Title: Sensitive periods for the effect of childhood adversity on DNA methylation: Updated results from a prospective, longitudinal study

Authors: Alexandre A. Lussier*[1,2,3], Yiwen Zhu[1,4], Brooke J. Smith[1], Andrew J. Simpkin[5], Andrew D.A.C. Smith[6], Matthew J. Suderman[7], Esther Walton[8], Caroline Relton[7], Kerry J. Ressler[2,9], Erin C. Dunn**[1,2,3,10]

Affiliations:

[1] Psychiatric and Neurodevelopmental Genetics Unit, Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA, 02114, USA.

[2] Department of Psychiatry, Harvard Medical School, Boston, MA, 02115, USA.

[3] Stanley Center for Psychiatric Research, The Broad Institute of Harvard and MIT, Cambridge, MA, 02142, USA.

[4] Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA, 02114, USA

[5] School of Mathematics, Statistics and Applied Mathematics, National University of Ireland, Galway, Ireland.

[6] Mathematics and Statistics Research Group, University of the West of England, Bristol, UK.

[7] MRC Integrative Epidemiology Unit, Population Health Sciences, Bristol Medical School, University of Bristol, Bristol, UK.

[8] Department of Psychology, University of Bath, Bath, UK.

[9] McLean Hospital, Belmont, MA, 02478, USA.

[10] Center on the Developing Child at Harvard University, Cambridge, MA, 02138, USA.

Corresponding authors:

*Alexandre A. Lussier: alussier@mgh.harvard.edu

**Erin C. Dunn: edunn2@mgh.harvard.edu

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Childhood adversity is a potent, but preventable risk factor for numerous long-term physical and mental illnesses (1-3). Although the mechanisms underlying these associations remain unknown, DNA methylation (DNAm) and other epigenetic modifications have emerged as potential mechanisms for the biological embedding of early-life environments (4).

In a recent publication (5), we showed that seven types of childhood adversity had time-dependent effects on DNAm at age 7, using data from the Avon Longitudinal Study of Parents and Children (ALSPAC) in combination with a novel structured life course modeling approach (SLCMA) (6, 7). Specifically, we identified potential **sensitive periods** in development, largely occurring in the first three years of life, when exposure to childhood adversity might have greater differential effects on DNAm (5).

Since the publication of this research, there have been several notable updates, including revisions to individual-level DNAm data provided by ALSPAC and refinements to improve the utility of the SLCMA, which had not been previously applied to high-dimensional data (8). Elsewhere, we describe how these updates impacted the original results, with an emphasis on how changes to epigenetic data and analyses shape the replicability of epigenome-wide associations (9). In the current report, we summarize and interpret the biological relevance of these updated results.

Our updated analyses revealed 48 loci showing time-dependent associations between childhood adversity and DNAm levels at a 5% false-discovery rate (FDR; **Figure 1**; **Table 1**) (9). As previously shown (5), we continued to find evidence in support of sensitive periods among this new set of FDR-significant loci. However, exposure to adversity during *early childhood*, meaning between ages 3-5, was most frequently associated with DNAm differences (39 of 48 loci), rather than adversity between ages 0-3 as previously reported (5). Exposure to

adversity during other sensitive periods before age 7 was associated with DNAm differences at 8 loci, while a recency model (i.e., the cumulative number of exposed time points from age 0-7 weighted by exposure timing) was associated with DNAm differences at one locus; an accumulation model (i.e., the cumulative number of exposed time points) was not associated with any DNAm differences. These results recapitulate the main finding by Dunn et al. (2019) in showing that sensitive periods in development play an important role in the biological embedding of childhood adversity.

Most associations in the updated results came from family instability (43 loci), followed by sexual/physical abuse (2 loci), financial hardship (2 loci), and caregiver physical/emotional abuse (1 locus). Exposures to maternal psychopathology, neighborhood disadvantage, or one adult in the household were not associated with any DNAm differences (FDR<0.05). Similar to our earlier study, we observed more associations than a traditional epigenome-wide association study (EWAS) comparing ever- to never-exposed (9). We did not detect any of these associations in DNAm measured from cord blood at birth, suggesting that our observed differences in DNAm likely resulted from postnatal exposures.

Childhood adversity was generally associated with a decrease in DNAm levels (85.4% negative beta coefficients; χ^2=24.1; p=9.2x10^{-7}). On average, childhood adversity exposure was linked to a 3.2% absolute difference in DNAm levels (range 0.4-9.6%). For the locus associated with the recency of exposure to financial hardship, adversity was linked to a decrease in DNAm.

From a biological standpoint, the 48 FDR-significant loci were more likely to be located within predicted enhancer regions (χ^2=11.5; p=7.1x10^{-4}) and were slightly less often located in gene promoters (χ^2=2.2; p=0.14). FDR-significant loci were also more likely to be located away from, rather than inside or near, CpG islands compared to all loci tested (χ^2=22.9; p=3.6x10^{-4}).

These findings suggest that enhancers and regions of low CpG density may be more responsive to childhood adversity than promoters and CpG-dense regions.

To further explore the biological relevance of these FDR-significant loci, we examined the correlation between DNAm levels in blood and brain using publicly-available data (10). Two-thirds of loci (32/48) showed small but positive correlations between blood and brain region DNAm (prefrontal cortex $r_{avg\_positive}$=0.12; entorhinal cortex $r_{avg\_positive}$=0.13; superior temporal gyrus $r_{avg\_positive}$=0.15; cerebellum $r_{avg\_positive}$=0.1), providing some evidence that adversity-induced changes in blood could reflect parallel changes in the brain.

We next assessed the biological functions of genes near these FDR-significant loci (n=44 genes) using the DAVID gene ontology tool, identifying 14 clusters of processes involved in metabolism, cell death, and epigenetic regulation (11, 12). Only the top cluster – glucose transport – was significantly enriched in our top loci (p=0.028), highlighting potential mechanisms linking childhood adversity to metabolic syndrome, diabetes, and obesity (13). Although the lack of overrepresentation in other biological processes suggests childhood adversity may have broader biological effects, it is more likely a consequence of the small number of target genes analyzed.

To further understand the broader epigenetic context of these FDR-significant loci, we investigated their likely chromatin context (14). Although there was no overrepresentation of DNase I hypersensitive regions or coincident histone H3 marks in top loci (FDR<0.05), significant loci were slightly overrepresented within histone H3 marks in Primary T cells from cord blood (p=0.008). These findings suggest a potential link between childhood adversity and immune functioning, consistent with prior literature (15).

Finally, we did not find any evidence for increased evolutionary conservation of genes with FDR-significant loci, as measured by intolerance to loss-of-function variation probabilities from the Exome Aggregation Consortium (p=0.55) (16). However, 8 loci were in genes with high probability of intolerance to loss-of-function (pLI>0.9), suggesting that certain genes associated with childhood adversity may be under higher evolutionary constraint for human survival and reproduction (**Table 1**).

Overall, our updated results parallel the primary finding from our original manuscript (5) in showing that sensitive periods play an important role in the DNAm differences resulting from exposure to postnatal adversity. However, these current findings emphasize the salience of adversity exposure during *early childhood* (between the ages of 3 and 5), rather than exposure during *very early childhood* (before age 3), as identified in prior analyses. Future studies are needed to replicate these findings in other datasets, assess the persistence of these effects into adolescence and adulthood, and further examine their role in psychiatric disease risk.

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FIGURE LEGEND

Figure 1. Frequency of model selection for each type of adversity. The number of loci for which adversity significantly predicted DNA methylation levels at 5% false discovery rate (FDR) is shown here. Sensitive period hypotheses included very early (0-3 years), early (3-5 years), or middle childhood (6-7 years), while additive hypotheses included accumulation (not shown, as there were no significant associations) and recency.

Table 1. Time-dependent associations between childhood adversity and DNA methylation at age 7.

CpG	Adversity	Hypothesis (age in years)	DNAm (unexp.)[1]	DNAm (exp.)[2]	DNAm diff.[3]	Effect estimate[4]	SE	P-value	FDR	Nearest gene	pLI[5]
cg12023170	**Caregiver physical or emotional abuse (n= 698)**	**Middle childhood (6)**	**0.091**	**0.125**	**0.034**	**0.034**	**0.005**	**1.25E-09**	**5.48E-04**	**TCEA3**	**9.66E-04**
cg20369299	Physical or sexual abuse (n= 681)	Early childhood (4.75)	0.724	0.628	-0.096	-0.091	0.018	2.12E-07	4.67E-02	MIR4776-1	NA
cg13817046		**Early childhood (4.75)**	**0.483**	**0.411**	**-0.072**	**-0.075**	**0.013**	**5.07E-08**	**2.23E-02**	**PRR15**	**4.47E-01**
cg04908608	**Financial hardship (n= 752)**	**Recency**	**0.639**	**0.622**	**-0.017**	**-0.003**	**0.001**	**1.12E-07**	**2.47E-02**	**PRKCE**	**1.00E+00**
cg19219503		**Middle childhood (7)**	**0.879**	**0.843**	**-0.036**	**-0.039**	**0.007**	**1.08E-07**	**2.47E-02**	**ANKRD30A**	**1.26E-23**
cg01407460	Family instability (n= 681)	**Early childhood (4.75)**	**0.025**	**0.021**	**-0.004**	**-0.004**	**0.001**	**3.10E-08**	**6.82E-03**	**CORO7-PAM16**	**1.63E-12**
cg01587190		Early childhood (4.75)	0.067	0.078	0.010	0.011	0.002	1.11E-06	2.53E-02	ERCC2	3.14E-13
cg06770536		Middle childhood (5.75)	0.740	0.688	-0.052	-0.051	0.010	4.31E-06	4.51E-02	C1orf127	1.33E-05
cg22346081		Early childhood (4.75)	0.863	0.844	-0.019	-0.019	0.004	3.48E-06	3.92E-02	SORT1	1.00E+00
cg14948379		Early childhood (4.75)	0.855	0.822	-0.034	-0.035	0.006	4.10E-07	2.07E-02	MRPS9	3.36E-06
cg17134302		Early childhood (4.75)	0.851	0.834	-0.017	-0.018	0.004	2.91E-06	3.92E-02	FBXO36	3.23E-06
cg22060367		Early childhood (4.75)	0.887	0.867	-0.020	-0.020	0.004	1.84E-07	1.62E-02	TANK	9.44E-01
cg01100868		Early childhood (4.75)	0.903	0.885	-0.018	-0.018	0.003	2.07E-06	3.37E-02	SLIT3	9.92E-01
cg16338178		Early childhood (4.75)	0.830	0.800	-0.030	-0.030	0.006	3.22E-06	3.92E-02	LINC01845	NA
cg27639644		Early childhood (4.75)	0.858	0.827	-0.031	-0.031	0.006	5.64E-07	2.07E-02	RAB9BP1	NA
cg00943585		Early childhood (4.75)	0.834	0.785	-0.049	-0.048	0.010	3.48E-06	3.92E-02	LOC154449	NA
cg27061903		Early childhood (4.75)	0.052	0.070	0.018	0.018	0.003	2.23E-06	3.51E-02	COX7A2	3.76E-01
cg01023798		Early childhood (4.75)	0.865	0.839	-0.026	-0.026	0.005	1.54E-06	2.82E-02	SDK1	5.02E-03
cg02886132		Early childhood (4.75)	0.885	0.868	-0.017	-0.017	0.004	1.18E-06	2.53E-02	TYW1B	8.12E-09
cg10571837		Early childhood (4.75)	0.901	0.888	-0.014	-0.014	0.003	5.35E-07	2.07E-02	ZNF713	1.12E-03
cg17719337		Middle childhood (5.75)	0.036	0.045	0.009	0.009	0.002	2.48E-06	3.65E-02	CNPY1	8.75E-03
cg19569074		Middle childhood (6.75)	0.673	0.594	-0.079	-0.079	0.017	3.03E-06	3.92E-02	SDK1	5.02E-03
cg23184756		Early childhood (4.75)	0.847	0.822	-0.025	-0.025	0.005	4.89E-07	2.07E-02	ZNF735	NA
cg01654242		Early childhood (4.75)	0.802	0.752	-0.050	-0.050	0.009	1.95E-06	3.31E-02	FBXO43	1.50E-04

cg16231917		Early childhood (4.75)	0.211	0.268	0.057	0.057	0.012	4.09E-06	4.39E-02	PVT1	NA
cg27457457		**Early childhood (4.75)**	**0.695**	**0.623**	**-0.073**	**-0.075**	**0.013**	**2.77E-08**	**6.82E-03**	**RIPK2**	**5.54E-01**
cg13876553		Early childhood (4.75)	0.812	0.775	-0.037	-0.039	0.008	3.88E-06	4.27E-02	DOCK8	1.35E-04
cg21172807		Early childhood (4.75)	0.104	0.128	0.024	0.024	0.005	3.14E-06	3.92E-02	BRINP1	9.95E-01
cg05886789		Early childhood (4.75)	0.845	0.817	-0.028	-0.029	0.005	2.57E-07	1.89E-02	PLXDC2	6.13E-01
cg07206497		Early childhood (4.75)	0.876	0.854	-0.022	-0.023	0.004	1.16E-06	2.53E-02	USP6NL	1.02E-01
cg08971940		Early childhood (4.75)	0.784	0.741	-0.043	-0.045	0.009	1.73E-06	3.05E-02	FZD8	NA
cg01504589		Early childhood (4.75)	0.851	0.809	-0.043	-0.042	0.008	8.53E-07	2.53E-02	ZC3H12C	9.99E-01
cg22011436		Early childhood (4.75)	0.855	0.826	-0.029	-0.030	0.005	1.21E-06	2.53E-02	SYT13	2.20E-01
cg26997966		Early childhood (4.75)	0.853	0.829	-0.025	-0.025	0.005	3.14E-07	1.97E-02	RNF214	8.30E-01
cg00967695		Early childhood (4.75)	0.885	0.847	-0.038	-0.039	0.007	1.30E-06	2.56E-02	CHFR	6.37E-01
cg01267076		Early childhood (4.75)	0.865	0.841	-0.024	-0.024	0.004	1.34E-06	2.56E-02		
cg10940545		Middle childhood (6.75)	0.819	0.750	-0.069	-0.073	0.015	3.47E-06	3.92E-02	BRI3BP	1.25E-01
cg13706680		**Early childhood (4.75)**	**0.881**	**0.859**	**-0.021**	**-0.022**	**0.004**	**7.98E-08**	**8.79E-03**	**KITLG**	**5.09E-01**
cg14637285		Early childhood (4.75)	0.855	0.828	-0.027	-0.027	0.005	2.82E-06	3.92E-02		
cg04079399		**Very early childhood (2.5)**	**0.891**	**0.870**	**-0.021**	**-0.020**	**0.004**	**6.15E-08**	**8.79E-03**	**LINC00398**	**NA**
cg12188526		Early childhood (4.75)	0.889	0.873	-0.016	-0.016	0.003	3.24E-06	3.92E-02	SNORD56B	NA
cg01841772		Early childhood (4.75)	0.806	0.772	-0.035	-0.036	0.009	4.52E-06	4.63E-02	NOB1	8.61E-08
cg09305491		Early childhood (4.75)	0.913	0.899	-0.015	-0.015	0.003	1.02E-06	2.53E-02	PRKCB	1.00E+00
cg27567416		Early childhood (4.75)	0.891	0.876	-0.015	-0.015	0.003	1.12E-06	2.53E-02	ADCY9	9.74E-01
cg05353659		Early childhood (4.75)	0.897	0.881	-0.016	-0.017	0.003	1.02E-06	2.53E-02	G6PC	1.81E-03
cg11438065		Early childhood (4.75)	0.901	0.886	-0.015	-0.016	0.003	5.06E-07	2.07E-02	ZNF780B	3.90E-13
cg14401897		Early childhood (4.75)	0.830	0.784	-0.046	-0.044	0.009	2.34E-06	3.56E-02	SBNO2	2.44E-02
cg26848593		Middle childhood (5.75)	0.025	0.030	0.005	0.005	0.001	1.10E-06	2.53E-02	UBOX5	1.79E-03

FDR = False-discovery rate; SE = standard error. Bold = significant at a genome-wide Bonferroni-corrected threshold $p<1.13x10^{-7}$.

[1]Mean DNA methylation levels in individuals unexposed to adversity.

[2]Mean DNA methylation levels in individuals exposed to adversity.

[3]Differences in DNA methylation levels between exposed and unexposed individuals.

[4]Effect estimates from linear regression of exposure to adversity and DNA methylation, adjusting for cell types, child sex, race/ethnicity, birth weight, maternal education, maternal age at birth, number of previous pregnancies, and maternal smoking during pregnancy.

[5]Probability of intolerance to loss-of-function from the Exome Aggregation Consortium (ExAC) for the gene nearest to the significant locus(12). NA means that the nearest gene was not in the ExAC list.

Figure 1