Isolating the effects of HIV infection and HIV exposure on epigenetic profiles in infants using historical data from the Mothers and Infants Cohort Study

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Summary

Background Epigenetics offers insight into the mechanisms by which early life HIV infection and HIV exposure *in utero* affects offspring health. However, due to the widespread use of antiretroviral therapy (ART) during pregnancy/infancy, contemporary studies are unable to disentangle effects of HIV from ART exposure on epigenetic profiles.

Methods Using historical specimens collected before widespread use of ART (1985–1991), we compared DNA methylation (DNAm) profiles among infants with perinatally-acquired HIV (PHIV), HIV-exposed but uninfected (HEU), and HIV-unexposed uninfected (HUU). DNAm in peripheral blood mononuclear cells collected at 3 and 12 months of age (36 PHIV, 33 HEU, and 33 HUU) was profiled using the Illumina Infinium MethylationEPIC BeadChip. We tested for differentially methylated (DM) CpG sites between groups at 3 and 12 months, adjusting for sex, race/ethnicity, and cell type proportions. Biological pathway enrichment analyses were conducted.

Findings Comparing PHIV to HEU, there were 2 DM sites at 3 months and 11 at 12 months. Comparing PHIV to HUU, there was 1 DM CpG site at 3 months and 6 at 12 months. Immune-related pathways, including interferon-mediated signalling pathways were enriched. HIV exposure was not associated with any variation in DNA methylation, as no differences were detected between HEU vs. HUU at 3 or 12 months.

Interpretation HIV infection (in the absence of ART during pregnancy/infancy) was associated with DNA methylation changes at 3 and 12 months of life in infants. Differential methylation in PHIV is related to immune processes and HIV exposure in the absence of infection does not contribute to differential methylation.

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Keywords: Epigenetics; DNA methylation; HIV; Antiretroviral therapy

Introduction

With increasing access to antiretroviral therapy (ART), programs to prevent mother-to-child transmission of HIV have expanded, leading to a reduction in the number of infants living with perinatally-acquired HIV (PHIV).[1] At the same time, the number of infants exposed to HIV while *in utero* but born uninfected (HEU) is increasing worldwide. Both PHIV and HEU children face a lifetime of challenges to their health and well-being associated with HIV infection and/or HIV exposure, including deficits in growth and neurodevelopment.[2,3] There remain gaps in our understanding of how HIV infection and/or HIV exposure directly impact health at birth and throughout life.

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Research in context

Evidence before this study

We searched PubMed using the terms "HIV or PHIV or perinatally acquired HIV" AND "DNA methylation or DNAm or EWAS" AND "children or infants" prior to initiating the study. The search resulted in few studies that evaluated the association between HIV infection and/or exposure and DNA methylation in infants. Studies in infants or children also included other exposures in addition to HIV, such as ART exposure or other environmental exposures, limiting understanding of HIV-specific effects on the epigenome during pregnancy/early infancy. Our prior work using data from a cohort of children in South Africa (4–9 years of age) found widespread DNA methylation differences associated with HIV and ART relating to pathways involved in B-cell maturation and regulation of the adaptive immune system. Further research is required to understand if these DNA methylation changes occur in the absence of ART.

Added value of this study

In conclusion, our study demonstrates that HIV infection is associated with the infant epigenome in the absence of ART. Our findings are strengthened by consistency with prior evidence reported in adults with HIV pre-ART. We also found no effect of *in utero* HIV exposure in the absence of infection on the infant epigenome. Together, these findings enhance our knowledge of the biology of early-life HIV infection and exposure on DNA methylation.

Implications of all the available evidence

Our findings suggest HIV infection is associated with DNAm changes in early infancy in the context of no ART. Further investigation of gene pathways enriched for differential methylation in the ART-naïve epigenome can shed light on the biology of early-life HIV exposure and infection.

Epigenetics offers tremendous opportunities to understand the mechanisms by which early HIV infection and HIV exposure affect long-term health. A growing body of evidence implicating epigenetic mechanisms as a link between the *in utero* environment and long-term health outcomes in offspring, including growth, neurodevelopmental deficits, behaviour, metabolic disorders, and obesity,[4–7] supports a rationale to investigate HIV-induced DNA methylation (DNAm) changes in early life.

Our prior work provides compelling evidence that changes in the epigenome, specifically in DNAm profiles, are detectable in children aged 4–9 years with PHIV on suppressive ART.[8] Widespread DNAm differences associated with HIV and ART were detected in cytosine-phosphate-guanine (CpG) sites on genes involved in B-cell maturation and adaptive immune system regulation.[8] Because of the nearly universal use of ART today, studies in contemporary populations are typically unable to disentangle effects of HIV from effects of ART exposure on DNAm profiles, yet understanding these independent effects is critical for defining mechanisms underlying long-term health consequences and potential points of intervention.

This study leverages historical stored blood biospecimens and data from the Mothers and Infants Cohort Study (MICS), a prospective, epidemiologic cohort study of pregnant people with and without HIV and their offspring conducted in New York City (NYC) between 1985 and 1991 when ART use was not widespread.[9] Using these data and specimens, we examined the role of HIV infection and exposure on DNAm profiles in infants at 3 and 12 months of age. Our central hypothesis is that DNA methylation profiles in infants are associated with HIV infection and/or HIV exposure in the context of no ART.

Methods

Study population and sample selection

The MICS was a prospective, epidemiologic cohort study of the natural history of HIV infection in people during and after pregnancy and in their offspring.[10–18] In brief, pregnant people living with HIV were enrolled through four medical centres/clinics in NYC: Albert Einstein College of Medicine, Bronx–Lebanon, the Special Substance Abuse Pregnancy Clinic at SUNY, and the Haitian Prenatal Clinic at SUNY. Subjects also entered through outside referral. Pregnant people without HIV with similar demographic characteristics were enrolled concurrently from the same centres to serve as a comparison group. Between December 1985 and May 1991, 276 pregnant people with HIV and 213 without HIV were enrolled and followed prospectively; protocol details can be found elsewhere.[9] Both groups were examined and interviewed in an identical manner. Visits included a clinical evaluation by a paediatrician, laboratory testing, and blood processing and storage at −80 °C. Investigators were blinded to the infant's HIV status. Data and biospecimens for this analysis were obtained from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) Data and Specimen Hub (DASH).[9] All infants with blood specimens available in the biorepository close to 3 and 12 months of age were selected for analysis.

Ethics

This study was approved by the Rutgers Institutional Review Board (IRB) (reference number Pro2020002280) and deemed non-human subjects research.

Assessment of DNA methylation

Frozen cell pellet biospecimens from NICHD DASH were sent to Emory University, where DNA was

extracted using DNeasy Blood and Tissue Kits (Qiagen, Inc) following reproducible standardized protocols, quantified via Qubit Fluorometer (RRID: SCR_020311) using the Qubit dsDNA BR Assay kit (Invitrogen, Inc, Cat #Q32853), aliquoted, and stored at −80 °C until analysis. DNA samples were randomly distributed across 96-well plates by HIV group, timepoint (3 or 12 months), and biological sex to minimize potential batch effects.[19] DNAm was assessed at the Emory University Integrated Genomics Core (RRID: SCR_023529) using the Illumina Infinium MethylationEPIC BeadChip v1 (catalogue WG-317-1003), which interrogated the methylation status of >850,000 CpG sites (Illumina, Inc., San Diego, CA).

Pre-processing and analyses were performed using R (version 4.1.2).[20–22] Standard pre-processing procedures, including filtering, quality control, and dye-bias correction were performed using the *ewastools* package (version 1.7).[21,22] Control metrics and sex information were checked; no samples failed. Dye-bias correction was done for quality control. The threshold of SNP outliers was set at −3.5; one sample was dropped. A total of 189 samples from 102 unique infants (36 PHIV, 33 HEU, and 33 HUU infants) were included in the analysis. Overall, 843,393 CpG sites were retained after removing the control probes, non-CpG probes, failed probes with detection p-value ≥0.05, SNP-enriched probes, probes demonstrated to cross-hybridize non-specifically in the genome, and sex chromosome probes.[21,22] Cell type proportions (CD4+ T-cells, CD8+ T-cells, granulocytes, monocytes, B-cells, natural killer cells) were estimated using a validated method[23] based on the Reinius et al. reference dataset.[24] EPIC methylation annotation was performed using the *minfi* package and the *IlluminaHumanMethylationEPICanno.ilm10b4.hg19* package.[25,26]

Statistical analysis

Descriptive characteristics

Descriptive characteristics were analysed and summarized using appropriate statistical methods. Continuous variables were presented as means and standard deviations and categorical variables expressed as frequencies and percentages. Comparisons of continuous variables between groups were conducted using analysis of variance (ANOVA), with post hoc analyses performed using Tukey's Honest Significant Difference test for pairwise differences. For categorical variables, group differences were assessed using the Chi-square tests or Fisher's exact tests.

Epigenome-wide association study (EWAS): site and regional analyses

The epigenome-wide association of perinatal HIV infection or exposure with DNAm at each individual CpG site was evaluated using linear regression models adjusted for sex (male vs. female), race/ethnicity (Black vs. other), and cell type proportions, using the *CpGassoc* package (version 2.6) and *limma* package (version 3.48.3).[27,28] Linear model assumptions were tested using the *gvlma* package (version 1.0.0.3).[29] For comparison purposes, in supplemental analyses we also evaluated models adjusted only for sex and race/ethnicity. Additional sensitivity analyses were performed to adjust for age in months and clinic site. CpG sites were considered significantly differentially methylated (DM) by controlling the family-wise error rate at 5% (Bonferroni corrected p-value <5.928 e−8) and $|\Delta\beta|> 0.05$, where $\Delta\beta$ is the mean difference between the average DNAm of the groups.

Similarly, since regional EWAS approaches can be used to aggregate evidence of association across multiple CpG sites in a region, a regional analysis was conducted to determine significant DM regions (DMRs) associated with perinatal HIV infection or exposure using the *DMRcate* package (version 2.6.0).[30,31] *DMRcate* applies Gaussian kernel smoothing (bandwidth = 1000, scaling factor = 2) to t-statistics for individual CpG sites. A Stouffer transformation was applied to p-values. Regions were considered significantly DM if the Stouffer p-value was <0.05, $|\Delta\beta|>0.05$, and the region contained at least 2 CpG sites. For all analyses, we compared PHIV vs. HEU, PHIV vs. HUU, and HEU vs. HUU groups, at 3 months and 12 months, respectively.

Pathway enrichment analysis

Biological pathway enrichment analyses were conducted for genes associated with DM CpG sites and regions from the adjusted linear regression models using the *missMethyl* (version 1.26.1) package.[32] Pathways with Bonferroni corrected p-value <0.05 were considered enriched.

Analysis of genes selected a priori

CpG sites on a set of genes selected a *priori* based on a previous EWAS of children with HIV on ART,[8] were tested for associations with HIV infection or exposure using linear regression adjusted for sex, race/ethnicity, and cell type composition, as in the EWAS analyses. We also evaluated models adjusted only for sex and race/ethnicity. Genes of interest included: *EBF4, XDH, SPERT, NLRC5, FOXP1, FNDC3B, RPS6KA2, DLL1, HCG22*. Bonferroni-corrected p-values (0.05/# of CpG sites per gene on the MethylationEPIC array) were used to identify significant sites comparing PHIV vs. HEU and PHIV vs. HUU groups at 3 and 12 months. Hyper- or hypomethylation at gene-specific CpG sites were noted for the PHIV group (hypermethylation indicates higher average methylation for the PHIV group when compared to the HEU or HUU group).

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the manuscript.

Results

Characteristics

Table 1 depicts the demographic characteristics of the 102 mother–infant pairs included in this study, including 36 PHIV, 33 HEU, and 33 HUU infants. By design, there were some differences in the distribution of the clinical site where mothers were recruited.[11] There were no differences in other infant or maternal characteristics between the three groups. For the 36 PHIV, a total of 24 3-month and 33 12-month biospecimens were available for DNAm analyses. All 33 HEU and HUU infants had specimens available at 3- and 12 months. The PHIV group was slightly older at the time of the closest collected 12-month biospecimen than the HEU and HUU groups. As expected, differences in estimated cell type proportions were observed in those with and without HIV, including lower estimated CD4 T-cell proportions at 3 and 12 months (Table 1; Supplemental Table S1).

EWAS: site analyses

First, we conducted EWAS site analyses comparing PHIV vs. HEU, PHIV vs. HUU and HEU vs. HUU groups at 3 and 12 months. Given the differences in cell type proportions between groups, primary comparisons between PHIV vs. HEU and PHIV vs. HUU were adjusted for sex, race/ethnicity, and cell type proportions. In these adjusted analyses, comparing PHIV to HEU, there were 2 DM CpG sites at 3 months (Fig. 1a and b), corresponding to 2 unique genes - *IFIT3* and *ADAR* (Supplemental Table S2). At 12 months there were 11 DM CpG sites when comparing PHIV to HEU (Fig. 2a and b). These sites corresponded to 7 unique genes–*ADAR, ELAVL3, LAP3, PARP14, PARP9/DTX3L,* and *SMAD3*—(Supplemental Table S3). Distributions of DNAm at the identified sites are presented in Supplemental Figure S1A and B. Additional adjustment for age resulted in similar findings at both time points (Supplemental Tables S4 and S5). Additional adjustment for clinic site also resulted in similar findings (data not shown). Of note, analyses that only adjusted for sex and race/ethnicity and did not account for cell type proportions resulted in a larger number of DM CpG sites (93 at 3 months and 27,187 at 12 months) (Supplemental Table S6).

Comparing PHIV to HUU, there was 1 DM CpG site located at *XRN1* at 3 months (Figure 1C and D; Supplemental Table S7) and 6 DM CpG sites (4 unique genes—*ADAR, PARP9/DTX3L, FLT1*) at 12 months in adjusted analyses (Figure 2C and D; Supplemental Table S8). Distributions of DNAm at the identified sites are presented in Supplemental Figure S1C and D. Similarly, further adjustment for age resulted in similar findings at both time points (Supplemental Tables S9, S10). Additional adjustment for clinic site also resulted in similar findings (data not shown). Analyses that only adjusted for sex and race/ethnicity and did not account for cell type proportions resulted in a larger number of DM CpG sites (1138 at 3 months and 7902 at 12 months) (Supplemental Table S6).

Pathway enrichment analyses for genes associated with DM CpG sites did not result in any significant pathways at 3 months for either comparison of PHIV vs. HEU or PHIV vs. HUU (Table 2). At 12 months, pathway enrichment analyses identified 20 pathways related to response to virus and external stimuli, as well as regulation of interferon-mediated signalling pathways for PHIV vs. HEU. The comparison of PHIV vs. HUU resulted in three pathways related to chromatin and protein binding.

Comparison of HEU and HUU groups showed no significant difference in average methylation between groups at both timepoints (Figs. 1e, f and 2e, f).

EWAS: regional analyses

In regional analyses adjusted for sex, race/ethnicity, and cell type composition, there were no significant DMRs when comparing PHIV to HEU or HUU at 3 months (Supplemental Table S11). However, at 12 months comparing PHIV to HEU, there were 250 DMRs (212 unique genes). Nine of these DMRs had a mean difference in methylation greater than 0.10, overlapping the following genes: *IFI44L, PARP9, AC067945.2, STAT1, DDX60, LY6E, STAT1, SP100, XX-C283C717.1, DENND6B, DUSP5P1*. Comparing PHIV to HUU at 12 months, there were 7 DMRs (9 unique genes, including *PARP9, ADAR, PSMB9, BCL2L14, PSMB8, ZNF727, IRF7, TAP1, RP11-3N2.13*). Similar to site analyses, there were a larger number of DMRs in analyses that only adjusted for sex and race/ethnicity and did not account for cell type proportions.

We used pathway enrichment analysis to better understand the genes associated with DMRs. No pathways were significant at 3 months comparing PHIV vs. HEU or PHIV vs. HUU in primary analyses adjusted for sex, race/ethnicity, and cell type proportions. At 12 months, for PHIV vs. HEU, pathway enrichment analysis of DM CpG sites resulted in 79 pathways (Supplemental Table S12). The top 10 pathways by Bonferroni significance included those related to organismal, anatomical, and nervous system development as well as cell adhesion via plasma-membrane adhesion molecules. For PHIV vs. HUU, we found 1 enriched pathway—regulation of response to biotic stimulus.

Analysis of CpGs on genes associated with ART and HIV

Table 3 shows the number of significant DM CpG sites associated with a subset of genes we selected *a priori* (due to prior associations with HIV and ART in children with HIV) at 3 and 12 months for PHIV vs. HEU and PHIV vs. HUU. After adjustment for sex, race/ethnicity, and cell type proportions, no DM sites were observed at 3 months. However, at 12 months we identified DM

Characteristic	PHIV, N = 36	HEU, N = 33	HUU, N = 33	p-value[a]
Infants				
Sex, N (%)				0.820
Female	16 (44%)	17 (52%)	15 (45%)	
Male	20 (56%)	16 (48%)	18 (55%)	
Race/Ethnicity, N (%)				0.187
Hispanic or Latino	12 (33%)	10 (30%)	8 (24%)	
Non-Hispanic black	14 (39%)	20 (61%)	21 (64%)	
Non-Hispanic white	6 (17%)	2 (6.1%)	4 (12%)	
Unknown	4 (11%)	1 (3.0%)	0 (0%)	
Gestational age (weeks), N (%)				0.718
<37 = Preterm	7 (21%)	4 (13%)	4 (13%)	
37–38 = Early term	9 (26%)	8 (25%)	6 (19%)	
39–40 = Full term	16 (47%)	15 (47%)	16 (50%)	
≥40 = Late/post term	2 (5.9%)	5 (16%)	6 (19%)	
Unknown	2	1	1	
Birth weight (g), N (%)				0.817
<1500 = Very low	2 (5.9%)	2 (6.1%)	0 (0%)	
1500–2500 = Low	6 (18%)	6 (18%)	7 (21%)	
2500–4000 = Normal	25 (74%)	23 (70%)	23 (70%)	
≥4000 = High	1 (2.9%)	2 (6.1%)	3 (9.1%)	
Unknown	2	0	0	
Head Circumference (cm), Mean (SD)	36.8 (16.00)	43.1 (24.04)	37.9 (15.89)	0.360
Unknown	2	0	0	
Apgar score–total at 1 min, mean (SD)	7.5 (2.28)	7.2 (2.34)	7.8 (1.63)	0.545
Apgar score–total at 5 min, mean (SD)	8.5 (1.42)	8.7 (1.12)	8.7 (1.20)	0.834
Selected Biospecimens				
Number of 3 month Biospecimens	24	33	33	
Age (months) at month 3 Biospecimen, mean (SD)	3.1 (0.91)	3.4 (0.53)	3.6 (0.93)	0.080
Age (months) at month 3 Biospecimen, range	(0.9, 5.1)	(2.1, 4.2)	(2.0, 6.3)	
Proportion CD4 T-Cells at month 3 Biospecimen, mean (SD)	0.25 (0.10)	0.37 (0.06)	0.40 (0.06)	<0.001
Proportion CD8 T-Cells at month 3 Biospecimen, mean (SD)	0.17 (0.07)	0.14 (0.04)	0.16 (0.03)	0.046
Proportion natural killer cells at month 3 Biospecimen, mean (SD)	0.07 (0.05)	0.06 (0.06)	0.03 (0.04)	0.026
Proportion Monocytes at month 3 Biospecimen, mean (SD)	0.05 (0.04)	0.03 (0.03)	0.04 (0.03)	0.020
Proportion Granulocytes at month 3 Biospecimen, mean (SD)	0.17 (0.15)	0.09 (0.06)	0.08 (0.04)	<0.001
Proportion B-Lymphocytes at month 3 Biospecimen, mean (SD)	0.30 (0.08)	0.32 (0.06)	0.30 (0.06)	0.398
Number of 12 month Biospecimens	33	33	33	
Age (months) at month 12 Biospecimen, mean (SD)	14.6 (6.13)	12.0 (0.27)	12.0 (0.47)	0.004
Age (months) at month 12 Biospecimen, range	(9.0, 42.0)	(11.4, 12.7)	(10.3, 12.8)	
Proportion CD4 T-cells at month 12 Biospecimen, mean (SD)	0.20 (0.10)	0.34 (0.07)	0.33 (0.09)	<0.001
Proportion CD8 T-Cells at month 12 Biospecimen, mean (SD)	0.22 (0.09)	0.14 (0.05)	0.15 (0.06)	<0.001
Proportion natural killer cells at month 12 Biospecimen, mean (SD)	0.09 (0.08)	0.07 (0.06)	0.07 (0.06)	0.185
Proportion Monocytes at month 12 Biospecimen, mean (SD)	0.04 (0.04)	0.04 (0.05)	0.05 (0.06)	0.971
Proportion Granulocytes at month 12 Biospecimen, mean (SD)	0.15 (0.11)	0.09 (0.08)	0.11 (0.10)	0.040
Proportion B-Lymphocytes at month 12 Biospecimen, mean (SD)	0.29 (0.07)	0.31 (0.07)	0.30 (0.06)	0.449
Mothers				
Clinic site, N (%)				<0.001
Haitian prenatal clinic	1 (2.8%)	3 (9.1%)	16 (48%)	
AECOM	11 (31%)	9 (27%)	7 (21%)	
Bronx-Lebanon	0 (0%)	1 (3.0%)	0 (0%)	
Outside referrals	16 (44%)	16 (48%)	7 (21%)	
SSAPC	8 (22%)	4 (12%)	3 (9.1%)	
Race/Ethnicity, N (%)				0.365
Hispanic or Latino	12 (38%)	10 (31%)	8 (24%)	
Non-Hispanic black	14 (44%)	20 (63%)	21 (64%)	
Non-Hispanic white	6 (19%)	2 (6.3%)	4 (12%)	
Unknown	4	1	0	
BMI (kg/m^2), mean (SD)	26.9 (4.89)	24.8 (6.01)	30.2 (25.73)	0.400

(Table 1 continues on next page)

Characteristic	PHIV, N = 36	HEU, N = 33	HUU, N = 33	p-value[a]
(Continued from previous page)				
Delivery type, N (%)				0.209
Vaginal	29 (85%)	25 (76%)	24 (73%)	
Other	2 (5.9%)	7 (21%)	4 (12%)	
Cesarean	3 (8.8%)	1 (3.0%)	5 (15%)	
Unknown	2	0	0	

Abbreviations: PHIV, perinatally-acquired HIV; HEU, HIV exposed but uninfected; HUU, HIV unexposed and uninfected; SD, standard deviation; BMI, body mass index; AECOM, Albert Einstein College of Medicine; SSAPC, Special Substance Abuse Pregnancy Clinic at SUNY. [a]Pearson's Chi-squared test; Fisher's exact test; One-way ANOVA.

Table 1: **Characteristics of the 102 mother–infant pairs of the mothers and infants cohort study (MICS), by infant HIV status.**

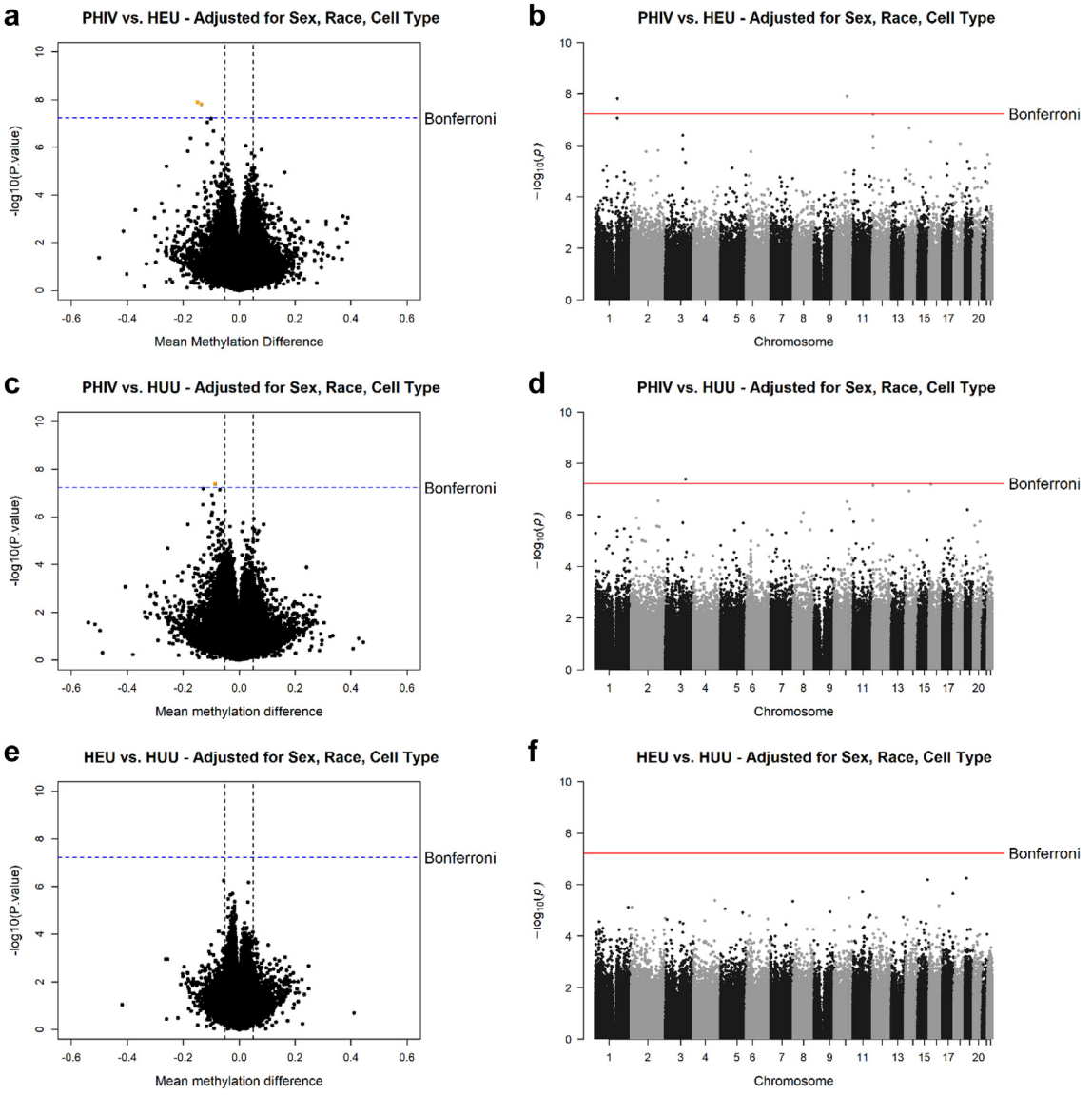

Fig. 1: Volcano and Manhattan plots for mean differential methylation at CpG sites for PHIV vs. HEU (a and b), PHIV vs. HUU (c and d), and HEU vs. HUU (e and f) infants, adjusted for sex, race/ethnicity and cell type proportions at 3 months. Abbreviations: PHIV, perinatally-acquired HIV; HEU, HIV exposed but uninfected; HUU, HIV unexposed and uninfected; Orange CpG sites–hypomethylated on average for infants with PHIV when compared to HEU (a) or HUU (c) infants at 3 months. Blue CpG sites–hypermethylated on average for infants with PHIV when compared to HEU (a) or HUU (c) infants at 3 months.

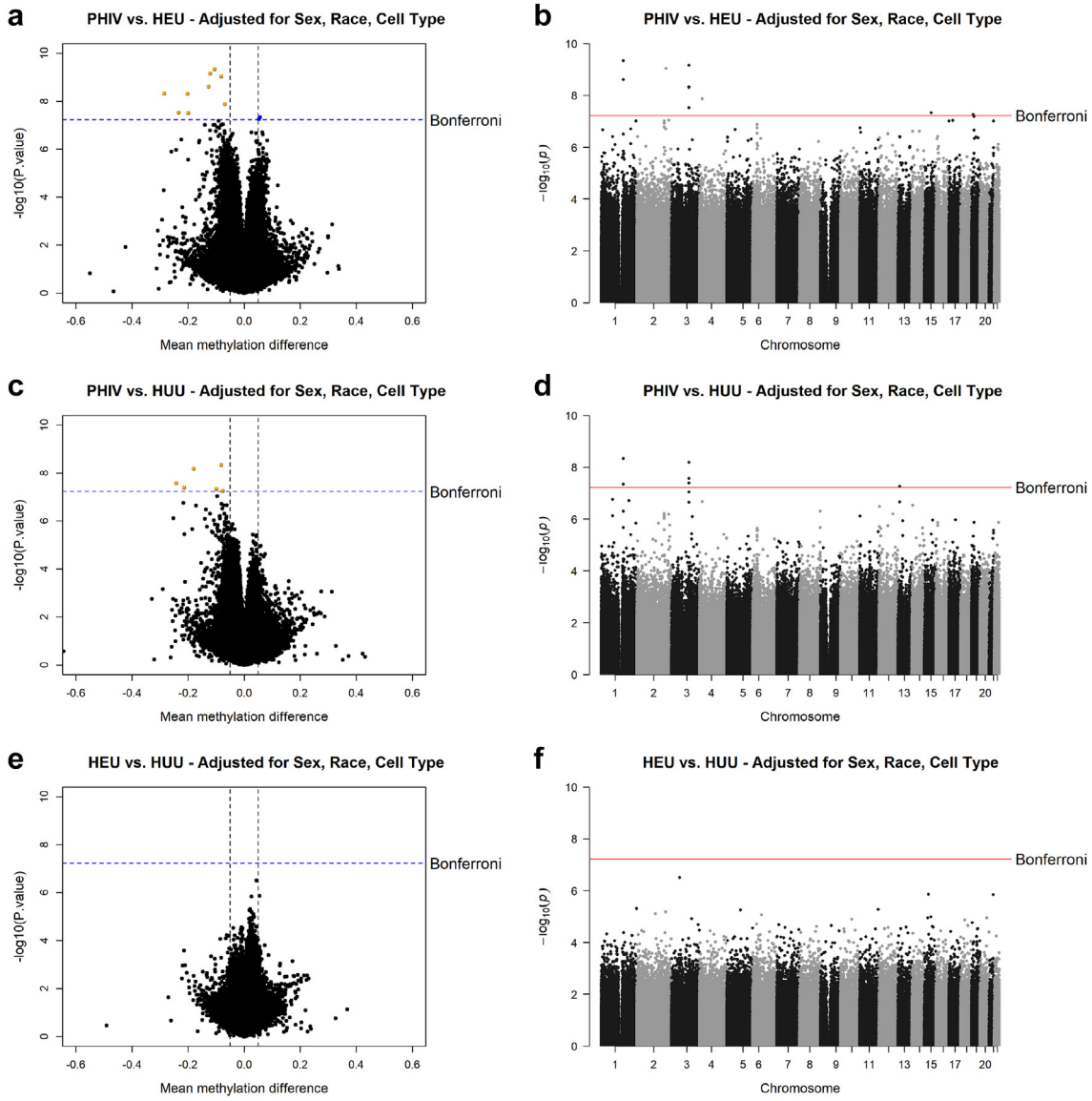

Fig. 2: Volcano and Manhattan plots for mean differential methylation at CpG Sites for PHIV vs. HEU (a and b), PHIV vs. HUU (c and d), and HEU vs. HUU (e and f) infants, adjusted for sex, race/ethnicity and cell type proportions at 12 months. Abbreviations: PHIV, perinatally-acquired HIV; HEU, HIV exposed but uninfected; HUU, HIV unexposed and uninfected; Orange CpG sites–hypomethylated on average for infants with PHIV when compared to HEU (a) or HUU (c) infants at 12 months. Blue CpG sites–hypermethylated on average for infants with PHIV when compared to HEU (a) or HUU (c) infants at 12 months.

CpG sites at CpG sites on *XDH*, *SPERT*, *NLRC5*, *FOXP1*, and *HCG22* in both comparisons (PHIV vs. HEU and PHIV vs. HUU). In addition, for the comparison of PHIV vs. HEU we identified 2 DM CpG sites on *RPS6KA2* at 12 months.

Discussion

In this study, we found that perinatal HIV infection directly affects the infant epigenome at 3 and 12 months of life at a small number of CpG sites in the absence of ART during pregnancy/infancy, after adjusting for cell type composition. These findings provide important insights into the pathways through which untreated HIV can affect host DNAm profiles in infants with PHIV. In addition, we found no effect of *in utero* HIV exposure in the absence of infection on the infant epigenome, contrary to our hypothesis. Absence of an observed effect indicates that HIV exposure alone in the absence of ART during pregnancy and infancy may not lead to significant DNAm changes in the epigenome in HEU infants. Together, these findings enhance our

Comparison	ID	Ontology	Term	N	DE	Significant genes in set	p-value	Adjusted p-value
PHIV vs. HEU at month 3	NA	NA	NA	NA	NA	NA	NA	NA
PHIV vs. HUU at month 3	NA	NA	NA	NA	NA	NA	NA	NA
PHIV vs. HEU at month 12	GO:0001959	BP	regulation of cytokine-mediated signalling pathway	169	3	ADAR, PARP14, PARP9	2.081×10^{-05}	4.163×10^{-04}
	GO:0002831	BP	regulation of response to biotic stimulus	501	4	ADAR, DTX3L, PARP14, PARP9	1.602×10^{-05}	3.204×10^{-04}
	GO:0003950	MF	NAD + ADP-ribosyltransferase activity	23	2	PARP14, PARP9	3.601×10^{-05}	7.201×10^{-04}
	GO:0009607	BP	response to biotic stimulus	1506	5	ADAR, DTX3L, SMAD3, PARP14, PARP9	4.079×10^{-05}	8.158×10^{-04}
	GO:0009615	BP	response to virus	398	4	ADAR, DTX3L, SMAD3, PARP9	4.802×10^{-06}	9.605×10^{-05}
	GO:0031347	BP	regulation of defence response	795	5	ADAR, DTX3L, SMAD3, PARP14, PARP9	2.112×10^{-06}	4.223×10^{-05}
	GO:0032101	BP	regulation of response to external stimulus	1097	5	ADAR, DTX3L, SMAD3, PARP14, PARP9	1.310×10^{-05}	2.619×10^{-04}
	GO:0035561	BP	regulation of chromatin binding	11	2	DTX3L, PARP9	7.625×10^{-06}	1.525×10^{-04}
	GO:0035563	BP	positive regulation of chromatin binding	9	2	DTX3L, PARP9	3.945×10^{-06}	7.890×10^{-05}
	GO:0043207	BP	response to external biotic stimulus	1472	5	ADAR, DTX3L, SMAD3, PARP14, PARP9	3.553×10^{-05}	7.106×10^{-04}
	GO:0051707	BP	response to other organism	1470	5	ADAR, DTX3L, SMAD3, PARP14, PARP9	3.540×10^{-05}	7.080×10^{-04}
	GO:0060330	BP	regulation of response to type II interferon	14	2	PARP14, PARP9	1.075×10^{-05}	2.149×10^{-04}
	GO:0060334	BP	regulation of type II interferon-mediated signalling pathway	14	2	PARP14, PARP9	1.075×10^{-05}	2.149×10^{-04}
	GO:0060759	BP	regulation of response to cytokine stimulus	181	3	ADAR, PARP14, PARP9	2.455×10^{-05}	4.910×10^{-04}
	GO:0080134	BP	regulation of response to stress	1350	5	ADAR, DTX3L, SMAD3, PARP14, PARP9	3.665×10^{-05}	7.330×10^{-04}
	GO:0097677	MF	STAT family protein binding	11	2	DTX3L, PARP9	6.323×10^{-06}	1.265×10^{-04}
	GO:0140888	BP	interferon-mediated signalling pathway	106	3	ADAR, PARP14, PARP9	3.299×10^{-06}	6.599×10^{-05}
	GO:1900180	BP	regulation of protein localization to nucleus	141	3	DTX3L, SMAD3, PARP9	2.237×10^{-05}	4.475×10^{-04}
	GO:1900182	BP	positive regulation of protein localization to nucleus	92	3	DTX3L, SMAD3, PARP9	5.497×10^{-06}	1.099×10^{-04}
	GO:1990404	MF	NAD + -protein ADP-ribosyltransferase activity	23	2	PARP14, PARP9	3.399×10^{-05}	6.797×10^{-04}
PHIV vs. HUU at month 12	GO:0035561	BP	regulation of chromatin binding	11	2	DTX3L, PARP9	2.048×10^{-06}	6.144×10^{-06}
	GO:0035563	BP	positive regulation of chromatin binding	9	2	DTX3L, PARP9	1.363×10^{-06}	4.089×10^{-06}
	GO:0097677	MF	STAT family protein binding	11	2	DTX3L, PARP9	1.833×10^{-06}	5.500×10^{-06}

Abbreviations: PHIV, perinatally-acquired HIV; HEU, HIV exposed but uninfected; HUU, HIV unexposed and uninfected; ID, Gene Ontology (GO) ID; Term, Gene Ontology of GO term ("BP", biological process; "CC", cellular component; "MF", molecular function); N—number of genes in GO term; DE—number of differentially methylated genes in GO term; p-value—overrepresentation of GO term p-value; adjusted p-value—Bonferroni-corrected p-value (0.05/# of GO terms tested).

Table 2: **Gene ontology (GO) pathway enrichment for genes associated with differentially methylated (DM) CpG sites identified from EWAS for PHIV vs. HEU and PHIV vs. HUU, adjusted for sex, race/ethnicity, and cell type proportion at 3 and 12 months.**

knowledge of the biology of early-life HIV infection and exposure on DNA methylation.

Since DNAm signals from bulk tissues are known to be influenced by the underlying cell type composition and cell type proportions are known to be different in those with and without HIV, an inherent consequence of the infection, our primary models adjusted for estimated cell type proportions.[23,33] As expected, the large differences in DNAm between groups we observed in unadjusted analyses did not persist after adjustment for estimated cell type proportions, demonstrating that DNAm differences are largely due to HIV-related changes in cell type composition. Of particular interest are the DM sites and regions that remained in adjusted analyses, as they represent patterns of differential methylation directly associated with HIV infection, independent of changes in cell type composition.

Differential methylation associated with untreated HIV infection has been reported in a study of adults with HIV (mean age 38 years).[34] To date, no studies have examined the influence of untreated HIV infection during pregnancy and infancy on genome-wide DNA methylation profiles in infants. We identified DM sites and regions associated with HIV in the context of PHIV very early in life. Enrichment results show that later in infancy (i.e. 12 months of age), identified DM sites and regions were enriched in pathways related to viral response and regulation of interferon mediated pathways as well as biological processes that aligned with

Gene names	# CpG sites associated with gene	Bonferroni p-value cut off	PHIV vs. HEU—adjusted for sex and race/ethnicity				PHIV vs. HUU—adjusted for sex and race/ethnicity				PHIV vs. HEU—adjusted for sex, race/ethnicity, and cell type proportions				PHIV vs. HUU—adjusted for sex, race/ethnicity, and cell type proportions			
			#Bonferroni CpG sites at month 3		#Bonferroni CpG sites at month 12		#Bonferroni CpG sites at month 3		#Bonferroni CpG sites at month 12		#Bonferroni CpG sites at month 3		#Bonferroni CpG sites at month 12		#Bonferroni CpG sites at month 3		#Bonferroni CpG sites at month 12	
			Higher in PHIV	Lower in PHIV	Higher in PHIV	Lower in PHIV	Higher in PHIV	Lower in PHIV	Higher in PHIV	Lower in PHIV	Higher in PHIV	Lower in PHIV	Higher in PHIV	Lower in PHIV	Higher in PHIV	Lower in PHIV	Higher in PHIV	Lower in PHIV
EBF4	56	0.000892857	0	0	5	11	0	2	4	7	0	0	0	0	0	0	0	0
XDH	21	0.002380952	0	1	0	9	0	3	0	8	0	0	1	0	0	0	1	0
SPERT	17	0.002941176	0	1	0	5	0	3	0	3	0	0	2	0	0	0	2	0
NLRC5	62	0.000806451	3	5	5	12	5	10	4	12	0	0	4	0	0	0	2	0
FOXP1	299	0.000167224	19	1	43	18	30	9	27	14	0	0	2	0	0	0	1	0
FNDC3B	152	0.000328947	0	5	1	22	1	29	1	9	0	0	0	0	0	0	0	0
RPS6KA2	362	0.000138121	1	2	1	66	3	18	1	48	0	0	2	0	0	0	0	0
DLL1	38	0.001315789	0	1	2	3	0	2	1	2	0	0	0	0	0	0	0	0
HCG22	30	0.001666667	0	2	0	12	0	5	0	9	0	0	1	0	0	0	1	0

Abbreviations: PHIV, perinatally-acquired HIV; HEU, HIV exposed but uninfected; HUU, HIV unexposed and uninfected.

Table 3: **Differentially Methylated CpG sites on Targeted Genes for PHIV vs. HEU Infants and PHIV vs. HUU Infants at 3 and 12 months.**

organism development. Meanwhile, prior findings in youth with PHIV on ART found methylation changes affected genes involved in regulation of the adaptive immune system and B cell maturation.[8] These differences could be due to ART exposure, longer HIV infection, or other unknown reasons. Of interest, the gene ontology enrichment analysis of the DM sites reported in the study of ART-naïve adults was primarily enriched in biological processes related to the regulation of the immune system responses, even after adjustment for cell type composition.[34] The additional enriched processes in organism development identified in our study may be due to early life infection with HIV in our study population.

Several of the DM sites were mapped to interferon-stimulated genes (ISGs) in comparisons of PHIV to HEU and HUU groups at 3 months (*IFIT3*, *XRN1*), 12 months (*LAP3*, *PARP14*, *PARP9/DTX3L*), and both 3 and 12 months (ADAR). All sites were hypomethylated. Many of these ISGs play a role in defence against viruses, by triggering early host responses,[35–39] and epigenetic modification of ISGs have been found to be associated with pre-ART CD4/CD8 ratio, viral loads, and mortality.[34,40,41] This finding is consistent with other epigenome-wide association studies of adults with HIV.[34,41,42] In particular, *PARP9/DTX3L* were identified as top mapped genes in a study of 184 participants with HIV pre-ART.[34] CpG sites mapped to these genes were also hypomethylated; an examination of gene expression in a subgroup analysis of 23 individuals before starting ART revealed higher mRNA levels of *PARP9*, suggesting it is upregulated during untreated HIV infection.[34] Our finding suggests these pathways are similarly affected in the context of perinatal HIV infection.

We examined DM at CpG sites on a targeted set of genes identified from an epigenome-wide association analysis in a prior study of children with HIV on ART.[8] DM was observed on at least one CpG site on several of these targeted genes, supporting that untreated HIV affects methylation at these sites. Of particular interest were *RPS6KA2*, *FOXP1*, *SPERT*, and *NLRC5*, which all had two or more implicated CpG sites.

NLRC5, a transcriptional regulator of the MHC class I pathway[43] has been implicated in several epigenome-wide association studies in adults with HIV pre- and post-ART.[8,34,44] Our finding that untreated HIV is associated with methylation on NLRC5, combined with prior evidence of DM on sites mapped to NLRC5 in children with PHIV on ART, indicates that ART may not resolve epigenetic alterations on this gene. Additional analysis of pre- and post-ART samples are needed to better understand these DNA methylation dynamics. *FOXP1* is a transcription repressor gene that is linked to the development of multiple tissues, such as cranial nerves, lungs, heart, and B cells.[45,46] Gene silencing of *SPERT*, or spermatid-

associated protein, has been linked to tumour growth inhibition, but its role in HIV remains unclear.[47] *RPS6KA2*, also known as *RSK3*, plays a role in neuronal development and synaptic plasticity, and its dysregulation can disrupt neurological function. This aligns with pathways enriched at 12 months, which were related to development processes and cell communication, and may be a signal that HIV disrupts processes underlying typical neurodevelopment.

In this study, we did not observe dysregulation to the epigenome due to HIV exposure alone in our comparison of HEU to HUU infants at 3 or 12 months. The widespread use of ART in pregnancy has significantly reduced vertical HIV transmission, resulting in a growing population of HEU infants. Despite HEU children exhibiting poorer developmental outcomes compared to HUU children in the context of no ART, our study suggests that epigenetic mechanisms may not be a contributing factor.[48] However, further investigations are needed to comprehensively understand the impact of ART exposure on DNA methylation given that epigenetic variability associated with exposure to antiretrovirals *in utero* has been previously observed.[49]

Our study had some limitations that should be acknowledged. First, identifying pathways enriched for differential methylation does not confirm differential expression or whether specific genes are upregulated or down regulated. Further studies, including gene expression analyses, are needed to better understand the downstream impact of methylation changes on growth, neurological, or other development outcomes in early life. Second, our study extracted DNA from peripheral blood mononuclear cells, which consists of different types of cells that can affect variability in DNA methylation.[50] Since cell type proportions are known to be different in those with and without HIV, an inherent consequence of the infection, we adjusted our primary models to account for these differences to identify patterns of differential methylation associated with HIV infection independent of changes in cell type proportions. However, it would be of interest to investigate these associations in sorted cell types.[23] Third, samples from the MICS were stored for >30 years, which may have affected results; however DNA quality was adequate for all samples and it has been shown that blood processing/sample storage has negligible effects on methylation.[51] Fourth, the design of the study using historical data did not allow us to evaluate what happens to methylation patterns after ART is initiated. Studies of methylation profiles in adults with HIV initiating ART have found mixed results in restoration of DNA methylome, likely due to differences in ART duration and populations.[34,52] This will be important to understand in the context of PHIV infection. In addition, these analyses do not account for the possibility that infants with PHIV may have received antiretrovirals, e.g. zidovudine, in the first year of life, which may influence DNAm. However, much of this study was conducted before zidovudine was available.[53] Finally, we adjusted for sex, race/ethnicity, and cell type proportions, but were unable to control for other potential unmeasured confounders (e.g. exposure to tobacco or alcohol *in utero*) that have been shown to be associated with DNA methylation changes. The comparison population in the MICS was recruited from the same clinical sites.

In conclusion, to our knowledge, our study demonstrates that HIV infection is associated with the infant epigenome in the absence of ART. Our findings are strengthened by consistency with prior evidence reported in adults with HIV pre-ART. We identified biological pathways for further validation and exploration. Future studies will need to elucidate whether these changes are partially or fully restored with ART, and how the dysregulated epigenome may impact health outcomes, such as growth and neurodevelopment, for those living with PHIV.

Contributors

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All authors read and approved the final version of the manuscript.

Data sharing statement

Data from the Mothers and Infants Cohort Study (MICS) are available on NICHD DASH (https://dash.nichd.nih.gov/). Source code are available at https://github.com/jdougl3/EWAS_PHIV_EBioMedicine.

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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2025.105696.

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