discovery-driven epigenome-wide analyses (EWAS) may have limited interpretability, as they do not specify or test *a priori* hypotheses.

Gene set-based analyses of DNAm data combine the advantages of discovery- and hypothesis-driven studies by investigating *biological pathways* rather than specific genes. As such, gene set-based approaches can complement EWAS by providing additional new insights into mechanisms implicated in the pathophysiology of neuropsychiatric disorders (Do et al.,

2015). For example, by considering the DNAm patterns of genes linked to sensitive periods, researchers can investigate the extent to which exposure to childhood adversity has time-dependent associations with epigenetic modifications that might disrupt processes involved in developmental plasticity. In this study, we analyzed data from a large, population-based sample to identify if and when childhood adversity exerts stronger effects on DNAm during the first seven years of life. We analyzed DNAm for loci mapped to three gene sets shown to regulate sensitive periods. Gene sets were identified from a literature review of animal and human studies on known genetic components governing the *opening* ($n_{\text{genes}}=15$), *closing* ($n_{\text{genes}}=36$), and *expression* ($n_{\text{genes}}=8$) of sensitive periods for brain development (**Figure 1**; Takesian and Hensch, 2013; Zhu et al., 2022). To our knowledge, our study is the first to implement LDA with functionally grouped epigenetic data to examine associations between time-varying exposures and DNAm.

2. Materials and Methods

2.1 Sample and Procedures

Data came from a UK-based prospective birth cohort, called the Avon Longitudinal Study of Parents and Children (ALSPAC) (Boyd et al., 2013; Fraser et al., 2013). The ALSPAC is a longitudinal population-based cohort based in Avon, UK. Pregnant women residing in the area with estimated delivery dates between April 1, 1991, and December 31, 1992, were invited to participate in the study. The initial sample included 14,514 women who returned at least one questionnaire or attended one focus clinic by July 19, 1999. Out of the initial sample, a total of 14,676 fetuses and 13,988 children who were alive at 1 year of age were included. A second wave of participants were recruited when the oldest children in the cohort reached approximately

7 years of age, which consisted of eligible individuals who did not join the study initially, resulting in the enrollment of an additional 913 children. With the two waves combined, the ALSPAC sample consisted of 14,901 children alive at age 1 year (Boyd et al., 2013; Fraser et al., 2013).

Our analytic sample came from a subsample of ALSPAC, called the Accessible Resource for Integrated Epigenomics Studies (ARIES). The ARIES subsample includes 1,018 mother-child pairs from whom blood-based DNAm data were collected. Participants in the ARIES subsample were randomly selected from ALSPAC participants with complete data across at least five timepoints of data collection (Relton et al., 2015). There were 785 singleton children with DNAm at age 7 in our analytic sample, who had complete data on all covariates and at least one type of childhood adversity across all time points.

Ethical approval for ALSPAC and ARIES was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committee. Consent for biological samples was collected in accordance with the Human Tissue Act (Human Tissue Act, 2004). Please note the study website contains details of all the data that is available through a fully searchable data dictionary and variable search tool (<http://www.bristol.ac.uk/alspac/researchers/our-data/>). Secondary analyses of ALSPAC data were approved with oversight by the Mass General Brigham Institutional Review Board (IRB) (Protocol 2017P001110).

2.2 Measures

2.2.1 Exposure to Childhood Adversity

We analyzed seven commonly-occurring types of childhood adversity: (a) caregiver physical or emotional abuse; (b) sexual or physical abuse (by anyone); (c) maternal

psychopathology; (d) one adult in the household; (e) family instability; (f) financial hardship; and (g) neighborhood disadvantage. These adversities were selected because they were frequently examined in prior studies of early life stress on epigenetic profiles (Barker et al., 2018; Dunn et al., 2019; Krause et al., 2020). They also had at least four repeated assessments before age 7 in ALSPAC, enabling analysis of adversity timing. Maternal psychopathology was assessed using two psychometrically validated instruments (namely, the Crown-Crisp Experiential Index (Crown and Crisp, 1979) and the Edinburgh Postnatal Depression Scale (Cox et al., 1987)) and one item evaluating suicidality. The other six adversities analyzed were measured using maternal and/or partner prospective self-assessments. **Table 1** presents a summary of the respondent(s), instrument(s) or questionnaire items, exposure definition, and available time points for each adversity.

To identify potential sensitive period effects of childhood adversity on DNAm, we created three exposure variables per adversity, based on whether children were exposed to that type of adversity, during each of three developmental periods: very early childhood (0-2 years), early childhood (3-5 years), and middle childhood (6-7 years). The developmental periods were defined to be consistent with our prior research on epigenetic markers of childhood adversity (Dunn et al., 2019), and were based on subject matter knowledge only, independent of any results. For a given adversity, *any* reporting of exposure (within one or more developmental periods) led us to code a child as exposed during that developmental period(s); reporting of no exposure (at *all* assessment periods) led us to code a child as unexposed. Overall, 21 variables were created in total, for three developmental periods and seven types of adversity.

2.2.2 DNA Methylation

DNAm profiles were obtained from blood samples collected at age 7 and assayed using the Illumina Infinium Human Methylation 450k BeadChip microarray, which measured DNAm at 485,577 CpG sites (Illumina, San Diego, CA). The 450K microarray captures DNAm variation at 99% of RefSeq genes. To minimize batch effects, samples were semi-randomly assigned across time points to different plates (Relton et al., 2015). Beta values for DNAm were analyzed, which represent the proportion of cells methylated at each CpG. Data quality control was performed using the protocol described by Min et al. (Min et al., 2018). The pipeline applied functional normalization and background correction using the *meffil* R package. Additionally, cross-hybridizing CpGs, polymorphic CpGs, and CpGs located in sex chromosomes were removed before analyses. To reduce the impact of outliers, beta values at each CpG site were winsorized by setting values outside the 5th and 95th percentiles to the 5th and 95th cutoffs, respectively.

We focused on DNAm in promoter regions, as promoters show more robust associations with transcription levels (Vialou et al., 2013). Promoters were defined as the genomic regions 1500 base pairs upstream of the transcription start site and from the 5'-untranslated region through the first exon (Lokk et al., 2014). A total of 530 CpG sites were annotated to the promoters of the 58 sensitive period regulating genes. The number of CpG sites annotated to each gene promoter varied from 1 (*PILRB*) to 31 (*PTPRS*) (**Figure 2**). A full list of CpG sites examined (and their annotations) is available in **Table S1**.

Of note, although our list of sensitive period genes came from published studies on plasticity in the brain, DNAm was measured in blood by ALSPAC researchers. Correlation analyses between DNAm levels in blood and brain at these 530 CpG sites were modestly

concordant (**Supplemental Materials**), similar to the distribution across the entire genome (Hannon et al., 2015; Braun et al., 2019).

2.2.3 Covariates

To control for potential confounding of the association between childhood adversity and DNAm by sociodemographic characteristics, we adjusted for the following covariates: child race/ethnicity (White; non-White); birth weight (continuous, in grams); maternal age (ages 15-19; ages 20-35; ages 35+); number of previous pregnancies (0;1;2; 3+); sustained maternal smoking during pregnancy (yes; no); and maternal education (levels corresponding to schooling before age 16, until age 16, through ages 16-18, and university degree or above). Prior studies showed these variables could influence both exposure to childhood adversity and epigenetic modifications (Dunn et al., 2019; Marini et al., 2020).

2.3 Data Analysis

Primary analysis

Figure 1 displays the analytic steps of our study. In step 1, technical variables were regressed on DNAm levels at each CpG site (estimated cell proportions and sample type, i.e., whole blood or white blood cells). In step 2, we used PCA as a data reduction strategy, as including all 530 CpGs was computationally challenging. PCA extracts linear combinations (i.e., principal components, or PCs) of DNAm values at the promoter of each CpG site within a certain gene. The goal was for these PCs to explain the maximal amount of variance across individual CpGs, and create parsimony in subsequent regression models. With one exception (*PILRB*), we retained two PCs per gene, which on average explained 75% of variability in

DNAm in each gene. For *PILRB*, only one CpG was available for analysis, thus only one PC (explaining 100% of variability in DNAm) was retained. Using PCA, we reduced the original 530 individual CpGs to 16-77 PCs per analysis, which correspond to 8-39 genes in each gene set. In step 3, we adjusted for confounding by regressing the PCs on all pre-specified covariates listed under section 2.2.3.

In step 4, we used linear discriminant analysis (LDA) to test associations between childhood adversity and the PCs in each gene set. LDA provides a measure of association between a binary exposure, in this case childhood adversity exposure status within one of the three developmental periods, and a multivariate outcome, here the DNAm PCs in a gene set. LDA is mathematically equivalent to canonical correlation analysis (Bach and Jordan, 2005), as both methods assess multivariate associations. The strength of association was calculated as the squared canonical correlation (canonical R^2), which is a multivariate equivalent of a Pearson's correlation coefficient. We calculated the canonical R^2 for each of the three developmental periods and selected the developmental period with the largest canonical R^2 for reporting. To account for the selection of the largest canonical R^2 , we constructed an empirical null distribution for the largest R^2 through 5,000 bootstrapping iterations and reported the bootstrap p-value.

Notably, the LDA models were adversity- and timing-specific. In other words, we tested a simple model, where only exposure to a specific type of adversity during the tested developmental period had an association with the outcome. Therefore, the comparison group was defined as children unexposed to the tested exposure during that period, because our hypothesis focused on the presence of effect at a given time point above and beyond exposures at other time periods. Construction of hypotheses and comparison groups in this manner is consistent with the

structured life course modeling approach (SLCMA; pronounced slick-mah) (Smith et al., 2022).

Step 4 was repeated for each adversity and gene set, summing to 21 tests of association. We therefore assessed p-values after Bonferroni corrections ($\alpha=0.05/21=0.002$).

Secondary analysis

To further interpret our results, we pursued a secondary analysis using data available from the EWAS Catalog (<http://www.ewascatalog.org>). The EWAS catalog is a well-curated database containing published results from EWAS of DNAm and phenotypes spanning multiple domains, including biological functioning, disease risk, and social characteristics (Battram et al., 2021). We annotated the 530 probes analyzed here to any trait associations reported in the catalog. All associations from published analyses with a p-value $< 1 \times 10^{-4}$ were reported.

Sensitivity analyses

To evaluate the robustness and generalizability of our results, we pursued four sets of sensitivity analyses. First, DNAm patterns in different genomic regions (e.g., promoters vs. gene bodies) may be involved in different regulatory processes, sometimes in divergent directions (Jones, 2012). We therefore evaluated if our primary results, which were based on promoter regions, were similar to analyses examining variation in gene bodies of the three sensitive period gene pathways. The same analytic procedure was followed for these analyses: beta values at CpG sites in each gene body were summarized into two principal component scores per gene, and a LDA model was fit to each exposure time point.

Second, it is possible that any exposure to adversity before age 7 impacts DNAm, regardless of when the exposure occurred. Thus, we tested a basic model, comparing the presence (versus absence) of exposure to each type of adversity before age 7 on DNAm variation in promoters of sensitive period gene sets. Again, we pursued the same analytic approach, except

that no selection of time period was examined, only the ever versus never exposure variable for each adversity type.

Third, there are ongoing debates regarding the benefits and drawbacks of lumping (versus splitting) of adversity by type (Smith and Pollak, 2021). Some argue that different types of childhood adversity may impact epigenetic modifications through distinct pathways and thus should be examined separately (Dunn et al., 2019). Others suggest that early life stress, regardless of adversity type, could have similar effects on underlying biological processes (Heim and Binder, 2012); therefore, these effects can be captured under a broad classification of childhood adversity. To provide a contrast for our results, we investigated whether aggregating exposure status across the seven types of adversity, instead of examining each type separately as in the primary analysis, changed the results. During each developmental period (very early childhood, early childhood, and middle childhood), children exposed to any of the seven types of childhood adversity were coded as exposed, resulting in three models for each gene set (i.e., one for each sensitive period).

Fourth, as the gene set-based approach was meant to complement EWAS, meaning the agnostic search for signals across the genome, we were interested in determining if combining CpGs into genes and pathways yielded more information than CpG-level analyses. Therefore, we compared our findings to those obtained from a previous EWAS of the same dataset (Lussier et al., 2022). Specifically, Lussier et al. performed a SLCMA to compare multiple encoded exposures of childhood adversity and identify the life course hypothesis most strongly associated with DNAm at each locus across the epigenome using the ALSPAC cohort.

All analyses were performed using software package R. We made the scripts publicly available on GitHub (<https://github.com/thedunnlab/geneset>).

3. Results

3.1 Sample Characteristics

Compared to the entire ALSPAC, children in the ARIES analytic sample (n=785) were more likely to be born to mothers who were older, had fewer previous pregnancies, less likely to smoke during pregnancy, and better educated at baseline. Children in the analytic sample were also less likely to experience childhood adversity (**Table 1**).

The prevalence of childhood adversity ranged from 2.2% to 24% across adversities and time periods (**Figure S1**). Overall, 496 children (63.2%) experienced at least one type of childhood adversity before age 7. Across time points, exposure to any type of childhood adversity was most prevalent during very early childhood (48.2%), followed by early childhood (37.7%) and middle childhood (29.0%). Within a specific type of adversity, exposures were moderately to strongly correlated across time periods (**Figure S2**). Across adversities, exposures were weakly to moderately correlated within a period (**Figure S3**).

3.2 Principal Component Analysis, Linear Discriminant Analysis, and Model Selection

Primary Results

For 53 of 58 genes, the first two PCs accounted for more than 50% of the variation in promoter DNAm (**Figure 2**). Adversity during middle childhood (6-7 years) was most frequently associated with DNAm in the promoters of genes involved in regulating sensitive period functioning (selected for 12 out of the 21 LDA models). Two associations between the timing of adversity and DNAm of gene sets were identified at a bootstrap p-value < 0.05, which was corrected for the three sensitive period hypotheses tested per gene set and adversity type. Specifically, exposure to sexual or physical abuse in middle childhood was associated with

differences in DNAm among *opening* genes ($p=0.019$). Exposure to financial hardship in middle childhood was associated with DNAm differences in *expression* genes ($p=0.028$). After applying a Bonferroni correction for multiple testing, no association remained significant.

Secondary Analysis Results

CpGs annotated to promoters of the sensitive period genes in our study were linked to other traits in previous EWAS. Queries of the 530 CpG sites using results from the EWAS Catalog yielded 2000 entries across 503 CpGs and 58 genes (**Table S1**). The number of studies reported for each CpG ranged from 14 (cg16762684, annotated to the closing gene, *MBP*, encoding myelin basic protein) to 1, capturing 79 unique traits. Out of the 503 CpGs, the majority (89%; $n=446$) reported associations with biological age, an unexpected finding given that these gene sets are implicated in developmental plasticity and sensitive period timing. The second most common trait was tissue type ($n=393$), followed by sex ($n=78$), gestational age ($n=73$), and rheumatoid arthritis ($n=61$). Notably, seven CpGs also appeared in study results of schizophrenia.

Sensitivity Analysis Results

Results from sensitivity analyses were as follows. First, DNAm annotated to gene bodies did not yield even a nominal signal after bootstrapping (**Table S2**). Second, analysis of lifetime exposure to adversity suggested sexual or physical abuse was nominally associated with DNAm in the *opening* gene promoters ($p=0.041$), but this association was not statistically significant after multiple test correction (**Table S3**). The canonical R^2 associated with sexual or physical abuse was also attenuated from 0.08 to 0.06, and the corresponding Wilks' p -value increased from 0.004 to 0.041, suggesting any signal was weakened by combining exposures across time points. Third, the composite measure of any type of childhood adversity before age 7 was also

unassociated with DNAm PCs (**Table S4**). Fourth, there was little overlap in our gene set-based analyses and the CpG-level associations shown by Lussier et al. (2022). No association emerged at $FDR < 0.05$ among all 530 CpGs (**Table S5**).

4. Discussion

In the current study, we implemented a novel analytic approach combining PCA and LDA to interrogate the joint contribution of time-varying exposures to childhood adversity and epigenetic modifications of pathways implicated in sensitive period functioning. As is often the case for an early work, particularly in the 'omics setting, our analyses did not detect any strong signals between adversity and DNAm in these gene sets. Nevertheless, our analytic approach provides proof-of-concept for a gene set-based approach to examine time-dependent exposures and pathway-specific DNAm. Specifically, our approach and results contribute to the existing literature in several meaningful ways.

First, our analyses demonstrate how PCA and LDA can be implemented to examine a pathway-specific hypothesis about time-varying environmental experiences and downstream differences in epigenetic profiles. The combination of PCA and LDA allowed us to achieve the goals of data reduction and hypothesis testing effectively, which has also been demonstrated in a study exploring biotypes of post-traumatic stress disorder (Yang et al., 2021). Compared to EWAS, candidate gene, or other research designs in the literature of social and psychiatric epigenetics, our approach has several unique strengths. For example, our framework extends existing pathway-based analyses, including enrichment or network analyses (Brown et al., 2020; Grillault Laroche et al., 2020), by examining gene sets pertaining to specific biological hypotheses and sourced from literature reviews or subject matter knowledge. Our approach also

leverages perspectives from the SLCMA to account for time-dependent effects of correlated exposures via model selection and bootstrapping (Smith et al., 2022).

Second, although no conclusive evidence emerged from our results, the suggestive associations shed light on the value of considering developmental timing and sensitive periods at both the genetic and phenotypic level. After correcting for the number of sensitive period hypotheses tested via bootstrapping, exposure to adversity during middle childhood (ages 6-7), namely physical or sexual abuse and financial hardship, was potentially linked to differences in DNAm patterns within the promoters of genes involved in the onset or duration of sensitive periods. Middle childhood is a critical stage of development: key structures of neural plasticity, such as perineuronal nets, reach maturation around age 8 in brain regions implicated in psychopathology, including the medial prefrontal cortex and hippocampus (Mauney et al., 2013; Rogers et al., 2018). Adverse environmental stimuli during this period of rapid growth and maturation could lead to disruptions of experience-expectant mechanisms. Epigenetic modifications might also influence the activity of genes involved in neuronal plasticity, further disrupting molecular and behavioral responses. However, we emphasize our analyses were exploratory in nature and no association persisted after accounting for the number of adversities and gene sets examined. Repeated investigation of these findings in larger, more diverse samples, as well as studies focusing on the cascading effects of adversity later in life, are needed to further disentangle the role of epigenetic mechanisms in the biological embedding childhood adversity during sensitive periods.

Further, considering the time-sensitive nature of exposures allowed us to detect a few, albeit weak, signals compared to a traditional classification of ever versus never exposed. Although the decrease in effect estimate for physical or sexual abuse was not striking, as modest

relationships between exposure to adversity and DNAm overall were expected, it nevertheless suggests meaningful gains from a developmentally informed approach. Our sensitivity analysis looking at the combined effects of all types of adversity also revealed no association across time periods. Early life stress in different forms may produce unique biological signatures and alter functioning differently even within the domain of sensitive period regulation. Therefore, as discussed in prior reviews and empirical articles, biological signatures resulting from childhood adversity could potentially be revealed through alternate conceptualizations of adversity, such as “splitting” rather than “lumping” adversity measures (Sumner et al., 2019; Colich et al., 2020; Smith and Pollak, 2021).

Lastly, our analyses serve as an important proof-of-concept for showing the feasibility of applying a developmentally informed gene set-based approach. We tested hypotheses about whether and how childhood adversity may influence epigenetic changes of pathways involved in genetic orchestration of developmental plasticity. The lack of strong support for our hypothesis requires additional investigation. It is unclear if the absence of findings could be attributed to inadequate sample sizes to identify modest effects, or true absence of association for our hypothesis. Although very few existing studies on DNAm collect longitudinal data on childhood adversity with the level of granularity that our analysis included, replication of these possibly null results in another independent sample is needed. If the effects of childhood adversity on DNAm at sensitive period-related genes were too small to be detected or diluted by measurement error, larger data collection effort with prospective reporting of adversity may need to be prioritized.

Future research should also test alternative hypotheses. It may be that genetic factors regulating sensitive periods and childhood adversity jointly influence developmental outcomes

through other mechanisms than DNAm in promoter regions. As shown in a previous study, genetic risk conferred by common variants in specific sensitive period pathways modified the association between child abuse and depressive symptoms in adolescence (Zhu et al., 2022). Moreover, genetic or epigenetic variation in sensitive period-regulating components could modulate the timing and extent to which other key pathways were modified epigenetically, instead of directly affecting the sensitive period gene sets *per se*. Beyond hypotheses about developmental plasticity, our gene set-based approach can also be extended to test hypotheses about alternate neurobiological or physiological pathways underlying the effect of adversity, such as inflammation or functionalities of the hypothalamic-pituitary-adrenal axis (Cecil et al., 2020).

Our analytic process was straightforward to implement, and the scripts we provide can be adapted to assess other pathways of interest. Preliminary data on the statistical power of the method are also provided. These data may offer guidance for future applications, especially with more refined gene sets or modest sample sizes (**Figure S4**). We also encourage simulation-based power calculations to be performed for other pathways of interest in specific study contexts.

This study has several limitations. First, the sensitive period gene sets analyzed here may not be exhaustive. The current analysis primarily focuses on the genetic basis of neural plasticity regulated by GABAergic processes, whereas the time-varying effects of early life adversity may operate through other biological pathways. Thus, we may have potentially omitted possible genes implicated in sensitive period processes, which relate adversity to DNAm. As the field continues to identify molecular regulators of sensitive periods, our analyses can be expanded to include additional genes or pathways. Second, the definitions of adversity exposure and corresponding comparison groups did not encapsulate the full range of plausible mechanisms.

Because we were interested in prospectively reported adversity in childhood and DNAm measured at age 7, we did not examine exposures to adversity after 7 years, due to considerations about temporality (i.e., exposure assessment should precede DNAm sample collection). Thus, our analyses omitted exposures occurring in adolescence, a potential sensitive period linked to changes in brain functions and behavioral outcomes (Fuhrmann et al., 2015). Follow-up analyses investigating adversity exposure across development and later DNAm time points can more thoroughly examine the importance of exposure timing. Additionally, we examined each sensitive period exposure separately, following the parsimonious framework of the SLCMA. Our approach represents an initial step towards a more comprehensive understanding of adversity, sensitive periods, and epigenetic modifications in development. In future studies, potential interactions between time points and adversity types should be more carefully assessed. Third, our analyses may have been underpowered, due to the low prevalence of adversity during certain developmental periods, modest expected effect sizes, and the fact that statistical power of LDA decreases with the number of features included (Helmer et al., 2021). Although our sample size per feature was in line with prior recommendations for sufficient power and estimation stability (Barcikowski and Stevens, 1975; Helmer et al., 2021), we were inherently less powered to detect associations with larger gene sets, such as the *closing* set, due to the higher number of PCs analyzed. Thus, using our approach with larger datasets or conducting meta-analyses are critical next steps.

Finally, several factors could impact the generalizability of our results. For instance, we analyzed DNAm data from blood samples, despite our gene sets capturing developmental plasticity in the brain. While brain and blood DNAm levels are generally only modestly correlated (Braun et al., 2019), DNAm variations in peripheral tissues may still serve as

important biomarkers for brain-based disorders. In our analyses, almost half (28 out of the 58) of the genes included at least one CpG with strong blood-brain correlations ($\rho \geq 0.6$; **Figure S5**).

Thus, analyses of blood DNAm can allow identification of potential targets for follow-up experiments, where brain tissues might be accessible. Furthermore, ALSPAC participants are predominantly of European ancestry and the subsample with epigenetic data had lower levels of adversity compared to the larger cohort. It remains an important objective of future work to examine the generalizability of the current findings in children from minority groups, who often disproportionately experience childhood adversity (Slopen et al., 2016).

In conclusion, our study outlines a gene set-based approach to studying time-varying exposures and pathway-level epigenetic modifications during development. This analytic approach offers a useful complement to current epigenome-wide analyses and could ultimately improve the interpretability of molecular findings in neuropsychiatric research.

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Table 1. Summary of childhood adversity measures analyzed in the current study.

Adversity	Respondent	Instrument or questionnaire items	Exposure definition	Time points of assessment
Caregiver physical or emotional abuse	Mother and partner	1) your partner was physically cruel to your children; 2) you were physically cruel to your children; 3) your partner was emotionally cruel to your children; 4) you were emotionally cruel to your children	Children were coded as exposed if either the mother, the partner, or both, endorsed any of the items. Children were coded as unexposed if any negative response was available and no positive response was provided.	8 months, 1.75 years, 2.75 years, 4 years, 5 years, and 6 years

Sexual or physical abuse	Mother	An item asking whether or not the child had been exposed to either sexual or physical abuse from anyone	Children were coded as exposed if an affirmative response was provided to the item. Children were coded as unexposed if any negative response was available and no positive response was provided.	1.5 years, 2.5 years, 3.5 years, 4.75 years, 5.75 years, and 6.75 years
Maternal psychopathology	Mother	1) the Crown-Crisp Experiential Index (CCEI), assessing anxiety and depression; 2) the Edinburgh Postnatal Depression Scale (EPDS); and 3) a question asking about suicide attempts in the past 1.5 years	Consistent with prior studies and established cutoffs, children were coded as exposed if one or more of the following criteria was met: 1) CCEI depression score > 9; 2) CCEI anxiety score > 10; 3) EPDS score > 12; or the 4) a suicide attempt since the time of the last interview. Children were coded as unexposed if none of the criteria above were met and none of the scores were missing.	8 months, 1.75 years, 2.75 years, 5 years, and 6 years
One adult in the household	Mother	An item asking about the number of adults (>18 years of age) living in the household	Children were coded as exposed if fewer than two adults were residing in the household. Children were coded as unexposed if two or more adults resided in the household.	8 months, 1.75 years, 2.75 years, 4 years, and 7 years
Family instability	Mother	Child had 1) been taken into care; 2) been separated from their mother for two or more weeks; 3) been separated from their father for two or more weeks; or 4) acquired a new parent.	Children were coded as exposed if at least two of these events occurred at a single time point. Children were coded as unexposed if none of the events occurred and no questions were missing.	1.5 years, 2.5 years, 3.5 years, 4.75 years, 5.75 years, and 6.75 years
Financial	Mother	The family had	Children were coded as	8 months,

hardship	difficulty affording the following: 1) items for the child; 2) rent or mortgage; 3) heating; 4) clothing; 5) food. Each of the 5 items was coded on a Likert-type scale (1=not difficult; 2=slightly difficult; 3=fairly difficult; 4=very difficult)	exposed if their mothers reported at least fair difficulty for three or more items at each time point. Children were coded as unexposed if mothers provided responses to all five items and the above criterion was not met.	1.75 years, 2.75 years, 5 years, and 7 years	
Neighborhood disadvantage	Mother	There were problems in the neighborhood: 1) noise from other homes; 2) noise from the street; 3) garbage on the street; 4) dog dirt; 5) vandalism; 6) worry about burglary; 7) mugging; and 8) disturbance from youth. Response options to each item were: 2=serious problem, 1=minor problem, 0=not a problem or no opinion	A sum score was derived, ranging from 0-16. Children with scores ≥ 8 , generally corresponding to the 95th percentile, were classified as exposed. Children were coded as unexposed if the sum scores were below 8 and no questions were missing.	1.75 years, 2.75 years, 5 years, and 7 years

Table 2. Distribution of covariates and childhood adversity in the ARIES analytic sample (n=785), compared to the full ALSPAC sample (n=14,901).

	ALSPAC full sample (n=14,901)	ARIES analytic sample (n=785)	Comparison p-value
<i>Covariates</i>			
	<i>n (%)</i>	<i>n (%)</i>	
Race			
Non-White	611 (5.1)	24 (3.1)	0.01
White	11,488 (94.9)	761 (96.9)	
Sex			
Male	7542 (51.3)	405 (51.6)	0.91
Female	7152 (48.7)	380 (48.4)	
Age of mother at child's birth			
Ages 15-19	650 (4.6)	<5 (0.5)	<0.001
Ages 20-35	12363 (88.4)	698 (88.9)	
Age 36+	968 (6.9)	83 (10.6)	
Number of previous pregnancies			
0	5800 (44.7)	357 (45.5)	0.02
1	4550 (35.0)	295 (37.6)	
2	1860 (14.3)	104 (13.2)	
3+	772 (5.9)	29 (3.7)	
Sustained smoking during pregnancy			
No	9565 (78.8)	704 (89.7)	<0.001
Yes	2577 (21.2)	81 (10.3)	
Maternal education at baseline			
Below O-level	3735 (30.0)	101 (12.9)	<0.001
O-level	4303 (34.6)	268 (34.1)	
A-level	2795 (22.5)	244 (31.1)	
Degree or above	1603 (12.9)	172 (21.9)	
	<i>Mean (SD)</i>	<i>Mean (SD)</i>	
Birth weight, grams	3391.65 (560.04)	3488.56	<0.001
<i>Exposure prevalence</i>			
	<i>n (%)</i>	<i>n (%)</i>	
Physical or sexual abuse	1564 (23.4)	122 (18.2)	0.001
Financial hardship	3273 (41.0)	164 (24.1)	<0.001
One adult in the household	2336 (32.1)	108 (16.0)	<0.001
Family instability	1760 (23.8)	125 (18.0)	<0.001
Neighborhood disadvantage	1815 (24.2)	112 (16.0)	<0.001
Caregiver physical or emotional abuse	1944 (28.6)	147 (21.0)	<0.001
Maternal psychopathology	5350 (60.3)	297 (43.4)	<0.001

Note. P-values were obtained from χ^2 tests or independent t-tests comparing distributions of covariates and exposure variables in the analytic sample to distributions in the entire ALSPAC sample.

Table 3. Associations between time-dependent exposure to childhood adversity and DNA methylation in promoter regions of three gene sets known to regulate sensitive period

Childhood adversity	Sensitive period	Canonical R²	Wilks' lambda	Wilks'	Bootstrap
<i>Opening genes</i>					
Maternal psychopathology	Very	0.06	0.94	0.118	0.307
Financial hardship	Very	0.05	0.95	0.427	0.766
Sexual or physical abuse	Middle	0.08	0.92	0.004	0.019
One adult in the household	Middle	0.04	0.96	0.633	0.904
Family instability	Middle	0.05	0.95	0.257	0.583
Neighborhood disadvantage	Middle	0.05	0.95	0.171	0.388
Caregiver physical or emotional	Middle	0.04	0.96	0.549	0.872
<i>Closing genes</i>					
Family instability	Early	0.13	0.87	0.096	0.284
Neighborhood disadvantage	Early	0.09	0.91	0.817	0.977
Caregiver physical or emotional	Early	0.12	0.88	0.155	0.387
Sexual or physical abuse	Middle	0.12	0.88	0.122	0.339
Financial hardship	Middle	0.12	0.88	0.063	0.401
One adult in the household	Middle	0.10	0.90	0.433	0.753
Maternal psychopathology	Middle	0.13	0.87	0.046	0.127
<i>Expression genes</i>					
Sexual or physical abuse	Very	0.04	0.96	0.077	0.214
Neighborhood disadvantage	Very	0.04	0.96	0.069	0.178
Family instability	Early	0.04	0.96	0.029	0.090
Caregiver physical or emotional	Early	0.03	0.97	0.260	0.567
Financial hardship	Middle	0.05	0.95	0.008	0.028
One adult in the household	Middle	0.03	0.97	0.327	0.634
Maternal psychopathology	Middle	0.03	0.97	0.370	0.706

Bootstrap p-values accounted for the number of hypotheses tested per gene set and adversity (i.e., three sensitive period hypotheses). Associations with bootstrap p-values <0.05 were in bold.

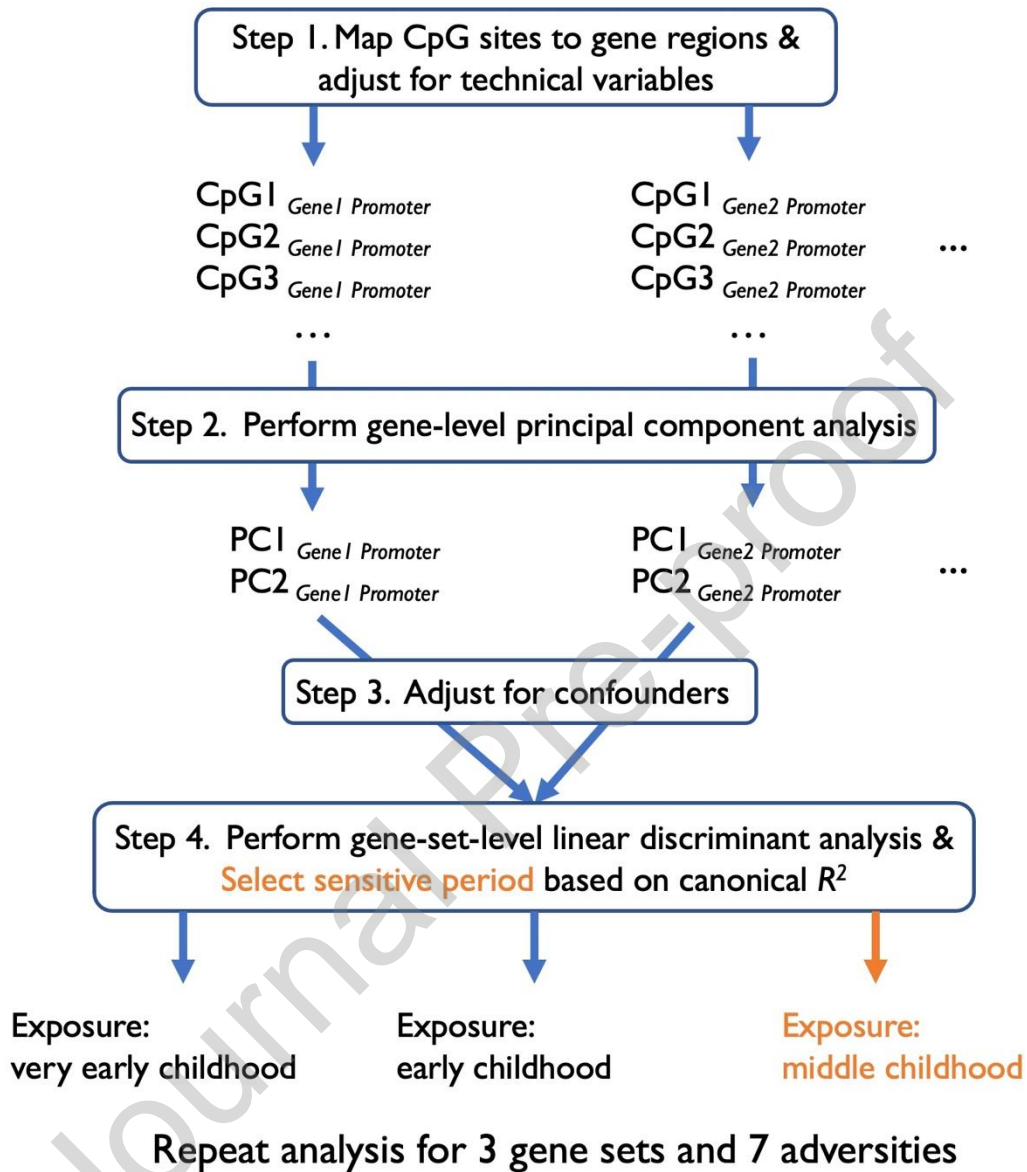


Figure 1. Flowchart of analyses. After adjusting for technical variables (Step 1), variation among CpG sites annotated to the promoter of a given gene was summarized at the *gene* level into two principal component (PC) scores (Step 2). PCs were then adjusted for confounders (Step 3). Finally, at the *gene set* level, associations between time-varying exposures to childhood adversity and PCs of all genes within a gene set were assessed using a linear discriminant analysis (Step 4). The sensitive period most strongly associated with the PCs was selected based on canonical R^2 , as highlighted in orange in this example.



Figure 2. Variances of CpGs located in promoters of three sets of sensitive period genes, as explained by the first two principal components (PCs). The number of CpGs annotated to the promoter of each gene is noted in parentheses. The red dashed line indicates 50% of the variance. For 53 of 58 genes, variance explained by the first two PCs exceeded 50

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Early life adversity may affect epigenetic regulation of developmental plasticity
- The effects of adversity may be strongest during sensitive periods of development
- Analyzing the timing and type of childhood adversity could reveal new findings
- Gene set analyses complement genome-wide studies, yielding functional insights

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