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Original Article

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Newborn blood DNA methylation and childhood asthma: findings from the ECHO program

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Abstract

Background: DNA methylation (DNAm) at birth has been linked to childhood asthma in epigenome-wide association studies (EWASs). However, existing EWASs have limited representation of non-European and extremely preterm participants and have not explored sex-specific DNAm differences. This study examined the association between DNAm in newborn blood and subsequent childhood asthma risk in a diverse population.

Methods: Data from the Environmental influences on Child Health Outcomes (ECHO) Program were used for EWAS meta-analyses in United States (US) cohorts of children born before and after 28 weeks of gestation. DNAm was measured in newborn blood using Illumina arrays. Childhood asthma was defined as provider-diagnosed asthma with persistent symptoms beyond age 5. Linear regression was used to identify differentially methylated positions (DMPs), and "comb-p" was used to identify differentially methylated regions (DMRs). Sex-stratified analyses were performed.

Results: The meta-analysis included 942 children (369 asthma cases) born after 28 weeks of gestation. We identified a novel DMP (cg24749470 in *CADM1*, $P = 9.31 \times 10^{-8}$) and 18 DMRs (Šidák *P*-value <.001) associated with asthma, with four DMRs in the human leukocyte antigen region. At these four DMRs, the association between DNAm and asthma differed by sex. In the extremely preterm cohort ($n = 271$, 106 asthma cases), we identified 20 DMRs, with two novel asthma-associated DMPs (cg03237868 in *SPATA18*, $P = 2.71 \times 10^{-8}$; cg20681219 in *IRF2*, $P = 5.18 \times 10^{-8}$) identified in males.

Conclusion: In US children born before and after 28 weeks of gestation, we discovered novel genomic loci linking newborn blood DNAm to childhood asthma, suggesting DNAm involvement in early asthma development.

Keywords: childhood asthma; DNA methylation; newborn blood; epigenome-wide association study.

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Key Messages

- We sought to identify neonate DNA methylation changes associated with childhood asthma risk in a large US based cohort study.
- We discovered methylation changes, present at birth, associated with prospective childhood asthma diagnoses and some of these changes were sex specific.
- Our findings suggest methylation, at specific positions in the genome, could serve as a biomarker of later asthma diagnoses and also provide insights into biology, at the earliest stages of life, i.e. relevant to asthma risk.

Introduction

Asthma is the most common chronic disease among children in the United States (US), affecting over six million children [1]. While genetic variants, environmental exposures, and gene–environment interactions are known contributors to childhood asthma development, the underlying mechanisms, including at the earliest stages of life, remain unclear. Emerging evidence indicates that DNA methylation (DNAm) may serve as a molecular link between prenatal exposures and asthma development [2].

Childhood asthma is thought to arise from immune dysregulation [3]. DNAm present in blood at birth may reflect early-life immunological disturbances that may contribute to asthma development later in childhood. Several European cohort studies, including children of reported White race, have reported newborn DNAm-associations with later, focusing on candidate genomic regions [3–6] or interrogating the entire genome [7–9].

We aimed to examine associations between DNAm in newborn blood and subsequent childhood asthma risk in a large, more diverse, US cohort study to identify asthma-associated methylation differences common across race groups. Leveraging data from the Environmental influences on Child Health Outcomes (ECHO), we performed an epigenome-wide association study (EWAS) meta-analysis to identify differentially methylated positions (DMPs) and regions (DMRs) associated with asthma, including among a subset of participants born extremely preterm (before 28 weeks), given their unique risk and the distinct etiology compared with infants born after 28 weeks [10].

Methods

The ECHO study

ECHO is a research study that leverages multiple existing cohorts in the US to investigate the effects of various early environmental exposures on child health and development. ECHO cohorts share a standardized data collection protocol, and data collected previously using cohort-specific protocols were harmonized. ECHO study design and data collection method details are described elsewhere [11].

Inclusion criteria

ECHO cohorts that met the following criteria were eligible for these analyses, had: (1) asthma outcome data and (2) DNAm measured in newborn blood (cord blood, cord blood mononuclear cells [CBMCs], or blood spot at/near birth). Five cohorts met these criteria: Healthy Start, Project Viva, Mothers and Newborns, Urban Environment and Childhood Asthma (URECA), and Extremely Low Gestational Age Newborns (ELGAN). Table 1 describes the recruitment methods and population characteristics of these cohorts. Notably, asthma cases were overrepresented in the subset with DNAm data in some cohorts.

Childhood asthma outcome

Asthma cases were defined based on parent/caregiver report or adolescent self-report of provider-diagnosed asthma ever (no age restriction). Additionally, cases had to have at least one of the following at age 5 or later: repeated asthma diagnosis, asthma symptoms, use of asthma medications, doctor/primary care provider visit due to asthma, emergency room/urgent care visit due to asthma, or hospitalization for asthma. Non-cases were children who were at least 5 years old at the last study visit and never had a diagnosis of asthma.

DNAm measures

DNAm was measured using the Illumina HumanMethylation450 BeadChip (450K) or the HumanMethylationEPIC BeadChip (EPIC) arrays (Table 1). Sample-level and probe-level quality control (QC) filters were applied using the *minfi* R/Bioconductor package [12] (see details in Supplementary Material). A total of 1213 samples and 405 478 cytosine–phosphate–guanine (CpG) sites for the 450K and 783 517 CpG sites on the EPIC passed QC and were included in downstream analyses.

Covariates

Covariates for all analyses included child sex, maternal age at delivery, maternal education, or marital status as indicators for prenatal socioeconomic status, and maternal pre-pregnancy overweight or obesity status. For cohorts with non-missing data, we also included prenatal maternal smoking, delivery type, maternal history of asthma, and prenatal antibiotics use covariates (Supplementary Table S1). Cell composition was estimated using either a reference panel [13, 14] method (Supplementary Fig. S1) or surrogate variable analysis (SVA) [15] (see details in Supplementary Material). SVA was also used to account for batch effects.

Cohort-specific EWAS

We performed cohort-specific EWAS using linear regression to assess the association between the newborn DNAm *M*-value at individual CpGs as the dependent variable and childhood asthma status as an independent variable, adjusting for cohort-specific covariates, cell type, and batch effects (see details in Supplementary Material). Complementary linear regression models using methylation beta-values as the dependent variable were also performed. Our primary approach examined all samples. However, due to variations in asthma incidence, subtypes, and risk factors between males and females and race groups [16], we also performed secondary sex-stratified and race-stratified analyses to assess whether methylation changes differ by these factors.

Table 1. Description of ECHO cohorts that met the inclusion criteria

ECHO cohort	Recruitment methods and population characteristics	Enrollment years	DNAm: tissue; array	Sample size	Average age (years) at last study visit
Healthy Start	Colorado community sample of mother–child dyads recruited in early- to mid-pregnancy from obstetrics clinics at a university hospital and by word of mouth, as well as medical university employees. Children of mothers with steroid-dependent asthma were not eligible	2009–2014	Cord blood; 450K	26 asthma cases 213 non-cases	Cases: 8.3 Non-cases: 7.9
Project Viva	Pregnant women receiving prenatal care at one of eight urban and suburban clinics in and around Boston, Massachusetts, and their offspring	1999–2002	Cord blood; 450K	96 asthma cases 141 non-cases	Cases: 18.3 Non-cases: 18.1
Mothers and Newborns	Dominican or African American low-income mothers and their offspring who resided in northern Manhattan or the South Bronx and who were recruited at OB/GYN clinics	1998–2006	Cord blood; 450K/EPIC	167 asthma cases 157 non-cases	Cases: 16.2 Non-cases: 15.1
Urban Environment and Childhood Asthma (URECA)	Children whose biological mothers were recruited in pregnancy from four hospitals in St. Louis, Boston, Baltimore, and New York; who have a parental history of asthma, allergic rhinitis, or eczema; and who reside in census tracts with at least 20% of residents with income below the poverty level	2004	CBMC; EPIC	80 asthma cases 62 non-cases	Cases: 15.2 Non-cases: 15.0
Extremely Low Gestational Age Newborn (ELGAN)	Preterm infants (<28 weeks gestation) recruited from 1 of 15 United States hospitals in North Carolina, Michigan, Illinois, Connecticut, and Massachusetts	2002–2004	Blood spot collected 1–3 days after birth; EPIC	106 asthma cases 165 non-cases	Cases: 15.5 Non-cases: 15.3

CBMC = cord blood mononuclear cell; DNAm = DNA methylation; ECHO = Environmental influences on Child Health Outcomes; EPIC = Illumina HumanMethylationEPIC BeadChip.

Meta-analysis to identify DMPs

We performed a fixed effects meta-analysis using the METAL software [17] for four cohort-specific EWAS results of participants born after 28 weeks of gestation. We did not include the EWAS results of the ELGAN cohort in the meta-analysis because all the participants in this cohort were born before 28 weeks of gestation (extremely preterm birth), given the unique clinical presentation of asthma in these participants and the potential distinct mechanisms underlying their asthma development [10, 18]; thus, they were analysed and results reported separately. A CpG site was considered a DMP if it had a *P*-value less than 1.23×10^{-7}or 6.38×10^{-8} among non-extreme and extreme preterm births, respectively.

Differentially methylated regions

To identify asthma-associated DMRs, we applied the "comb-p" method [19] to the meta-analysis results and, separately, to the cohort-specific results for ELGAN. The parameters used were: (1) a window size of 1 kb, (2) a minimum *P*-value of .01 to start a region, and (3) at least three CpGs in the region. The "comb-p" method applied a one-step Šidák multiple testing correction on the regional *P*-value. We considered regions with a Šidák *P*-value <.001 to be DMRs.

Results

The meta-analysis included 942 participants born after 28 weeks of gestation with 369 (39.2%) asthma cases. Each cohort had varying proportions of asthma cases, ranging from 10.9% to 56.3%, due to differences in study design (Table 1). For the extreme preterm birth cohort (ELGAN), 271 participants met our inclusion criteria, with 106 (39.1%) classified as asthma cases. Supplementary Table S2 shows the breakdown of asthma cases and non-cases by sex.

As shown in Table 2, among participants born after 28 weeks (four cohorts), 55% reported non-White race and 32.7% reported Hispanic ethnicity. Extreme preterm participants, 36.1% reported non-White race and 8.8% reported Hispanic ethnicity. Maternal age at delivery, education attainment, and marital status also differed by cohort.

DMPs in participants born after 28 weeks of gestation

In the meta-analysis ($\lambda = 0.97$, Supplementary Fig. S2) including participants born after 28 weeks of gestation, we identified one DMP at cg24749470 ($P = 9.31 \times 10^{-8}$); we provide a list of the top 100 CpGs in Supplementary Table S3. At this DMP, we observed a 1.06% (95% CI: 0.67%, 1.45%) higher DNAm level in the cord blood samples of newborns who later developed asthma compared with newborns who did not (Supplementary Fig. S3). The effect size at this DMP was consistent across all four cohorts and sex and gestational age groups (Table 3, Supplementary Fig. S4) except in those born extremely preterm (difference in DNAm: −0.31%, 95% CI: −1.07%, 0.46%). We did not find sex-specific asthma-methylation changes among participants born after 28 weeks of

Table 2. Descriptive characteristics of our analytic sample, by ECHO cohort

Variables	Meta-analysis	ECHO cohort included in meta-analysis (≥28 weeks of gestation)				Analysis for extremely preterm newborns (<28 weeks of gestation)
		Healthy Start	Project Viva	Mothers and Newborns	URECA	ELGAN
Number of participants	942	239	237	324	142	271
Age at last study visit (years) Median [25th percentile, 75th percentile]	15.3 [8.9, 19.1]	8.3 [6.8, 8.8]	19.2 [17.7, 19.5]	16.5 [12.0, 20.2]	15.2 [15.0, 15.9]	16.3 [14.9, 17.7]
Asthma cases, *N* (%)	369 (39.2%)	26 (10.9%)	96 (40.5%)	167 (51.5%)	80 (56.3%)	106 (39.1%)
Male, *N* (%)	444 (47.1%)	125 (52.3%)	103 (43.5%)	146 (45.1%)	70 (49.3%)	148 (54.4%)
Non-White race, *N* (%)	526 (55.8%)	48 (21.3%)	38 (16.5%)	319 (98.5%)	121 (96.8%)	96 (36.1%)
Hispanic ethnicity, *N* (%)	308 (32.7%)	53 (22.2%)	27 (11.4%)	200 (61.7%)	28 (19.7%)	24 (8.8%)
Gestational age at birth (weeks), Median [min, max]	39 [30, 43]	39 [34, 42]	40 [30, 42]	39 [31, 43]	39 [34, 42]	26 [23, 28]
Preterm birth (gestational age between 30 and 37 weeks), *N* (%)	61 (6.5%)	6 (2.5%)	11 (4.6%)	36 (11.1%)	8 (5.6%)	N/A
Cesarean section, *N* (%)	N/A	49 (20.5%)	35 (15.5%)	N/A	34 (23.9%)	180 (66.2%)
Maternal age at delivery (year), Median [25th percentile, 75th percentile]	28 [23, 33]	30 [26, 33]	33 [30, 37]	24 [21, 28]	24 [20, 29]	29 [24, 34]
Maternal overweight/obese before pregnancy, *N* (%)	452 (49.2%)	105 (44.1%)	94 (41.6%)	163 (51.1%)	90 (60.0%)	110 (41.2%)
Mother did not attend college, *N* (%)	408 (44.5%)	52 (21.8%)	14 (6.4%)	231 (73.0%)	107 (75.9%)	106 (39.7%)
Mother not married nor living with a partner, *N* (%)	380 (43.2%)	46 (19.3%)	15 (6.9%)	238 (73.9%)	81 (78.6%)	68 (25.0%)

ECHO = Environmental influences on Child Health Outcomes; ELGAN = Extremely Low Gestational Age Newborn; URECA = Urban Environment and Childhood Asthma.
Note: The meta-analysis included participants from four ECHO cohorts: Healthy Start, Project Viva, Mothers and Newborns, and URECA. In the Mothers and Newborns cohort, 60% of participants had missing values for cesarean section. Due to the high missing rate, we did not report the statistics for cesarean section in this cohort and the meta-analysis.

Table 3. Association between newborn blood DNA methylation levels, at probe cg24749470 (*CADM1*), and later childhood asthma among participants born after 28 weeks of gestation

ECHO cohort	Sample size (asthma cases)	Average DNAm	Coefficient (95% CI): asthma cases versus non-cases	*P*-value	% DNAm difference (95%CI): asthma cases versus non-cases
Healthy Start	239 (26)	0.766	0.072 (−0.047, 0.164)	.1233	0.72% (−0.38%, 1.82%)
Project Viva	237 (96)	0.803	0.145 (0.065, 0.224)	**.0004**	1.43% (0.57%, 2.29%)
Mothers and Newborns	324 (167)	0.784	0.084 (0.032, 0.137)	**.0016**	0.97% (0.39%, 1.56%)
URECA	142 (80)	0.797	0.105 (0.012, 0.199)	**.0272**	1.08% (0.11%, 2.06%)
Meta-analysis (≥28 weeks of gestation)					
Overall	942 (369)	0.786	0.098 (0.062, 0.134)	**9.31E−08**	1.06% (0.67%, 1.45%)
Term birth (≥37 weeks of gestation) only	881 (336)	0.786	0.095 (0.057, 0.132)	**5.77E−07**	1.03% (0.62%, 1.44%)
Male only	592 (189)	0.786	0.067 (0.013, 0.122)	**.0157**	0.73% (0.12%, 1.34%)
Female only	622 (180)	0.786	0.113 (0.061, 0.165)	**1.93E−05**	1.21% (0.62%, 1.80%)

CI = confidence interval; DNAm = DNA methylation; ECHO = Environmental influences on Child Health Outcomes; URECA = Urban Environment and Childhood Asthma.
Note: The coefficients and *P*-values were generated based on methylation *M*-value. %DNAm difference was generated based on methylation beta value. The meta-analysis included participants from four ECHO cohorts: Healthy Start, Project Viva, Mothers and Newborns, and URECA. Bold text indicates a *P*-value less than 0.03.

Table 4. Differentially methylated regions in newborn blood associated with childhood asthma among participants born after 28 weeks of gestation

DMRs (chromosome: position)	Locus	Width (bp)	Number of CpGs	Šidák *P*-value	Nearest gene	Distance to TSS (bp)	Overlap with promoter	Direction of effect
DMRs in all participants born after 28 weeks of gestation								
chr3: 113 160 183–113 160 490	3q13.2	308	5	1.84E−04	*CFAP44*	0	Yes	−
chr8: 1 765 066–1 765 679	8p23.3	614	11	1.07E−10	*MIR596*	0	Yes	−
DMRs in males born after 28 weeks of gestation								
chr2: 74 875 253–74 875 548	2p13.1	296	6	7.07E−04	*M1AP*	0	Yes	−
chr2: 164 204 628–164 205 032	2q24.3	405	5	2.44E−04	*FIGN*	387481	No	−
chr6: 29 601 398–29 601 705	6p22.1	308	5	9.05E−05	*GABBR1*	−436	Yes	−
chr6: 30 078 954–30 079 436	6p22.1	483	11	1.48E−07	*TRIM31*	1431	No	−
chr6: 33 048 254–33 048 879	6p21.32	626	14	1.50E−10	*HLA-DPB1*	0	Yes	−
chr17: 37 123 638–37 123 949	17q12	312	9	2.62E−04	*FBXO47*	0	Yes	−
chr17: 40 274 524–40 274 740	17q21.2	217	6	5.91E−05	*HSPB9*	−16	Yes	−
chr20: 57 427 556–57 427 973	20q13.21	418	10	5.69E−04	*GNAS*	10867	No	−
DMRs in females born after 28 weeks of gestation								
chr1: 27 683 139–27 683 501	1p36.11	363	5	3.62E−08	*MAP3K6*	9836	No	−
chr1: 223 566 643–223 567 002	1q41	360	6	2.83E−07	*CCDC185*	0	Yes	−
chr4: 186 732 926–186 733 060	4q35.1	135	6	3.79E−09	*SORBS2*	350	No	−
chr6: 30 039 132–30 039 801	6p22.1	670	17	2.63E−11	*RNF39*	3827	No	+
chr7: 27 170 241–27 170 832	7p15	592	10	3.71E−05	*HOXA4*	0	Yes	+
chr7: 27 183 375–27 184 667	7p15	1293	31	1.64E−12	*HOXA5*	3392	No	−
chr17: 40 935 998–40 937 365	17q21.2	1368	11	7.55E−19	*WNK4*	371	No	−
chr21: 15 645 787–15 646 312	21q11.2	526	5	3.37E−06	*ABCC13*	0	Yes	+

bp = base pair; DMR = differentially methylated region; ECHO = Environmental influences on Child Health Outcomes; URECA = Urban Environment and Childhood Asthma.
The DMRs were identified by the comb-p method based on the meta-analysis results from four ECHO cohorts of participants born after 28 weeks of gestations. The four ECHO cohorts are Healthy Start, Project Viva, Mothers and Newborns, and URECA. Direction of effect was obtained from linear regression. A + represents higher methylation in asthma cases compared with non-cases; a − represents a lower methylation level in asthma cases compared with non-cases.

gestation. In race-stratified analysis, we examined the direction and magnitude of effect in a subset of 27 CpGs (Supplementary Table S4) between newborns who later developed asthma and those who did not across self- reported race groups. Of these 27 CpGs (Supplementary Table S3), 19 (70%) were consistent in their direction and magnitude of effect across self-reported race groups (Supplementary Table S3). For example, cg25694755 showed an increase in methylation associated with asthma in participants reporting black (difference in DNAm: 0.884%, 95% CI: −0.0208, 1.889) and white (difference in DNAm 1.045, 95% CI: −0.0347, 2.101) race. The remaining eight CpGs (30%) showed an inconsistent direction or magnitude of effect across race groups, e.g. cg04892724.

DMRs in participants born after 28 weeks of gestation

We identified two DMRs (Table 4 and Supplementary Tables S5 and S6) associated with childhood asthma. For example, one DMR, located in the promoter region of *MIR596* (chr8: 1 765 066–1 765 679; Šidák *P*-value = 1.07×10^{-10}) includes 11 CpG sites showed 0.5%–1% lower methylation in asthma cases compared with non-cases in both males and females (Supplementary Fig. S5). Sex-stratified analysis revealed eight DMRs in males and another eight DMRs in females. For example, a DMR discovered in male-only analysis (chr6: 33 048 254–33 048 879, *HLA-DPB1*, Šidák *P*-value = 1.50×10^{-10}, Fig. 1A) exhibited 3%–4% lower methylation across all 14 CpGs among males who later developed asthma. Four of

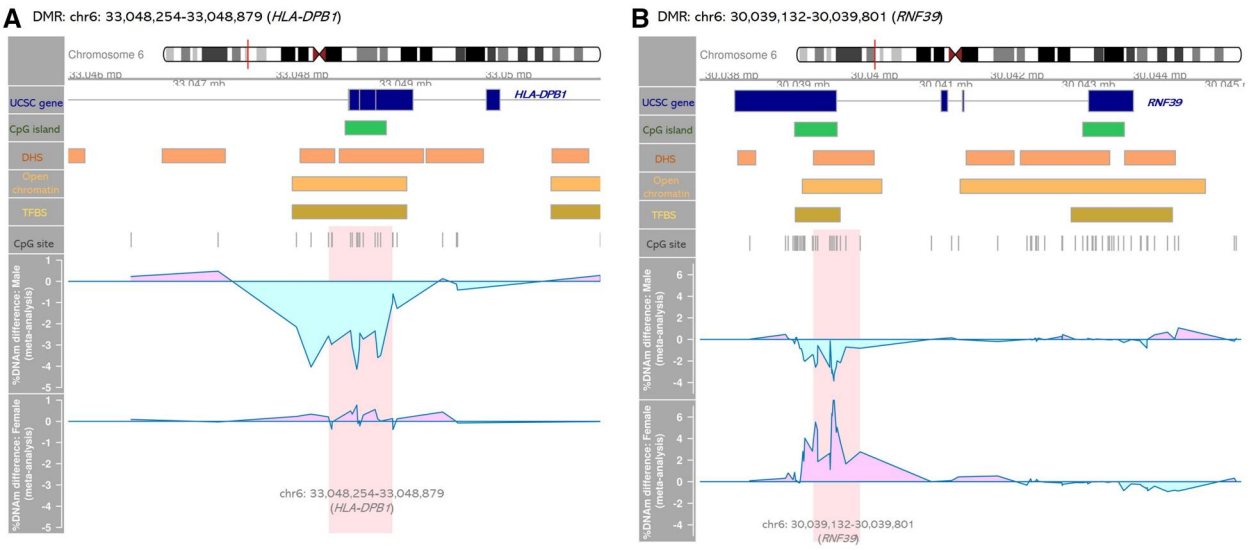

Figure 1. Sex-specific association between newborn DNA methylation (DNAm) level and childhood asthma at selected differentially methylated regions (DMRs) (6p21–6p22 locus) in participants born after 28 weeks of gestation. The two DMRs were selected as examples to visually demonstrate the difference in the association between DNAm and asthma between males and females. (A) The chr6:33 048 254–33 048 879 (*HLA-DPB1*) region. (B) The chr6: 30 039 132–30 039 801 (*RNF39*) region. The DMRs were identified by the comb-p method based on the sex-stratified meta-analysis results from four ECHO cohorts. The bottom panels show the effect size (%DNAm difference) for the association between DNAm (beta-value) at individual CpGs in the DMRs (highlighted by the rectangle shade in the background) and asthma among males and females, respectively. A %DNAm difference greater than 0 (pink area) represents hypermethylation in asthma cases compared with non-cases; a %DNAm difference less than 0 (blue area) represents hypomethylation in asthma cases compared with non-cases. Gene annotations were obtained from the UCSC Genome Browser (GRCh37/hg19). Abbreviations: DHS = DNase I hypersensitive sites; ECHO = Environmental Influences on Child Health Outcomes; TFBS = transcription factor binding site; UCSC = University of California, Santa Cruz, Genome Browser.

these 16 DMRs are situated within the human leukocyte antigen (HLA) region at the 6p21–6p22 locus, demonstrating sex-specific associations between DNAm and asthma. One DMR in the HLA region (chr6: 30 039 132–30 039 801, *RNF39*, Šidák *P*-value = 2.63×10^{-11}, Fig. 1B), discovered in females, showed an opposite direction of effect in males. The methylation level was 5%–7% higher in female asthma cases and 2%–4% lower in male asthma cases relative to those without asthma. Two DMRs at the 7p15 locus (*HOXA4* and *HOXA5* genes) also exhibited opposite directions of effect between sexes (Supplementary Fig. S6).

DMPs in extreme preterm birth participants

We did not find any CpGs showing associations with asthma among extremely preterm participants overall or in female-stratified analyses. We did however observe two DMPs associated with asthma in male participants ($\lambda = 1.05$, Supplementary Fig. S7). One DMP, cg03237868 ($P = 2.71 \times 10^{-8}$, *SPATA18*) showed higher methylation in newborns who later developed asthma compared with newborns who did not (%DNAm difference: 1.91%, 95% CI: 1.18%, 2.63%; Table 5, Supplementary Fig. S8). The other DMP (cg20681219, $P = 5.18 \times 10^{-8}$, *IRF2*) showed lower methylation among asthma cases than non-cases (%DNAm difference: −0.57%, 95% CI: −0.78%, −0.35%; Table 5, Supplementary Fig. S9).

DMRs in extremely preterm participants

Six regions exhibited asthma-associated differential methylation, with a Šidák *P*-value <.001, in newborns born before 28 weeks of gestation (Table 6). The two top DMRs overlapped with the promoter regions of *CCDC169* (chr13: 36 871 646–36 872 346, Šidák *P*-value = 1.10×10^{-9}) and *S100A13* (chr1: 153 599 479–153 600 064, Šidák *P*-value = 8.84×10^{-8}). The methylation level in *CCDC169* was higher in asthma cases compared with non-cases, with larger effect sizes observed in females (Fig. 2A). At the *S100A13* promoter, the methylation level was higher among asthma cases, with larger effect sizes in males (Fig. 2B). A third DMR located in the *CCDC185* gene promoter region (chr1: 223 566 447–223 567 002, Šidák *P*-value = 9.86×10^{-8}) showed higher methylation in asthma cases among all extremely preterm participants but the direction of effect differed by sex. Sex-stratified analyses revealed 10 additional DMR associations with asthma (Table 6). Among these, a DMR (chr6: 33 040 914–33 041 406, *HLA-DPA1*, Šidák *P*-value = 1.26×10^{-5}) within the HLA region at 6q21 was found among males and showed increased methylation in asthma cases.

Replication of previous findings

Previous work from the Pregnancy And Childhood Epigenetics (PACE) Consortium identified six DMPs associated with childhood asthma including at: cg13289553, cg13427149, cg16792002, cg17333211, cg21486411, cg02331902, cg07156990. We attempted to replicate these findings, mainly from European population data, in our US cohorts. As shown in Supplementary Figs. S10–S16, although all of the 95% confidence intervals crossed 1, we did observe a consistent direction and magnitude of effect between PACE and ECHO participants for two (cg07156990, cg13427149) of the six CpGs tested. We observed similar results in extremely preterm participants.

Discussion

This study of US children found associations between newborn blood methylation (DNAm) and prospective risk of childhood asthma including, for the first time, changes

Table 5. Sex-stratified association between newborn blood DNA methylation and later childhood asthma at differentially methylated positions cg03237868 (*SPATA18*) and cg20681219 (*IRF2*)

	Sample size (asthma cases)	Coefficient (95% CI): asthma cases versus non-cases	*P*-value	%DNAm difference (95% CI): asthma cases versus non-cases
cg03237868 (*SPATA18*)				
Extremely preterm/ELGAN (male)	148 (57)	0.152 (0.098, 0.206)	**2.71E−08**	1.91% (1.18%, 2.63%)
Extremely preterm/ELGAN (female)	124 (49)	0.012 (−0.052, 0.076)	0.7088	−0.09% (−0.91%, 0.74%)
≥28 weeks of gestation/meta-analysis (male)	444 (189)	0.045 (−0.002, 0.092)	0.0635	0.49% (−0.04%, 1.02%)
≥28 weeks of gestation/meta-analysis (female)	498 (180)	0.032 (−0.009, 0.073)	0.1299	0.41% (−0.08%, 0.90%)
cg20681219 (*IRF2*)				
Extremely preterm/ELGAN (male)	148 (57)	−0.284 (−0.387, −0.181)	**5.18E−08**	−0.57% (−0.78%, −0.35%)
Extremely preterm/ELAGN (female)	124 (49)	−0.081 (−0.215, 0.052)	0.2313	−0.20% (−0.48%, 0.08%)
≥28 weeks of gestation/meta-analysis (male)	444 (189)	−0.001 (−0.052, 0.050)	0.9660	0.04% (−0.08%, 0.16%)
≥28 weeks of gestation/meta-analysis (female)	498 (180)	−0.012 (−0.056, 0.033)	0.6075	−0.02% (−0.14%, 0.10%)

CI = confidence interval; DNAm = DNA methylation; ECHO = Environmental influences on Child Health Outcomes; ELGAN = Extremely Low Gestational Age Newborn; URECA = Urban Environment and Childhood Asthma.
Note: The coefficient and *P*-values were generated based on the methylation *M*-value. %DNAm difference was generated based on the methylation beta-value. The results for participants with an extremely preterm birth were obtained from the analysis of the ELGAN cohort. The results for participants with ≥28 weeks of gestation were obtained from the meta-analysis of four ECHO cohorts: Healthy Start, Project Viva, Mothers and Newborns, and URECA. Bold text indicates statistical significance.

Table 6. Differentially methylated regions identified in newborn blood associated with asthma in childhood among participants born before 28 weeks of gestation

DMRs (chromosome: position)	Locus	Width (bp)	Number of CpGs	Šidák *P*-value	Nearest gene	Distance to TSS (bp)	Overlap with promoter	Direction of effect
DMRs in all participants born before 28 weeks of gestation								
chr1: 153 599 479–153 600 064	1q21.3	586	13	8.84E−08	*S100A13*	0	Yes	+
chr1: 223 566 447–223 567 002	1q41	556	8	9.86E−08	*CCDC185*	0	Yes	+
chr6: 29 648 225–29 648 756	6p22.1	532	17	7.11E−05	*ZFP57*	−3294	No	+
chr11: 2 292 890–2 293 305	11p15.5	416	15	3.17E−07	*ASCL2*	−708	Yes	−
chr13: 36 871 646–36 872 346	13q13.3	701	12	1.10E−09	*CCDC169*	0	Yes	+
chr17: 170 875–171 257	17p13.3	383	7	5.72E−04	*RPH3AL*	6113	No	−
DMRs in boys born before 28 weeks of gestation								
chr1: 153 599 479–153 600 15	1q21.3	678	15	4.81E−15	*S100A13*	0	Yes	+
chr1: 155 005 957–155 006 412	1q21.3	456	7	9.74E−04	*DCST2*	0	Yes	+
chr4: 81 111 177–81 111 527	4q21.21	351	5	2.89E−04	*PRDM8*	4753	No	+
chr6: 29 648 161–29 648 901	6p22.1	741	19	1.00E−07	*ZFP57*	−3230	No	+
chr6: 33 040 914–33 041 406	6p21.32	493	7	1.26E−05	*HLA−DPA1*	0	Yes	+
chr11: 102 638 432–102 638 706	11q22.2	275	5	9.88E−05	*MMP10*	12653	No	−
chr14: 22 538 566–22 538 812	14q11.2	247	5	8.90E−04	*OR4E2*	405269	No	−
chr17: 1 508 432–1 508 723	17p13.3	292	5	3.70E−04	*SLC43A2*	22821	No	−
DMRs in girls born before 28 weeks of gestation								
chr1: 55 271 673–55 271 927	1p32.3	255	7	1.66E−06	*LEXM*	0	Yes	−
chr5: 135 416 205–135 416 613	5q31.1	409	8	6.17E−04	*VTRNA2 – 1*	0	Yes	+
chr13: 36 871 878–36 872 346	13q13.3	469	9	3.52E−05	*CCDC169*	0	Yes	+
chr17: 202 588–202 988	17p13.3	401	6	1.33E−06	*RPH3AL*	0	Yes	+
chr19: 2 256 468–2 256 673	19p13.3	206	5	9.24E−04	*JSRP1*	−46	Yes	−
chr22: 45 608 345–45 608 713	22q13.31	369	10	5.14E−07	*KIAA0930*	0	Yes	+

CI = confidence interval; DMR = differentially methylated region; ECHO = Environmental influences on Child Health Outcomes; ELGAN = Extremely Low Gestational Age Newborn; EWAS = epigenome-wide association study.
The DMRs were identified by the comb-p method based on the cohort-specific EWAS results from the ELGAN cohort of participants born before 28 weeks of gestation. A + represents higher methylation in asthma cases compared with non-cases; a − represents a lower methylation level in asthma cases compared with non-cases.

present in extremely preterm infants. These infants have a higher risk of developing asthma through unique etiological mechanisms due to premature lung development at birth [10], our findings shed crucial light on understanding asthma's early origins in this vulnerable population.

Prevalence of childhood asthma in our analytic sample was higher than in the general US population [1] due to inclusion of individuals with early asthma diagnosis before age 5 who may not have received another physician diagnosis later due to limited access to healthcare, despite continuing self- or parent-/caregiver-reported asthma symptoms after reaching school-age and due to oversampling of children at higher risk for asthma. Because our primary goal was to identify DNAm changes that contribute to asthma development, rather than to asthma incidence or prevalence, the overrepresentation of asthma cases in this study should not bias our results.

We conducted a fixed-effect meta-analysis of newborns born after 28 weeks of gestation from four ECHO cohorts. The fixed-effect model was selected based on the assumption that the DNAm difference at each CpG site has a same independent effect on later asthma risk after adjusting for covariates, as suggested by Nikolakopoulou *et al.* [20]. We assume that that observed heterogeneity across cohorts is largely attributed to specific covariates, such as sex and race, rather

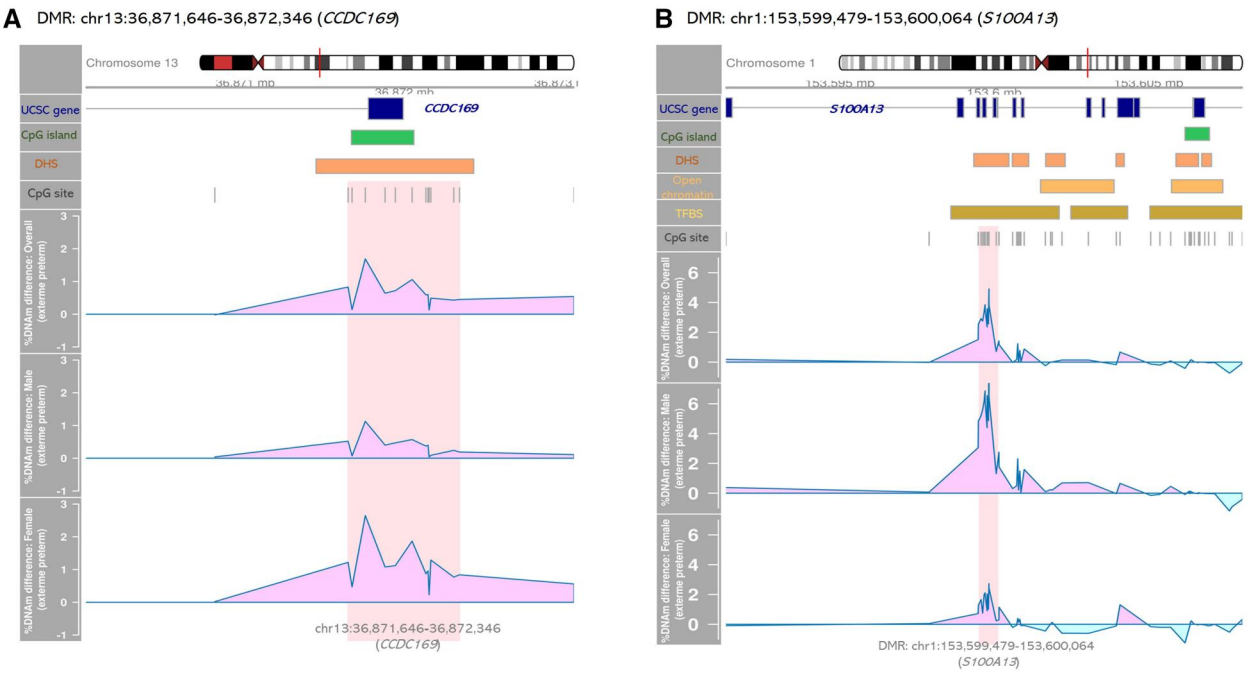

Figure 2. Association between newborn DNA methylation (DNAm) level and childhood asthma at selected differentially methylated regions (DMRs) in participants born before 28 weeks of gestation. The two DMRs were selected as examples for visual demonstration. (A) The chr1: 223 566 447–223 567 002 (*CCDC185*) region. (B) The chr1: 153 599 479–153 600 064 (*S100A13*) region. The DMRs were identified by the comb-p method based on the EWAS analysis of all participants in the ELGAN cohort. The bottom panels show the effect size (%DNAm difference) for the association between DNAm (beta-value) at individual CpGs in the DMRs (highlighted by the rectangle shade in the background) and asthma. A %DNAm difference greater than 0 (pink area) represents hypermethylation in asthma cases compared with non-cases; a %DNAm difference less than 0 (blue area) represents hypomethylation in asthma cases compared with non-cases. Gene annotations were obtained from the UCSC Genome Browser (GRCh37/hg19). Abbreviations: DHS = DNase I hypersensitive sites; ECHO = Environmental Influences on Child Health Outcomes; ELGAN = Extremely Low Gestational Age Newborns; EWAS = epigenome-wide association study; TFBS = transcription factor binding site; UCSC = University of California, Santa Cruz, Genome Browser.

than cohort design. Therefore, we used a fixed-effect model for the primary analysis, followed by sex- and race-stratified analyses to further explore heterogeneity across demographics. Our methylation results implicate biology relevant to asthma, expanding on previous findings. A CpG we identified at *CADM1* exhibited higher methylation in newborns who later developed asthma. Genetic variants in *CADM1* have been associated with chronic obstructive pulmonary disease [21], FEV1/FVC ratio [22], and COVID-19 severity [23]. *CADM1* is a pro-inflammatory adhesion molecule that plays a critical role in mast cell biology [24–26]. Mast cells play pivotal roles in asthma pathophysiology, acting both as effectors of the acute airway changes after allergen exposure and key contributors to the initiation and propagation of the disease process [24]. Our findings add to the current knowledge that DNAm at birth may regulate mast cell adhesion, potentially predisposing infants to later asthma risk. We also discovered a DMR at the microRNA *MIR596* promoter. While microRNAs have been implicated in allergic response and asthma development [27], microRNA-596 has not been previously implicated in allergy or asthma. Interesting, microRNA-596 negatively regulates the gene expression of *SMAD3* in bone marrow [28]; *SMAD3* has been associated with asthma risk in multiple genome-wide association studies (GWAS [29, 30] and EWAS [5] studies). It is possible that *MIR596* methylation contributes to asthma development through the same pathway as the *SMAD3* locus.

The magnitude of methylation changes we observed ranged from 1.06% to 3.56%. There are several reasons why changes of this seemingly small magnitude may be meaningful. First, it is possible that a subset of cells in the "bulk" blood samples we examined have large methylation changes, as detailed in Breton *et al.* [31]. Second, these changes could serve as an early-life biomarker for prospective asthma outcomes, which is useful regardless of whether they are part of the mechanism.

Several sex-specific asthma-associated methylation changes were located within the HLA chromosomal region. The HLA locus is a well-replicated asthma risk locus in genetic studies [32] and triggering immune responses [33]. A previous GWAS in Asian populations identified a genetic variant located between *HLA-DPA1* and *HLA-DPB1* that was associated with increased risk of pediatric asthma, independent from dust mite sensitization [34]. Taken together, this suggests molecular differences in HLA-DP at birth may predispose infants to asthma risk when they are later exposed to environmental antigens.

Our finding of differential methylation at *SPATA18* and *IRF2* in extremely preterm male infants align with reports of differential gene expression of *SPATA18* in bronchial biopsies of asthma patients [35] and *IRF2* genetic variation associations with asthma and lung function [36, 37]. Furthermore, multiple asthma-associated DMRs we identified have been associated with percent predicted FEV1 in children with atopic asthma [38] or multi-food allergy [39]. Our results provide new evidence that epigenetic regulation of these asthma/allergy-related genes contribute to asthma development from birth in this unique population of extremely preterm infants.

Given differences in asthma incidence, subtypes, and risk factors between by race and sex [16], we explored sex and race-specific effects. We identified several methylation changes showing different asthma associations by sex. Interestingly, one DMR at *RNF39* showed opposite directions of effect in children born after 28 weeks of gestation, with lower methylation in males and higher methylation in females associated with asthma. Methylation in this gene has previously been reported to show an association with lung functions but only in males [40]. The sex-specific associations we observed may be capturing differences in asthma subtypes (e.g. atopic versus non-atopic) because their distribution also varies by sex. Unfortunately, data to distinguish asthma subtypes, such as allergen sensitization or IgE level, were not available for the current analysis, which we recognize as a limitation in our ability to test this. To gain further insights, future studies incorporating more comprehensive clinical and molecular data to better distinguish asthma phenotypes/endotypes are warranted.

Among the top ranked 27 CpGs that we examined, most (70%) showed a consistent effect across self-reported black and white race groups. This suggests methylation at these CpGs occurs across diverse subpopulations in the US and could provide a common robust biomarker or reveal shared etiologic pathways across sociodemographic groups. A subset of CpGs (30%) were inconsistent across racial groups, indicating there could also be some race-specific effects of methylation on asthma risk. Further supporting the existence of both common and subpopulation specific methylation changes, we identified some consistent and inconsistent effect magnitudes between our US ECHO participants and those reported previously in European cohorts from PACE and to expand the set of CpGs examined across the genome. Future studies are needed to elucidate how race subpopulation differences may influence methylation associations asthma.

Several other limitations should be acknowledged. First, despite employing various approaches to control confounding, residual confounding may still exist due to the nature of observational data. Second, while DNAm in newborn blood is ideal for detecting early immunological disturbances relevant to asthma, it may not correlate with DNAm in respiratory tissues. Additionally, since newborn blood comprises multiple cell types, the observed differential methylation represents a weighted average of cell-specific effects, potentially explaining the relatively small magnitude DNAm differences we observed. Future studies utilizing DNAm data in other relevant tissues and at single-cell resolution will be crucial in elucidating the biological mechanisms underlying our findings.

Ethics approval

The study protocols were approved by the local and/or single ECHO institutional review boards. Written informed consent or parent's/guardian's permission was obtained along with child assent as appropriate, for ECHO-wide Cohort Data Collection Protocol participation and for participation in specific cohorts. This study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki.

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Author contributions

Y.L. and C.L.-A. designed the study. Analyses were carried out by Y.L. and K.J. with oversight and mentorship from C. L.-A., L.J., and K.H. The article was written by Y.L. with specific contributions from K.J. A.C. and C.K. provided expertise on data harmonization for asthma outcomes. C.O., A. S., W.G., L.B., D.D., R.F., D.G., J.H., and M.-F.H., and R. M. provided cohort site-specific expertise and result feedback. Result interpretation and critical review was provided by Y.L., L.J., C.L.-A., C.O., A.S., K.J., M.-F.H., and W.G. All co-authors read, edited, and approved the article.

Supplementary data

Supplementary data is available at *IJE* online.

Conflict of interest: C.L.-A. reports receiving consulting fees from the University of Iowa for providing expertise on epigenetics outside of this work. C.K. reports receiving royalties from UpToDate, serving as Associate Editor at JACI, and as a board member of the ABAI. All other authors declare that they have no competing interests.

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Data availability

Select de-identified data from the ECHO Program are available through NICHD's Data and Specimen Hub (DASH). Information on study data not available on DASH, such as some Indigenous datasets, can be found on the ECHO study DASH webpage.

Use of artificial intelligence (AI) tools

No AI tools were utilized to draft or edit the manuscript text, tables, or figures. No AI tools were used in the collection, analysis, or interpretation of the data.

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