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Breastfeeding association with DNA methylation in the pregnancy and childhood epigenetics (PACE) consortium

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Abstract

Background Breastfeeding is associated with short- and long-term beneficial effects on child health, including greater cognitive development, and enhanced immune programming. However, the underlying biological mechanisms are only partially understood, with epigenetics emerging as a potential contributor. In this study, we aimed to investigate whether breastfeeding practices are associated with differential DNA methylation (DNAm) in childhood blood.

Results We conducted meta-analyses of epigenome-wide association studies (meta-EWASs) in 3421 children from eleven international population-based birth cohorts from the Pregnancy And Childhood Epigenetics (PACE) Consortium. Breastfeeding was assessed as "ever" being breastfed vs. "never", and duration of any and exclusive breastfeeding. DNAm was measured in childhood blood (ages 5–12 years) using the Illumina 450 K or EPIC arrays, with cord blood at birth used as negative outcome control. At False Discovery Rate (FDR) < 5%, positive associations

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at six cytosine-phosphate-guanine (CpG) sites were identified in childhood blood: four with duration of exclusive breastfeeding, and three with duration of exclusive breastfeeding of more than three months compared to never. The annotated genes (*ALAD*, *FNBP4*, and *CHFR*) are related to developmental and immune processes. None of these CpG sites were FDR-significant in cord blood prior to breastfeeding.

Conclusions Breastfeeding was associated with differential DNAm in childhood blood at a limited number of CpG sites. Future studies in diverse populations are needed to examine the robustness of these associations, the sources of heterogeneity, and the generalizability of the findings.

Keywords Breastfeeding, Exclusive breastfeeding, Epigenetics, DNA methylation, Epigenome-wide association study, Meta-analysis, Birth cohort, Children

Background

Breastfeeding has substantial short- and long-term beneficial effects on child health, for instance by lowering the risk of respiratory problems, reducing the risk of metabolic diseases, improving cognitive development, and driving immune programming [1–6]. Human milk is preferred compared to formula milk because of its nutritional content, the presence of immunoglobulins, and its antioxidant and anti-inflammatory properties [7]. The World Health Organization (WHO) recommends exclusive breastfeeding until 6 months, and then continuing alongside complementary foods until at least 2 years of age) [8, 9].

Breastfeeding may influence infant development through epigenetic processes. The epigenome, defined as the set of chemical modifications of the DNA or related proteins or RNAs that regulate gene expression, can affect developmental pathways, consistent with the early-life programming of health and disease [10, 11]. One of the most studied epigenetic processes in humans is DNA methylation (DNAm), the addition of a methyl group onto a cytosine in a cytosine-phosphate-guanine dinucleotide (CpG) [12].

Few studies have investigated the association between breastfeeding and DNAm in humans. Two candidate gene studies conducted in blood and buccal epithelial cells found associations with DNAm of the *Leptin* (*LEP)* gene, which encodes a hormone that controls appetite behavior [13, 14]. More recent epigenome-wide association studies (EWAS) showed a potential association of breastfeeding with DNAm in early life, but were limited as they were single cohort studies often with relatively small sample sizes, low statistical power and providing few consistent results, with almost no cross-cohort validation [15–23] .

We conducted the largest study to date using an EWAS meta-analysis (meta-EWAS) approach aimed at identifying robust associations of breastfeeding with child blood DNAm.

Methods

Study participants

The study overall included data from fourteen birth cohorts from the Pregnancy And Childhood Epigenetics (PACE) Consortium [24]. Blood DNAm was measured during childhood in eleven of these cohorts and at birth (in cord blood) in six of the cohorts (three cohorts both in childhood and at birth). Results from the analyses at birth were used as negative outcome controls (i.e. it is not expected that breastfeeding practices affect DNAm at birth). For details of the participating cohorts see Table 1 and Supplemental Methods (Supplementary Material 1).

Breastfeeding data

Data on breastfeeding were collected through questionnaires administered to the mothers. Any breastfeeding was defined as the practice where an infant received breast milk, including milk expressed or donated (human milk bank), regardless of the introduction of formula feed and/or solid foods. Exclusive breastfeeding was defined as the practice where an infant only received breast milk, including milk expressed or donated, but no other food or drink (including water), except for oral rehydration therapy (e.g. due to complications at birth), drops and syrups (e.g. vitamins, minerals and medicines) based on the WHO definitions (https://www.emro.who.int/nutrition/breastfeeding/index.html). The following breastfeeding variables were created: (i) any breastfeeding (0: never breastfed, 1: ever breastfed); (ii) any breastfeeding duration in months (to harmonize across cohorts, children breastfed for >12 months were assigned the value of 12, and children never breastfed were assigned the value of 0); (iii) any breastfeeding duration in four categories (0: not breastfed, 1: ≤4 months, 2: >4 months and <12 months, 3: ≥12 months); (iv) exclusive breastfeeding duration in months; and (v) exclusive breastfeeding in three categories (0: not breastfed, 1: ≤3 months, 2: >3 months). Any breastfeeding and exclusive breastfeeding categories were defined according to WHO recommendations, average maternity leave across countries, frequency and type of questions asked in each cohort, and reasonable sample size in each category.

Table 1 Characteristics of each cohort included in the meta-EWASs of breastfeeding practices

			Child blood DNAm														
			Any breastfeeding									Exclusive breatfeeding					
			Ever vs. Never			Duration (months)		Duration categories				Duration (months)		Duration categories			
Cohort	**Ancestry**	**Mean age (years)**	**N**	**Never**	**Ever**	**N**	**Median [IQR]**	**N**	**Never**	**≤4 m**	**>4–12 m**	**≥12 m**	**N**	**Median [IQR]**	**N**	**Never**	**≤3 m**	**>3 m**
ALSPAC	European	7.45	767	81	686	767	5.00 [1.00, 9.00]	767	81	294	276	116	766	2.00 [0.00, 3.00]	766	81	593	92
BAMSE.E	European	8.30	–	–	–	300	8.75 [7.00, 11.25]	–	–	–	–	–	302	5.62 [4.00, 6.00]	–	–	–	–
BAMSE.M	European	8.34	–	–	–	196	8.38 [6.00, 11.00]	–	–	–	–	–	197	5.75 [4.00, 6.00]	–	–	–	–
CHOP	European	11.12	373	106	267	–	–	342	106	143	60	33	–	–		–	–	–
GENR	European	9.77	339	36	303	339	5.00 [2.50, 8.50]	288	36	116	104	32	–	–		–	–	–
GLAKU	European	12.31	72	26	46	72	9.00 [0.00, 13.62]	–	–	–	–	–	87	4.00 [0.00, 4.75]	87	26	12	49
HELIX	European	8.33	788	128	660	788	4.93 [0.99, 10.84]	788	125	250	278	135	–	–		–	–	–
HOME	Diverse	12.31	157	28	129	157	3.25 [0.50, 12.00]	157	28	56	33	40	157	0.03 [0.03, 0.69]	157	29	110	18
IOW	European	10.00	83	18	65	83	1.87 [0.47, 7.00]	–	–	–	–	–	83	1.40 [0.35, 3.38]	83	20	39	24
POGO.GDM	European	5.28	78	10	68	78	5.00 [2.00, 9.00]	–	–	–	–	–	––	–		–	–	–
Viva	European	7.78	268	25	243	268	8.50 [3.00, 12.00]	–	–	–	–	–	259	2.79 [0.47, 4.50]	–	–	–	–
Total N			2925	458	2467	3048	–	2342	376	859	751	356	1851	–	1093	156	754	183
			Cord blood DNAm (negative outcome control)															
			Any breastfeeding										**Exclusive breatfeeding**					
			Ever vs. Never			**Duration (months)**		**Duration categories**					**Duration (months)**		**Duration categories**			
Cohort	**Ancestry**	**Mean age (years)**	**N**	**Never**	**Ever**		**Median [IQR]**	**N**	**Never**	**≤4 m**	**>4–12 m**	**≥12 m**	**N**	**Median [IQR]**	**N**	**Never**	**≤3 m**	**>3 m**
ALSPAC	European	0	710	76	634	710	5.00 [1.00, 9.00]	710	76	274	251	109	709	2.00 [0.00, 3.00]	709	76	553	80
BIS	European	0	745	13	732	745	4.60 [1.38, 8.28]	–	–	–	–	–	561	1.15 [0.23, 5.06]	–	–	–	–
DCHS	African and admixed	0	250	45	205	250	6.00 [1.39, 12.00]	250	45	62	57	86	250	1.45 [0.46, 3.68]	250	53	125	72
GENR	European	0	979	99	880	979	5.00 [1.50, 8.50]	844	99	366	294	85	–	–	–	–	–	–
HOME	Diverse	0	261	45	216	261	5.00 [0.75, 12.00]	261	45	82	64	70	261	0.03 [0.03, 0.69]	261	46	183	32
INMA	European	0	376	28	348	376	5.26 [2.92, 8.97]	373	28	110	174	61	371	2.07 [0.07-4.36]	371	82	143	146
Total N			3321	306	3015	3321	-	2438	293	894	840	411	2152	–	1591	257	1004	330

DNAm: DNA methylation, m: months, meta-EWASs: meta-analyses of epigenome-wide association studies

With a dash, if the cohort does not participate in that particular analysis because the low number of samples in some group

Note that some of the cohorts have lower N in the variable "duration categories" compared to the others due to how the questionnaire was formulated

INMA is part of the HELIX cohort

Blood DNA methylation data

DNA was extracted from whole blood or buffy coat and processed with the Illumina EPIC or the 450 K methylation arrays. Data quality control was performed according to each cohort's preferred procedures and included sample and probe quality control, normalization, and correction for technical batch effects. For cohort-specific details, see Supplementary Material 1. Extreme DNAm values outside the range of (25th percentile − 3 × interquartile range (IQR)) to (75th percentile + 3 × IQR) were removed. DNAm values were expressed as beta values ranging from 0 (completely unmethylated) to 1 (completely methylated).

Six blood cell type proportions (natural killers, monocytes, granulocytes, CD8T cells, CD4T cells, and B cells) were estimated from childhood DNAm data using the Reinius reference panel and the Houseman algorithm [25, 26]. For cord blood DNAm, the Gervin and Salas reference panel was used, which includes the same six cell types plus nucleated red blood cells [27].

Epigenome-wide association analyses

Each cohort estimated the association between breastfeeding practices and blood DNAm using an adjusted robust linear regression model for each CpG site, with DNAm as the outcome according to a predefined analysis plan and R code.

Covariates included in the models were: age at blood sampling (in years, only for the childhood EWAS), sex (male, female), maternal age at delivery (years), maternal education (primary, secondary, and university), parity (nulliparous, multiparous), pre-pregnancy or early pregnancy maternal body mass index (BMI) (kg/m^2), birth weight (grams), gestational age at delivery (weeks), type of delivery (vaginal, caesarean), sustained maternal smoking during pregnancy (non-sustained smoking, sustained smoking), and estimated blood cell type proportions at time of blood sampling. Optional covariates based on specific cohort characteristics and data availability were child's ancestry (from genome-wide genetic principal components (PCs) or from questionnaire data), technical covariates or selection factors.

Meta-analyses

Prior to the meta-analyses, we conducted stringent quality control of cohort-specific EWAS results. Probe filtering was done to remove control probes, probes to detect single nucleotide polymorphisms (SNPs), non-CpG probes, probes in sex chromosomes, probes that give non-consistent measures across arrays (EPIC versus 450 k) in blood [28], and problematic CpG probes [29].

Quality control of the results from each cohort consisted of checking the effect sizes, standard errors (SE), p-values (p), number of significant CpG sites and calculation of the lambda inflation factors.

Fixed-effects inverse variance-weighted meta-analyses were conducted independently by two researchers (AC and HL), using the *EASIER* R package (https://github.com/isglobal-brge/EASIER) [30] and the *metafor* R package [31]. Random-effects meta-analyses were also run for significant probes in the fixed effect meta-analyses.

Given that the EPIC array was only available in a subset of the cohorts (four out of eleven cohorts), which in addition had relatively small sample sizes (< 160 participants), we only meta-analysed the CpG sites present on both EPIC and 450 K arrays. CpG sites present in only one cohort were filtered out from the meta-analyses.

A main meta-analysis was run for each breastfeeding definition including all children from all available cohorts. We used False Discovery Rate (FDR) method at 5% to control for multiple-testing [32]. Effect size is reported as the difference in DNAm between breastfed and never breastfed infants (for categorical variables) or by month of any or exclusive breastfeeding (for continuous variables).

Sensitivity analyses were performed to examine heterogeneity across cohorts and to reduce the chance of false positives by re-running the models: (i) restricting to European ancestry from all cohorts; (ii) restricting to cohorts with a sample size > 100; and (iii) restricting to European ancestry from cohorts with a sample size > 100. Leave-one-out analyses were conducted for CpG sites that were FDR-significant in the main model.

Cohort-specific and meta-analysis results were summarized in tables and plots using *ggplot2* and *forestplot* R packages [33] (https://cran.r-project.org/web/packages/forestplot/index.html). All analyses were conducted in R environment version 4.2.1 (2022-06-23).

In silico analyses

CpG sites were annotated to genes using the *IlluminaHumanMethylation450kanno.ilmn12.hg19* R package. DNAm quantitative trait loci (meQTLs) were retrieved from the Genetics of DNAm consortium (GoDMC) database (http://mqtldb.godmc.org.uk/index) [34], and *cis* expression quantitative trait DNAm (eQTMs) from the Human Early Life Exposome (HELIX) database (https://helixomics.isglobal.org/) [35]. Previous associations with exposures or traits were searched in the EWAS catalog (https://www.ewascatalog.org/) [36]. We also conducted enrichment analyses of the suggestive CpG sites (p < 1E-05) associated with exclusive breastfeeding variables using the EWAS Toolkit (https://ngdc.cncb.ac.cn/ewas/toolkit). We tested enrichment for KEGG pathways and traits/exposures from the EWAS Atlas.

Finally, a look-up of previous associations from studies of breastfeeding practices with N > 100 [17, 19, 21], and a

candidate-gene look-up across the whole *LEP* gene were also carried out due to its role in appetite regulation and previous evidence linking it with breastfeeding.

Results

Participants' characteristics

The meta-EWAS of breastfeeding practices included childhood blood DNAm from eleven cohorts, for a total N of 3421 participants. The study sample is summarised in Table 1 and in Supplemental Tables S1A-B (Supplementary Material 2). Nine cohorts participated in the meta-analyses of any breastfeeding ($N = 2467$ ever breastfed and $N = 458$ never breastfed children). Any breastfeeding duration data were available for 3048 children across ten cohorts, with median durations ranging from 1.87 to 9.00 months. Exclusive breastfeeding data were available for 1851 children across seven cohorts, with median durations ranging from 0.03 to 5.75 months. Children from ten out of eleven cohorts were of European ancestry. Three cohorts had < 100 participants.

EWAS of any breastfeeding and childhood blood DNA methylation

We did not identify CpG sites at which childhood blood DNAm was associated with any breastfeeding (ever vs. never, duration or categories). When restricting the analysis to children of European ancestry, duration of any breastfeeding was inversely associated with DNAm at cg07954414 (at the *KIAA0922* gene) (effect= -0.0001, standard error (SE) = 0.0000, p_{FDR}= 0.04) (Supplemental Table S2 (Supplementary Material 3)). This CpG site showed a similar association in the main model, close to FDR-significance (effect= -0.0001, SE = 0.0000, p_{FDR}= 0.12). No FDR-significant associations were found when restricting to cohorts with > 100 participants.

The cohort-specific EWAS and the meta-EWAS models showed good performance, with limited genomic inflation (by cohort: Supplemental Tables S3A-C (Supplementary Material 4); meta-EWASs: Supplemental Tables S4A-C (Supplementary Material 5) and Supplemental Figures S1-S3 (Supplementary Material 6)). Top CpG sites ($p < 1E-05$) are shown in Supplemental Tables S5A-D, S6A-D and S7A-B (Supplementary Materials 7, 8 and 9, respectively).

EWAS of exclusive breastfeeding and childhood blood DNA methylation

Continuous duration of exclusive breastfeeding was positively associated with DNAm at four CpG sites: cg01257194 (*ALAD*), cg20053493 (*FNBP4*), cg04942655 (intergenic), and cg20702204 (*CHFR*) (Table 2). Cg01257194 (*ALAD*), cg20053493 (*FNBP4*), and cg04942655 (intergenic) remained FDR-significant in cohorts with > 100 participants, and cg01257194 (*ALAD*) in children of European ancestry. There was high heterogeneity across the cohorts, with values of $I^2 > 0.6$ for all CpG sites, except for cg20053493 (*FNBP4*) ($I^2 < 0.17$), and effect sizes were relatively small (Fig. 1). The leave-one-out analysis indicated that some of the associations in the main model were driven by the HOME study, which is the most ethnically diverse sample of participants (Supplemental Table S8A (Supplementary Material 10)). Three other CpG sites were associated with exclusive breastfeeding duration in the sensitivity models (Supplemental Table S2 (Supplementary Material 3)). When restricting to European ancestry, longer exclusive breastfeeding duration was associated with higher DNAm at cg02352945 (*ACVR2B*) (effect = 0.0004, SE = 0.0001, p_{FDR}= 0.008), and at cg02592586 (intergenic) (effect = 0.0029, SE = 0.0006, p_{FDR}= 0.035). The effect size for both of these CpG sites was similar in the main model (cg02352945, *ACVR2B*, effect = 0.0003, SE = 0.0001, p_{FDR}= 0.054; cg02592586, intergenic, effect = 0.0026, SE = 0.0005, p_{FDR}= 0.081). In the cohorts with > 100 participants, exclusive breastfeeding duration was associated with higher DNAm levels at cg06061442 (*PLB1*) (effect = 0.0017, SE = 0.0003, p_{FDR}= 0.022). In the main model the effect size was similar (effect = 0.0013, SE = 0.0003, p_{FDR}=0.190), with heterogeneity decreasing from $I^2 = 0.8$ to $I^2 = 0$ when restricting to cohorts with > 100 participants.

Exclusive breastfeeding duration longer than three months compared to never being breastfed was also associated with three CpG sites: cg20053493 (*FNBP4*), cg27663031 (intergenic), and cg00315563 (intergenic) (Table 2). Cg00315563 (intergenic) and cg20053493 (*FNBP4*) were also FDR-significant in sensitivity models when restricting to European ancestry and to cohorts with > 100 participants. Cg20053493 (*FNBP4*) was also associated in the meta-EWAS of exclusive breastfeeding duration in months. Cg27663031 (intergenic) presented high heterogeneity ($I^2 > 0.6$), although the forest plot showed effects in the same direction across cohorts (Fig. 2; Supplemental Table S8B (Supplementary Material 10)). Additionally, one CpG site was FDR-significant only in cohorts with > 100 participants (> 3 months vs. never: cg22941178, *LOC284837*, effect = 0.0152, SE = 0.0029, p_{FDR}= 0.024) (Supplemental Table S2 (Supplementary Material 3)). The association at cg22941178 (*LOC284837*) was similar in the main model, though not FDR-significant (effect = 0.0129, SE = 0.0027, p_{FDR}= 0.141).

The lambda inflation factors and EWAS results for exclusive breastfeeding are shown in Supplemental Tables S3D-E, S4D-E, S9A-D, S10A-C (Supplementary Materials 4, 11 and 12), and Supplemental Figures S4, S5A-B (Supplementary Material 6).

Table 2 Association of breastfeeding and DNAm at FDR-significant CpGs identified in the main analysis

			Main analysis						Annotation						
CpG	Breastfeeding	Comparison/ Units	N	Effect size	SE	p-value	FDR p-value	I^2	Chromosome	Position (hg37)	Gene	Gene relative position	SNPs probe (MAF)	GoDMC SNPs	EWAS catalog
cg01257194	exclusive BF duration	months	1852	0.0017	0.0003	**9.48E-09**	**3.93E-03**	0.67	chr9	116,161,247	*ALAD*	5'UTR	–	–	CRP↑, alcohol, sex, age↑
cg04942655	exclusive BF duration	months	1852	0.0004	0.0001	**3.69E-08**	**5.09E-03**	0.65	chr17	80,303,572	intergenic	–	rs9889704 (0.31)	rs537613896, others	age↓
cg20702204	exclusive BF duration	months	1852	0.0008	0.0002	**3.27E-07**	**3.39E-02**	0.75	chr12	133,430,077	*CHFR*	body	–	–	age*↑
cg20053493	exclusive BF duration	months	1852	0.0005	0.0001	**2.25E-08**	**4.66E-03**	0.17	chr11	47,776,175	*FNBP4*	body	–	rs7935528, others	age↓
cg20053493	exclusive BF duration categories	≤3 m vs. never	754 vs. 156	0.0015	0.0005	3.80E-03	7.42E-01	0.19							
		>3 m vs. never	183 vs. 156	0.0036	0.0006	**1.11E-09**	**4.27E-04**	0.00							
cg27663031	exclusive BF duration categories	≤3 m vs. never	754 vs. 156	0.0042	0.0011	1.07E-04	4.41E-01	0.51	chr5	105,791,135	intergenic	–	–	–	age↓
		>3 m vs. never	183 vs. 156	0.0080	0.0015	**1.08E-07**	**2.07E-02**	0.62							
cg00315563	exclusive BF duration categories	≤3 m vs. never	754 vs. 156	0.0170	0.0038	9.25E-06	2.60E-01	0.00	chr3	182,243,039	intergenic	–	–	rs2700866, others	age↓
		>3 m vs. never	183 vs. 156	0.0202	0.0038	**1.61E-07**	**2.07E-02**	0.10							

BF: breastfeeding, COPD: chronic obstructive pulmonary disease, CRP: C-reactive protein, EWAS: epingenome-wide association study, FDR: false discovery rate, m: months, MAF: minor allele frequency, SE: standard error, SNP: single nucleotide polymorphism

*All associations with age are based on the fixed effect model of the paper by Mulder et al., except for this one which is based on the random effect model

Associations in bold are FDR-significant

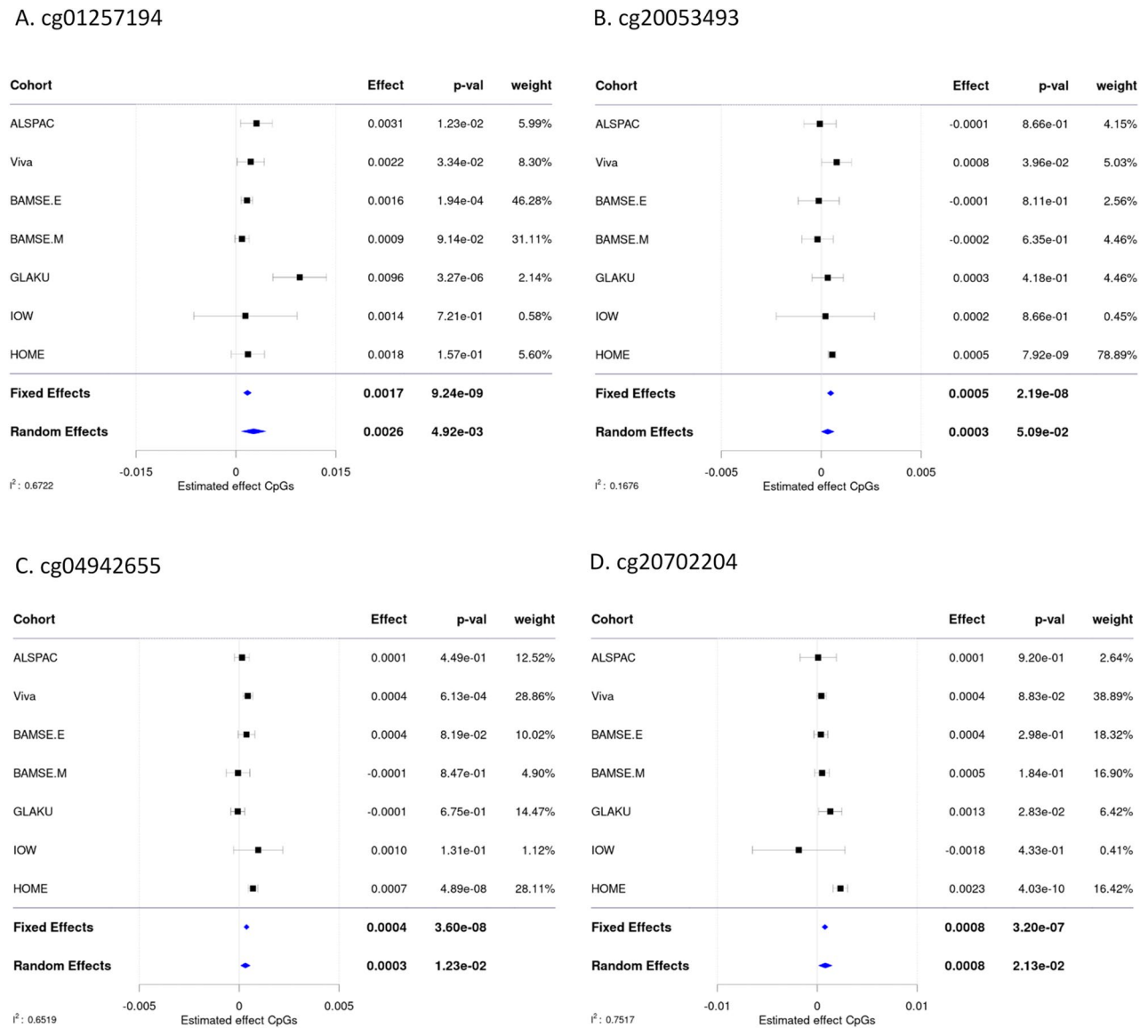

Fig. 1 Forest plots representing the association of exclusive breastfeeding duration (months) with FDR-significant CpG sites across cohorts ordered by age: **A** cg01257194 at the 5'UTR of *ALAD* gene, **B** cg20053493 at the *FNBP4* gene body, **C** cg04942655 intergenic, and **D** cg20702204 at the *CHFR* gene body. Both fixed and random effects are shown, as well as heterogeneity (I^2). Mean age at DNA methylation assessment in the different cohorts was: ALSPAC (7.45 years), Viva (7.78 years), BAMSE.E (8.30 years), BAMSE.M (8.34 years), IOW (10.00 years), HOME (12.31 years), and GLAKU (12.31 years)

Negative outcome control analysis

Six cohorts participated in the negative outcome control analyses focused on cord blood DNAm in relation to breastfeeding (Table 1 and Supplemental Tables S1C-D (Supplementary Material 2)). Four of these cohorts also participated in the meta-EWASs of breastfeeding and childhood blood DNAm, but with different sample sizes. The number of ever breastfed children was 3015 vs. 306 never breastfed. Data on the duration of any and exclusive breastfeeding were available for 3321 and 2152 children, respectively. Two cohorts were from non-European ancestry and all had > 100 participants.

None of the CpG sites identified in the main meta-EWAS of childhood blood DNAm were among the top CpG sites ($p < 0.05$) in cord blood. A summary of the full EWAS results is shown in Supplemental Tables S3 (by cohort: Supplementary Material 4) and Supplemental Tables S4 (meta-EWASs: Supplementary Material 5), volcano plots in Supplemental Figures S1-S5 (Supplementary Material 6), and top CpG sites in Supplemental Tables S5E-F, S6E-F, S7C-D and S9E-F, S10D-E (Supplementary Materials 7, 8, 11 and 12), for any and exclusive breastfeeding, respectively.

In the main model for cord blood, three CpG sites were FDR-significant for any breastfeeding and nine for

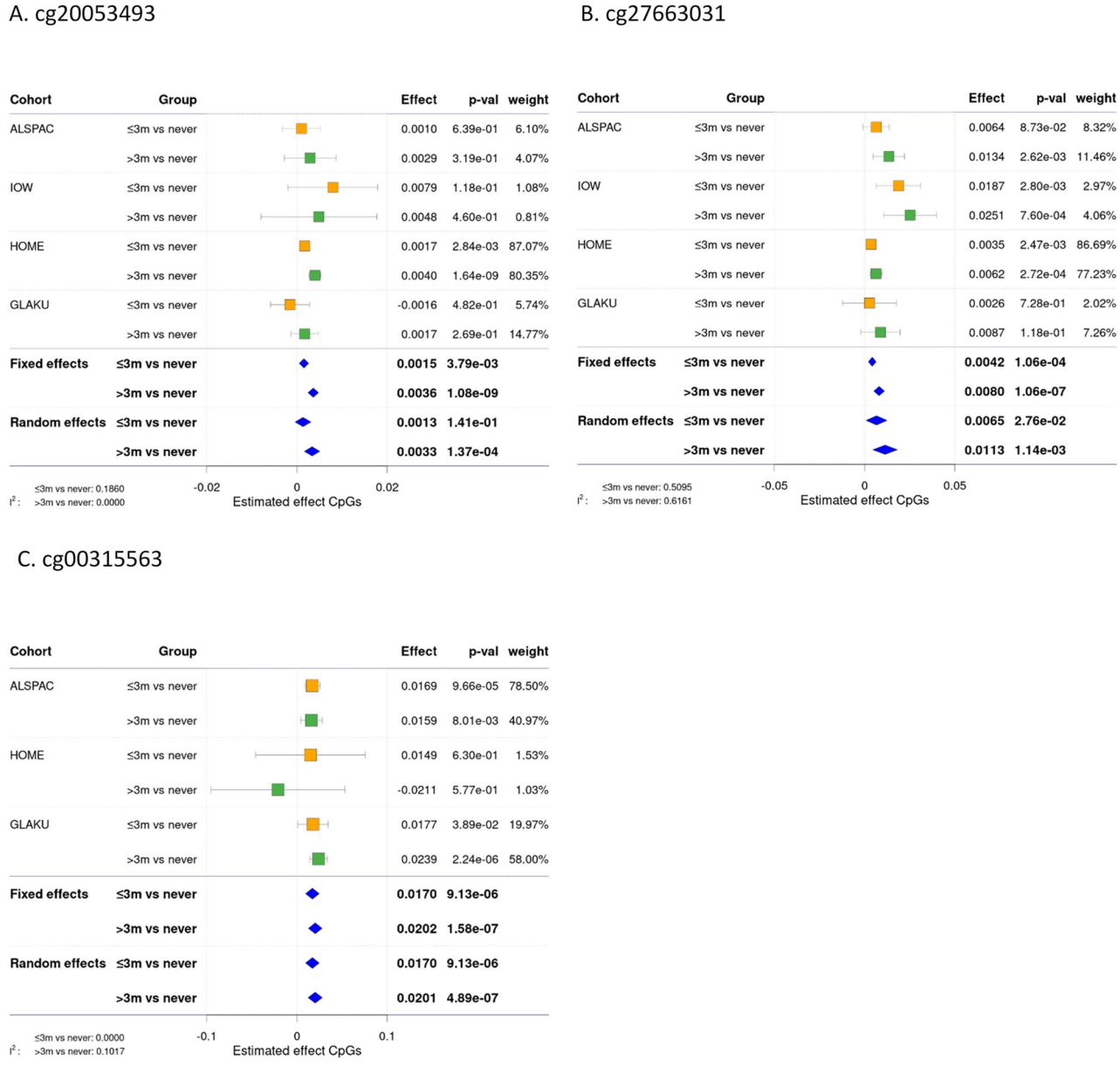

Fig. 2 Forest plots representing the association of exclusive breastfeeding categories (never, ≤3 months, >3 months) with FDR-significant CpG sites across cohorts: **A** cg20053493 at the *FNBP4* gene body, **B** cg27663031 intergenic, and **C** cg00315563 intergenic. Both fixed and random effects are shown, as well as heterogeneity (I^2). Mean age at DNA methylation assessment in the different cohorts was: ALSPAC (7.45 years), IOW (10.00 years), HOME (12.31 years), and GLAKU (12.31 years). IOW did not provide results for cg00315563

exclusive breastfeeding duration. When restricting to European ancestry, three CpG sites were FDR-significant, including one associated with any breastfeeding, one with duration of any breastfeeding, and one with exclusive breastfeeding for >3 months. None of these CpG sites were FDR-significantly associated with breastfeeding in the meta-EWAS of childhood blood (Supplemental Table S11 (Supplementary Material 13)).

Functional characterisation and comparison with previous studies

Three of the six CpG sites identified in the main model were located in the 5'UTR promoter and gene body of known genes (Table 2). None of the six CpG sites are *cis*-eQTMs, whereas three of them had mQTLs. Previous associations of these CpG sites with other traits or exposures included childhood age, alcohol, biological sex, and inflammation markers.

Functional enrichment analyses of suggestively associated CpG sites (p < 1E-05) for exclusive breastfeeding

definitions are presented in Supplemental Table S12 (Supplementary Material 14). Pathways enrichment emerging from these analyses included Ras signalling pathway, smoking, air pollution, and paracetamol exposure, as well as several diseases, including maternal depression and hypertensive disorders. A look up of the literature showed that out of nine CpG sites previously associated with breastfeeding [17, 19, 21, 37], one (cg11414913 – intergenic and previously described in the ALSPAC cohort) [37] was replicated in our study ($p < 0.05$ and same direction of effect) (Supplemental Table S13 (Supplementary Material 15)). We also found that four out of the sixteen CpG sites annotated to the *LEP* gene were inversely associated with any breastfeeding at nominal significance (effect range: -0.001 to -0.006, SE range: 0.0004 to 0.0039, all $p < 0.05$).

Discussion

In this meta-EWASs of breastfeeding practices involving eleven cohorts from the PACE Consortium, we identified six CpG sites at which DNAm was associated with exclusive breastfeeding. Five of them remained significant in at least one of the sensitivity analyses, and none were found in the negative outcome control meta-EWASs (cord blood). Substantial heterogeneity across cohorts was observed and the effects were generally of small magnitude, ranging from 0.36% to 2.02% of average DNAm differences between breastfeeding categories, and from 0.04% to 0.17% for each month of exclusive breastfeeding. Although small effect sizes are challenging to interpret biologically, they have been repeatedly reported in EWAS research, likely reflecting cell-specific effects that may nonetheless have a substantial impact on gene expression [38]. For example, studies in blood have reported differences of approximately 0.5% in DNAm between smokers and non-smokers [39], and of 0.07% per one-point increase in Mediterranean diet score [40].

Longer exclusive breastfeeding was associated with increased DNAm at cg01257194 (at the 5'UTR of the *ALAD* gene), cg04942655 (intergenic), cg20702204 (at the *CHFR* gene body) and cg20053493 (at the *FNBP4* gene body). *ALAD* encodes an enzyme that catalyses the second step in the porphyrin and heme biosynthetic pathway, *CHFR* is known to regulate cell cycle entry into mitosis, and *FNBP4* is involved in the regulation of cytoskeletal dynamics during cell division, migration and vesicle formation. Cg20053493 was also positively related to exclusive breastfeeding for ≥3 months, while two other intergenic CpG sites (cg00315563 and cg27663031) presented higher DNAm in the group of children exclusively breastfed for more than three months.

Of the six identified CpG sites, two are particularly noteworthy: cg01257194, previously related to higher levels of CRP and with alcohol consumption [41, 42], and cg20702204 at the *CHFR* gene body. *CHFR* gene variants have been reported for weight, height, BMI [43], cognitive abilities [44], and behavioural traits [45], suggesting that DNAm at this gene could be involved in developmental effects of breastfeeding. However, further studies need to confirm this and elucidate a potential mechanism, particularly as the association between breastfeeding and higher DNAm at cg01257194 seems to go in the opposite direction to the one expected for the known protective effects of breastfeeding on inflammation [46]. For the remaining CpG sites, it was not possible to estimate associations with gene or protein expression, making it more challenging to hypothesize their functional consequences.

The observation that all FDR-significant CpG sites were previously reported to show differences in DNAm levels with age from birth to adolescence is intriguing and could potentially have a biological explanation [47]. For instance, both breastfeeding and aging are dynamic processes that can influence DNAm patterns through various mechanisms, such as hormonal changes or maturation of the immune system (e.g. through changes in blood cellular composition) [7, 47]. However, the observed overlap could be also attributed to residual confounding or other unaccounted factors in the meta-EWASs, despite adjusting for child age at the moment of blood cell DNAm measurements in all our models.

We conducted functional enrichment analyses for suggestive CpG sites associated with exclusive breastfeeding. The annotation of CpG sites associated with the continuous exclusive breastfeeding variable showed enrichment in genes related to the Ras signalling pathway, which regulates cell growth, differentiation, and survival in response to external signals such as growth factors or cytokines. Enrichment was also observed for smoking, air pollution, and paracetamol exposure, as well as for several maternal conditions such as maternal depression and maternal hypertensive disorders during pregnancy. This could suggest potential residual confounding, but these analyses should be interpreted with caution, as they are based on CpG sites that were identified with $p < 1E\text{-}05$ threshold (classified as 'suggestive' only).

This study has several strengths. First, it is the largest investigation to date, incorporating data from eleven cohorts within the PACE Consortium for the primary meta-EWASs, and thus increasing the statistical power and robustness compared with previous studies. Second, we examined various definitions of both any and exclusive breastfeeding, allowing for a comprehensive investigation. Third, we used data from prospective studies, thereby reducing recall bias on breastfeeding practices. Fourth, we included a negative outcome control study. The aim of the negative outcome control analysis was to filter out potential non-causal associations; however, we

acknowledge that the only partial overlap of participants between the main and negative control analyses constrained this approach. Finally, we included several sensitivity analyses, thereby strengthening the robustness of the results.

The study also has some limitations. First, there were differences in exposure and outcome assessment across cohorts. Questionnaires on breastfeeding were administered at different postnatal visits in each cohort, and they did not account for potential use of donated human milk. Additionally, blood DNAm was measured at different ages across cohorts, including later childhood. Studies assessing blood DNAm closer to the time of breastfeeding initiation might be more likely to detect associations if effects on the epigenome are not sustained. Differences in both exposure definitions and age at sampling across cohorts could have contributed to the relatively higher heterogeneity observed in associations at some of the CpG sites. Second, the sample size of the study for certain categories of breastfeeding in some of the individual cohorts was still relatively small, thus not allowing us to test sub-categories for either any or exclusive breastfeeding. Breastfeeding categories were defined as a compromise between WHO recommendations, maternity leave, and available sample sizes. Third, while the statistical models were adjusted for several confounding variables, the possibility of residual confounding remains (e.g. by diet/food components, maternal lifestyle or socioeconomic factors). Fourth, we measured DNAm in blood cells, for reasons of accessibility. As DNAm is tissue-specific, this may not reflect associations with DNAm in other organs and tissues that may be relevant for breastfeeding-associated health outcomes. In addition, only DNAm was evaluated, while other epigenetic or molecular mechanisms, such as mitochondrial DNA content [48], could also contribute. Finally, although we included several cohorts spanning different continents, our sample was of predominantly European genetic ancestry, which may limit the extent to which our findings generalize to the full spectrum of human genetic and environmental diversity.

Conclusions

This meta-EWAS identified differential DNAm in childhood blood cells in relation to having been exclusively breastfed. Effect sizes observed were of small magnitude, and the results showed some degree of heterogeneity across cohorts. Consequently, the findings should be interpreted with caution. More diverse samples at younger ages, combined with further detailed examination of confounding structures and causal studies, are needed to fully understand potential underlying biological mechanisms.

Abbreviations

ALL	analysis conducted in all participants from all cohorts
ALL100	analysis conducted in all participants from cohorts with a sample size > 100
BMI	body mass index
CpG	cytosine-phosphate-guanine dinucleotide
DNAm	DNA methylation
EUR	analysis conducted in only children of European ancestry from all cohorts
EUR100	analysis conducted in only children of European ancestry from cohorts with a sample size > 100
EWAS	epigenome-wide association study
FDR	false discovery rate
IQR	interquartile range
Meta-EWAS	meta-analysis of epigenome-wide association studies
PC	principal component
SE	standard error
SNP	single nucleotide polymorphism
TSS	transcription start site
WHO	World Health Organization
3'UTR	3' untranslated region
5'UTR	5' untranslated region

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-025-02042-4.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

Supplementary Material 10

Supplementary Material 11

Supplementary Material 12

Supplementary Material 13

Supplementary Material 14

Supplementary Material 15

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Author contributions

BH, DCa, JFF, MB and SFB designed the study. BLG, BK, BH, BN, CLR, DJS, EM, EO, HJZ, IK, JFF, JL, JMB, JW, JWH, KMC, KR, KY, LD, MB, MFH, MK, MKa, MV, NK, RS, SH, SFB, TM, VG, VWVJ, and WK are the PIs of the cohorts or obtained data for the study. AC, AH, AM, AW, BN, DC, DCa, DP, EC, FIR, HL, HRE, JL, MES, OG, RF, RG, RMS, RO, SFB, SKM, SLR, and TM conducted the analyses in the individual cohorts. AC, HL and MB conducted the meta-analyses. DCa, JFF, MB, and SFB interpreted results and wrote the original draft, and all others contributed to the later drafts, and reviewed and approved the final version.

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Data availability

The full genome-wide DNAm meta-analysis summary statistics presented in this manuscript are available at ZENODO (https://doi.org/10.5281/zenodo.15624186). Cohort-level data may be available by direct contact to the authors. Participant-level data are not openly available due to informed consent coverage. Access to these data is managed at each institution's according to their policies.

Declarations

Ethics approval and consent to participate

Research was conducted in accordance with the Declaration of Helsinki. All participating studies received approval from their respective institutional ethics committees, and all participants provided written informed consent.

Consent for publication

Not applicable.

Competing interests

JMB has served as an expert witness on behalf of plaintiffs involved in litigation related to PFAS-contaminated drinking water.

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