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Association of exposure to second-hand smoke during childhood with blood DNA methylation

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ABSTRACT

Introduction: By recent estimates, 40% of children worldwide are exposed to second-hand smoke (SHS), which has been associated with adverse health outcomes. While numerous studies have linked maternal smoking during pregnancy (MSDP) to widespread differences in child blood DNA methylation (DNAm), research specifically examining postnatal SHS exposure remains sparse. To address this gap, we conducted epigenome-wide meta-analyses to identify associations of postnatal SHS and child blood DNAm.

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Methods: Six cohorts from the Pregnancy And Childhood Epigenetics (PACE) Consortium (total N = 2,695), with SHS data and child blood DNAm (aged 7–9 years) measured with the Illumina 450K array were included in the meta-analysis. Linear regression models adjusted for covariates were fitted to examine the association between the number of household smokers in postnatal life (0, 1, 2+) and child blood DNAm. Sensitivity models without adjusting for MSDP and restricted to mothers who did not smoke during pregnancy were evaluated.
Results: Our analysis revealed significant associations (False Discovery Rate < 0.05) between household postnatal SHS exposure and DNAm at 11 CpGs in exposed children. Nine CpGs were mapped to genes (*MYO1G, FAM184B, CTDSPL2, LTBP3*, *PDE10A*, and *FIBCD1*), while 2 CpGs were located in open sea regions. Notably, all except 2 CpGs (mapped to *FIBCD1* and *CTDSPL2*) have previously been linked to either personal smoking habits or *in utero* exposure to smoking. The models restricted to non-smoking mothers provided similar results. Importantly, several of these CpGs and their associated genes are implicated in conditions exacerbated by or directly linked to SHS.
Conclusions: Our findings highlight the potential biological effects of SHS on blood DNAm. These findings support further research on epigenetic factors mediating deleterious effects of SHS on child health and call for public health policies aimed at reducing exposure, particularly in environments where children are present.

1. Introduction

An estimated 40% of children worldwide were exposed to secondhand smoke (SHS) in 2004, one of the main contributors to indoor air pollution (Oberg et al., 2011). More recent surveys in the US give a similar prevalence of SHS exposure for children aged 3 to 11 years (Tsai, 2018; Tsai, 2021) and report that 25.3% of middle and high school students self-reported SHS exposure in the home (Walton, 2020). Although national smoking bans have reduced SHS exposure in the workplace and public places in recent years, there is little evidence to show a change in the prevalence or duration of reported exposure to SHS in the home (Callinan et al., 2010). SHS exposure has been associated with a number of health outcomes (Office on Smoking and Health (US), 2006), including elevated risk of cardiovascular disease and lung cancer, as well as lower lung function, respiratory conditions, and neurodevelopmental disorders in children and young people (Office on Smoking and Health (US), 2006; Lin, 2021; Fernández-Plata et al., 2016; Pavić, 2014; Hagstad, 2014; Merghani and Saeed, 2013; U.S. Department of Health and Human Services, 2014; Flor, 2024). This indicates the need to identify biomarkers that may predict future disease risk and better understand the biologic impacts of this common exposure.

Epigenetic mechanisms (including DNA methylation (DNAm), histone modifications, and regulatory RNAs) have been proposed as mediators of the effects of environmental exposures on disease outcomes (Cavalli and Heard, 2019). These mechanisms can regulate gene expression without affecting the actual DNA sequence, are sensitive to environmental factors, and partly controlled by genetics. DNAm is the most studied epigenetic mechanism in epidemiological settings.

DNAm has been extensively studied within the context of maternal smoking during pregnancy (MSDP), revealing substantial alterations predominantly observed in blood or placental tissues, as extensively reviewed by Cosin-Tomas et al. (Cosin-Tomas et al., 2022). Notably, a landmark meta-analysis conducted by the Pregnancy And Childhood Epigenetics (PACE) Consortium identified 6,073 CpGs differentially methylated in association with sustained MSDP (Joubert et al., 2016). Moreover, 73% of these CpGs were shown to have persistent effects at least until childhood (Joubert et al., 2016).

However, our understanding of maternal pregnancy exposures, such as smoking, is hindered by the lack of parallel investigations into other crucial exposures, including paternal influences and early postnatal exposures to offspring. This underscores the urgency to assess whether the impact of postnatal SHS exposure on offspring equals or exceeds that of prenatal exposure, potentially offering additional effective intervention targets beyond maternal pregnancy exposures (Sharp et al., 2019), and shedding light on critical periods of exposure.

Despite this need, research specifically focusing on SHS exposure remains limited, especially during the early years of life. Previous studies in this area have typically examined DNAm at a restricted number of cytosine-phosphate-guanine (CpG) sites, often with small sample sizes and/or without replication studies, and have predominantly involved adult populations (Hulls, 2020; Reynolds, 2017; Wilhelm-Benartzi, 2011; Hoang, 2024), with only one study conducted in children (Vives-Usano, 2020). For example, in adults, Reynolds et al., reported that indoor SHS exposure was inversely associated with DNAm of the *AHRR* gene in monocytes (n = 906) (Reynolds, 2017), whereas Hulls et al. performed an epigenome-wide association study (EWAS) of exposure to SHS with DNAm in peripheral blood and did not find any epigenome-wide significant association (n = 769) (Hulls, 2020). More recently, Hoang et al. conducted a meta-analysis including participants from 4 different cohorts and found that blood DNAm at 6 CpGs (2 mapping at *EDNRB* and *LPP* genes) was inversely associated with SHS in adults (n = 2,884) (Hoang, 2024). Finally, Wilhelm-Benartzi et al., found some evidence for an association between adulthood exposure to SHS and changes in DNAm of several CpG loci in bladder tumors (n = 43) (Wilhelm-Benartzi, 2011). In children, Vives-Usano investigated the associations of MSDP and childhood SHS exposure with molecular features measured in 1,203 European children (6–9 years old) from the Human Early Life Exposome (HELIX) project. Authors found significant associations with some serum and plasma metabolites, but did not find any with blood DNAm (Vives-Usano, 2020).

To address this gap comprehensively, we analysed the association between postnatal SHS exposure and genome-wide child blood DNAm using data from 2,695 children within 6 European cohort studies from the PACE Consortium. Additionally, we ran a secondary meta-analysis on the association of DNAm with MSDP as a positive control, and run two sensitivity meta-analyses (restricted to non-smoking mothers during pregnancy, and without adjusting by MSDP) to confirm the robustness of our findings.

2. Materials and methods

2.1. Participants

A total of 6 PACE cohorts (n = 2,695) participated in the meta-analyses. These cohorts, listed in alphabetical order, are the Avon Longitudinal Study of Parents and Children (ALSPAC) (Fraser, 2013), the Children's Allergy Environment Stockholm Epidemiology study (BAMSE_MeDALL and BAMSE_EpiGene) (Wickman et al., 2002), the Generation R Study (Kooijman, 2016), the Human Early Life Exposome (HELIX) (Maitre, 2018), and The Prevention and Incidence of Asthma and Mite Allergy (PIAMA) (Wijga, 2014). BAMSE comprises two different DNAm datasets from two different projects at the same age: Mechanisms of the Development of Allergy (MeDALL) and EpiGene, while HELIX comprises one DNAm dataset including data from 6 different cohorts: Born in Bradford (BIB) (United Kingdom), EDEN (France), Kaunas birth Cohort (KANC) (Lithuania), Infancia y Medio Ambiente (INMA) (Spain), The Norwegian Mother, Father and Child Cohort Study (MOBA) (Norway), and The Mother-Child Cohort in Crete (RHEA) (Greece). Ethical

approval for study protocols was obtained for all participating cohorts. Further information on this as well as the study methods for each cohort are described in detail in the Supplementary Methods. Cohorts excluded all multiple births and chose one random sibling per non-twin sibling pair. The analyses were restricted to participants of European ancestry.

2.2. Measures

2.2.1. DNA methylation measurements and quality control

Each cohort independently conducted laboratory measurements and quality control. Peripheral whole blood samples collected at ages 7–10 years underwent bisulfite conversion and were processed with the Illumina Infinium HumanMethylation450 (450K) BeadChip (Illumina) at cohort-specific laboratories. The cohorts performed sample processing, quality control (QC) and normalization based on their preferred protocols as described in the Supplementary Methods. We used normalized, untransformed beta values, ranging from 0 (fully unmethylated) to 1 (fully methylated). Methylation levels that fell outside of the range of the lower quartile minus 3 × interquartile to the upper quartile plus 3 × interquartile range were removed.

2.2.2. Postnatal second-hand smoke exposure

To assess postnatal SHS exposure to the child, we relied on smoking behavior reported by family members via questionnaires, presuming that they smoke in close enough proximity to the child to lead to actual exposure. We used the SHS exposure assessment conducted closest to the child's age at DNAm measurements. Household postnatal smoking was then treated as continuous variable coded as 0: No one smokes in the household, 1: Just one smoker living in the household, 2: More than one smoker living in the household. More details on the cohort-specific smoking variables are in the Supplementary Methods.

2.2.3. Maternal smoking during pregnancy

Maternal smoking during pregnancy (MSDP) was self-reported by the mothers during pregnancy and coded as a two-level category variable (0: No smoking during pregnancy; 1: Any smoking during pregnancy)). If a mother started smoking during the 2nd or 3rd trimester with no report of having smoked earlier in the pregnancy, she was removed from the analysis.

2.2.4. Covariates

The following covariates were used in the study: child sex, child age at DNAm measurement, maternal educational level (two categories (higher vs lower), or three categories (lower, medium, higher), depending on the cohort), blood cell type proportions (Natural killers, B cells, CD4T and CD8T lymphocytes, Monocytes, Granulocytes) estimated using standard algorithms for DNAm at childhood (Houseman, 2012; Reinius, 2012), technical covariates (optional, e.g., batch), selection factor (optional, e.g., if the study population was oversampled on a condition), and MSDP (0: No smoking during pregnancy; 1: Any smoking during pregnancy) (see Supplementary Methods for cohort specific details).

2.3. Statistical analyses

2.3.1. Descriptive statistics and correlations

Descriptive statistics of covariates and exposure were the number per category, percentage, or mean and standard deviation (SD). Polychoric correlation was used to assess the correlation between household postnatal SHS and MSDP.

2.3.2. Cohort-specific epigenome-wide association study

Each cohort ran the EWAS according to a predefined analysis plan, using linear regression models adjusting for covariates. The main model to investigate the effects of postnatal SHS and MSDP on child blood DNAm was:

(1) Child blood DNAm = postnatal SHS + MSDP + child sex + child age at DNAm measurement + maternal educational level + blood cell type proportions + technical covariates (optional) + selection factor (optional).

Additionally, two sensitivity models were examined in two of the largest cohorts (ALSPAC and HELIX). First, to investigate to which extent the association results were driven by those mothers who smoked during pregnancy, a model including only those mothers who did not smoke during pregnancy was evaluated:

(2) For postnatal SHS (only children of mothers who did not smoke in pregnancy):

Child blood DNAm = postnatal SHS + child sex + child age at DNAm measurement + maternal educational level + blood cell type proportions + technical covariates (optional) + selection factor (optional)

Second, another model without adjusting for by MSDP was tested:

(3) For postnatal SHS (without adjusting for MSDP, all children):

Child blood DNAm = postnatal SHS + child sex + child age at DNAm measurement + maternal educational level + blood cell type proportions + technical covariates (optional) + selection factor (optional)

2.3.3. Quality control and meta-analysis

We used the Quality Control module from the EASIER R package to perform the quality control of individual EWAS results, and annotate CpGs (according to hg19) (github). The quality control included the following steps: (1) identify and exclude CpGs with NA values, and those CpGs with call rate < 90%; (2) identify duplicated CpGs; (3) exclude CpGs in sex chromosomes, according to Zhou et al. (Zhou et al., 2017) (probes with poor base pairing quality (lower than 40 on 0–60 scale), probes with non-unique 30 bp 3′-subsequence (with cross-hybridizing problems), Infinium II probes with single nucleotide polymorphisms (SNPs) of global minor allele frequency (MAF) > 1% affecting the extension base, and probes with a SNP in the extension base that causes a color channel switch from the official annotation), and to Solomon et al. (probes that have shown to be unreliable in a recent comparison of the Illumina 450 K and EPIC BeadChips, Solomon et al., 2018); (4) visually inspect volcano plots for beta and standard errors (SEs); (5) distribution plots for p-values and betas; (6) box plots for betas and SEs; (7) precision plots showing 1/median SE vs sqrt (N); (8) calculate lambda values per study; (9) summarise SE, beta, lambda, and p-value; (10) save the cleaned files with harmonized headings before running *meta*-analysis.

Cohort-specific results were combined through inverse-variance weighted fixed effects *meta*-analysis, at ISGlobal (Barcelona) using the EASIER R package, which involves GWAMA for running the *meta*-analyses (github), and at the NIEHS (Research Triangle Park, North-Carolina) using the METAL tool (Willer et al., 2010). Results were compared and found to be identical.

Two main *meta*-EWAS were run as indicated above: (1) *meta*-analysis of EWAS of postnatal SHS in all children from all cohorts; and (2) *meta*-analyses of EWAS of MSDP in all children from all cohorts. Moreover, four sensitivity *meta*-analyses of EWAS were run in ALPSAC and HELIX as the two largest studies: (3) *meta*-analysis of EWAS of postnatal SHS in non-smoking mothers during pregnancy; (4) *meta*-analysis of EWAS of postnatal SHS in all children without adjusting for MSDP; (5) *meta*-analysis of EWAS of postnatal SHS in all children, as in the main analysis, but now including only data from these two cohorts; and (6) *meta*-analysis of EWAS of MSDP in all children, as in the main analysis, but now including only data from these two cohorts. These two last *meta*-analysis of EWASs were run to be able to compare the results of the sensitivity analyses with the same sample.

Genome-wide significance was defined using the False Discovery Rate (FDR) < 0.05 using the Benjamini & Hochberg method (Benjamini and Hochberg, 1995), Bonferroni at 1.21×10^{-7} ($p = 0.05/414{,}154$ CpGs),

and suggestive significance was established at $p < 1 \times 10^{-4}$ (a nominal p-value threshold of 10^{-4} was chosen to increase the number of CpGs and annotated genes available and displayed for the reader in the Supplementary Tables, but these set of CpGs was not used for any follow-up analysis). Effect sizes are expressed as the difference in DNAm (from 0 to 1) for each additional smoking person in the household (from 0 to 1, and from 1 to 2) for postnatal SHS, or the difference in DNAm (from No to Yes) for MSDP.

We performed leave-one-out *meta*-analyses for CpGs significantly associated (FDR < 0.05) with household postnatal SHS in the main model, to ensure that associations were consistent across cohorts. I^2 was estimated using (Q − df)/Q where Q is the Cochran's heterogeneity statistic.

QQ-plots, volcano plots, Manhattan plots and forest plots were created through the Meta-analysis module of the EASIER R package (github). Pearson's correlation and GGally R package were used to compare the resulting estimates between models. The difference in the effect size among main models and sensitivity models was calculated as (effect size main model − effect size alternative model)/effect size main model × 100. Venn diagrams to visualise the overlap between CpGs identified in the different models and between studies were conducted using the webtool https://bioinformatics.psb.ugent.be/webtools/Venn/. All the analyses were conducted with the R environment version R/4.1.2.

2.4. Follow-up analyses

FDR-significant and suggestive CpGs were annotated with the "IlluminaHumanMethylation450kanno.ilmn12.hg19″ Illumina R package, that annotates CpGs to proximal promoter (200 bp upstream the TSS − TSS200), distant promoter (from 200 to 1500 bp upstream the TSS − TSS1500), 5′UTR, first exon, gene body, and 3′UTR regions. CpGs further than 1,500 bp from the TSS were not annotated to any gene. Relative position to CpG islands (island, shelve, shore, and open sea) was also provided by the same R package. The UCSC Genome Browser (hg19) was used to further explore the genomic context of the identified CpGs.

To characterize potential genetic influences on these sites, we used the GoDMC database (https://mqtldb.godmc.org.uk/) for identifying methylation quantitative trait loci (mQTLs) (Min, 2021). GoDMC is a large-scale collaborative effort including 36 cohorts (5 of which participated in this study: BAMSE, ALSPAC, GENR, PIAMA, and part of HELIX (INMA and BiB)), based on whole blood from over 27,000 European samples.

To assess whether methylation levels of CpGs were associated with the expression levels of nearby genes in child blood, we consulted the HELIX Expression Quantitative Trait Methylation (eQTM) catalogue (https://helixomics.isglobal.org/), generated from samples overlapping with those included in this study (from the HELIX cohort).

Finally, FDR-significant CpGs were also looked up in the EWAS catalog (Battram, 2022) and EWAS atlas (Li, 2019) to examine potential associations with exposures and health outcomes based on existing literature.

3. Results

3.1. Study characteristics

The descriptive statistics across the six participating studies (ALSPAC, BAMSE_EpiGene, BAMSE_MeDALL, GenR, HELIX, and PIAMA) are shown in Table 1. Among a total of 2,695 children (47.8% females), 756 were exposed to postnatal SHS (28.1%) (544 with one smoker living in the household, 212 with more than one smoker living in the household), whereas 1,939 were classified as unexposed. Regarding MSDP, 628 children were exposed (23.3%) while 2,067 were not. Between 6.3 and 16.5% of the children exposed to postSHS were also exposed to MSDP depending on the cohort, and the correlation between MSDP and postnatal SHS exposure ranged from 0.56 (ALSPAC) to 0.76 (BAMSE_EpiGene) (Table S1). The age of the child at which DNAm was assessed ranged from 7.42 (ALSPAC) to 9.77 (GenR), and most (81–100%) of the participant mothers had a medium to high education level. All participants were of European ancestry. For more details on cohort characteristics and methodology please see Supplementary Methods.

3.2. Epigenome-wide meta-analyses of postnatal second-hand smoke

The main model examined the association between household postnatal exposure to SHS, continuous variable ranging 0 to 2, and genome-wide DNAm in child blood. To control for the known effect of *in utero* exposure of maternal smoking on child blood DNAm, we included MSDP as a covariate. The meta-analysis of this model had an inflation factor (lambda) of 0.99 (Figure S1), with inflation factors ranging from 0.91 to 1.09 for the cohort-specific results (Table S2). After correcting for FDR < 0.05, 11 CpGs were identified, 6 of which also met Bonferroni

Table 1
Descriptive statistics of study population (N = 2,695).

					Maternal education			*Household postnatal second-hand smoke (SHS)*			*Maternal smoking during pregnancy (MSDP)*		*Children exposed to both MSDP and household postnatal SHS (%)*
Cohort	***N***	***Ancestry***	***% Females***	***Child age at DNAm (SD)***	***High (%)***	***Medium (%)***	***Low (%)***	***None (%)***	***One person (%)***	***Two or more people (%)***	***No smoking (%)***	***Any smoking (%)***	
ALSPAC	772	European (UK)	49.6	7.4 (0.1)	172 (22.3)	505 (65.4)	95 (12.31)	592 (76.8)	126 (16.3)	54 (7.0)	479 (62.1)	293 (38)	117 (15.2%)
BAMSE_EpiGene	354	European (Sweden)	46.3	8.3 (0.5)	132 (37.3)	192 (54.2)	30 (8.47)	293 (82.8)	46 (13)	15 (4.2)	316 (89.3)	38 (10.7)	26 (7.3%)
BAMSE_MeDALL	260	European (Sweden)	46.5	8.3 (0.4)	105 (40.4)	133 (51.2)	22 (8.46)	214 (82.3)	41 (15.8)	5 (1.9)	229 (88.1)	31 (11.9)	18 (6.9%)
GenR	353	European (the Netherlands)	49	9.8 (0.3)	243 (68.8)	110 (31.2)	–	269 (76.2)	64 (18.1)	20 (5.7)	281 (79.6)	72 (20.4)	39 (11%)
HELIX	751	European (France, Greece, Lithuania, Norway, Spain and UK)	45.8	7.9 (1.7)	347 (46.2)	404 (53.8) (medium + low)	–	392 (52.2)	247 (32.9)	112 (14.9)	611 (81,4)	140 (18.6)	124 (16.5%)
PIAMA	205	European (the Netherlands)	50.2	8.1 (0.3)	74 (36.1)	92 (44.9)	39 (19.02)	179 (87.3)	20 (9.8)	6 (2.9)	177 (86.3)	28 (13.7)	13 (6.3%)

significance (Fig. 1, Table 2). Among the 11 FDR-significant CpGs, household postnatal exposure to SHS was associated with increased DNAm at 10 of them, and with decreased DNAm at 1 annotated to the *Phosphodiesterase 10A* (*PDE10A*) gene. Forest plots corresponding to the FDR-significant CpGs can be found in Figure S2. A total of 109 CpGs were found to be associated at a significance level of $p < 1x10^{-4}$, and these are summarized in Table S3.

We then performed leave-one-out meta-analyses for these 11 FDR-significant CpGs to check the consistency of results across cohorts (Table S4). The coefficients from the leave-one-out meta-analyses of 8 CpGs (with I^2 ranging from 0.0 to 0.7) fell within the coefficients of the main meta-analysis ± their SE (Figure S3). Regarding cg04180046 ($I^2 = 0.7$) and cg11166287 ($I^2 = 0.2$), the meta-analysis coefficient increased when removing the HELIX cohort, whereas for cg27649764 ($I^2 = 0.7$) the coefficient increased when removing HELIX and slightly decreases when removing the ALSPAC cohort from the meta-analysis. Overall, the leave-one-out analyses indicated consistent results across cohorts for most of the CpGs.

3.3. Epigenome-wide meta-analyses of any maternal smoking during pregnancy

The results of the meta-analysis of MSDP adjusted for postnatal SHS presented an inflation factor (lambda) of 1.00 (from 0.90 to 1.09 for the cohort specific results) (Figure S1, Table S2), and revealed 19 CpGs reaching FDR significance, 12 of which met the Bonferroni significance (Fig. 1, Table S5). Among the 19 FDR-significant CpGs, MSDP was associated with increased methylation in 13 of them, and with decreased methylation in 6. All 19 CpGs have been reported before as FDR-significant and following the same direction of effect in a meta-analysis of the effects of MSDP on cord and child blood DNAm (except for cg13399816, which was significant only in cord blood) (Joubert et al., 2016), supporting the reliability of our results. Within these 19 FDR-significant CpGs, 4 were annotated to the gene body and 3′UTR of *MYO1G* on chromosome 7. These 4 CpGs were also identified in the household postnatal exposure to SHS meta-analysis. In fact, among the 11 CpGs (8 loci) identified in the EWAS of postnatal SHS, 4 of them (1 locus) were also found associated with MSDP in our study with the same direction of effect. A total of 64 CpGs were found to be associated with MSDP at a significance level of $p < 1x10^{-4}$ (Table S5).

3.4. Sensitivity meta-analyses

To investigate to which extent the association results were driven by those mothers who smoked during pregnancy, we ran the models again in the two largest cohorts (HELIX and ALSPAC), now including only those mothers who did not smoke during pregnancy. This model includes a sample size of 1090 participants, 247 exposed to postnatal SHS with one smoker living in the house, 51 exposed to postnatal SHS with more than one smoker living in the house, and 792 unexposed (Table S6). Summary statistics of this sensitivity model and for the main models for postnatal SHS and MSDP run in ALSPAC and HELIX only, for direct comparison, are shown in Tables S7 and S8, respectively. There was no evidence of genomic inflation in the meta-analyses results from these two cohorts (postnatal SHS in non-smokers: λ=0.96, postnatal SHS: λ=0.99, MSDP: λ=1.04).

Correlation of effect sizes of suggestive CpGs ($p < 1x10^{-4}$) between this sensitivity model and the main model in the two cohorts was very high (correlation coefficient = 0.988) (Figure S4). Six out of the 11 FDR CpGs identified in the main model of postnatal SHS in the six cohorts, were also FDR-significant in the meta-EWAS of ALSPAC and HELIX. For all 11 CpGs, the direction and magnitude of effect was rather consistent between the main model and the model in non-smoking mothers during pregnancy: % effect difference ranged from 0.6% (cg22132788) to 36.0% (cg11549143) (Table S9). These results suggest that the main findings for SHS are not driven by the subset of mothers that smoked during pregnancy (Figure S5).

We also ran a meta-analysis of postnatal SHS without adjusting for MSDP in ALSPAC and HELIX the two largest cohorts in the study, including: 1523 participants, 373 exposed to postnatal SHS with one smoker living in the house, 166 exposed to postnatal SHS with more than one smoker living in the house, and 984 non-exposed (Table S6). Lambda was 1.03, suggesting no inflation issues. When comparing results with the meta-analysis of the main model adjusted by MSDP (in the same participants, only HELIX and ALSPAC cohorts), we observed that there were more FDR-significant CpGs associated with postnatal SHS in the unadjusted than in the adjusted model (24 vs 11) (Figure S5). The % difference of the effect size of the 11 CpGs between models (main and unadjusted for MSDP) ranged from 1.02% (cg2041852) to 54.12% (cg04180046) (Table S9). In 7 of the 11 CpGs, the effect was stronger in the unadjusted model compared with the adjusted model, suggesting an effect of MSDP on these CpGs. Summary statistics of main model run in

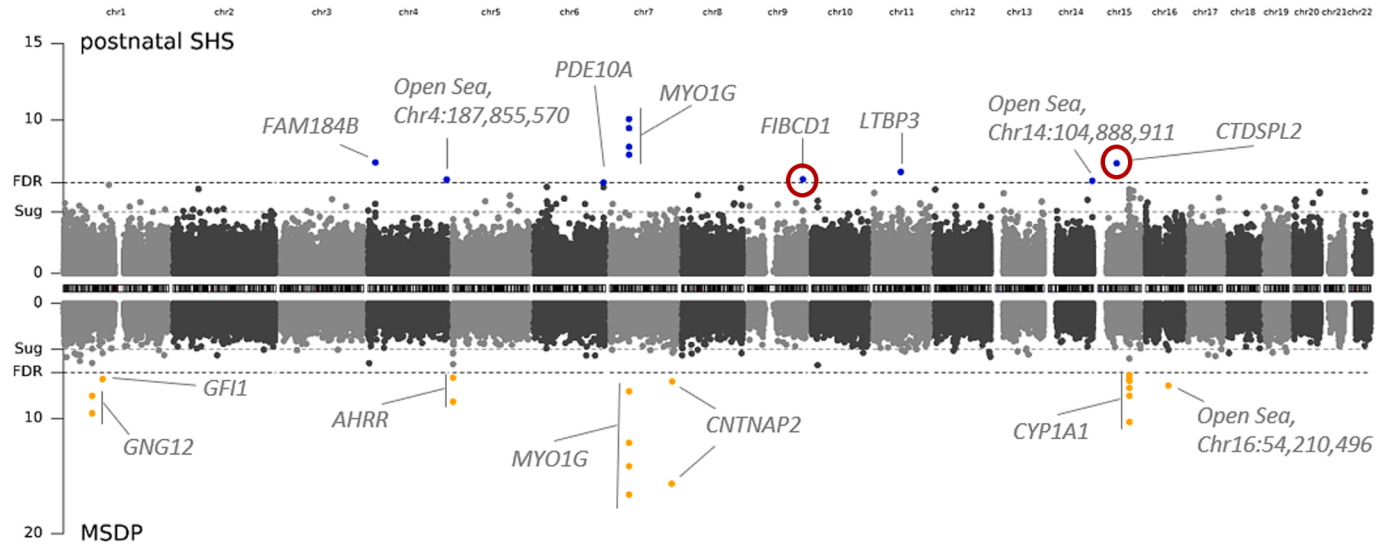

Fig. 1. Miami plot of the association between household postnatal exposure to second-hand smoke (SHS) and DNA methylation (DNAm) in child blood (top), and of the association between maternal smoking during pregnancy (MSDP) and DNAm in child blood (bottom). A total of 11 and 19 CpGs were considered statistically significant at FDR < 0.05 (blue and orange points), respectively. The light gray dashed line indicates the suggestive significance threshold, and the dark gray dashed line indicates the FDR significance threshold. CpGs that have not been previously associated with smoking-related exposures are highlighted in red.

Table 2
Meta-analysis results for False Discovery Rate-significant CpGs associated with household postnatal exposure to second-hand smoke (SHS). Cohorts are indicated in this order: GenR, PIAMA, HELIX, BAMSE_MeDALL, BAMSE_EpiGene, and ALSPAC. Effect size represents the difference in DNA methylation (DNAm) by number of household smokers (0, 1 or >=2 smokers). Annotated gene and CpG island relative position are indicated (TSS1500 covers from − 200 to − 1500 nt upstream of transcription start site (TSS); S_Shore corresponds to the 2 kb regions immediately downstream of the CpG island; N_Shore corresponds to the 2 kb regions immediately upstream of the CpG island). Chr stands for Chromosome, BN stands for Bonferroni, SE stands for standard error, I^2 stands for heterogeneity index, and FDR stands for False Discovery Rate.

CpG Id	Chr	Position	Coefficient	SE	P-value	I^2	N	N samples	Effects	BN	FDR	Relation to CpG island	UCSC RefGene Name	UCSC RefGene Group
cg04180046	7	45,002,736	0.012	0.002	8.58E-11	0.7	6	2695	++++-+	yes	3.6E-05	Island	*MYO1G*	Body
cg12803068	7	45,002,919	0.018	0.003	3.40E-10	0.5	6	2695	++++-+	yes	7.0E-05	S_Shore	*MYO1G*	Body
cg22132788	7	45,002,486	0.012	0.002	5.51E-09	0.5	6	2695	++++-+	yes	7.6E-04	Island	*MYO1G*	Body
cg19089201	7	45,002,287	0.011	0.002	1.79E-08	0.6	6	2695	++++-+	yes	1.9E-03	Island	*MYO1G*	3′UTR
cg16449012	4	17,781,880	0.007	0.001	5.80E-08	0.2	6	2695	++++++	yes	4.5E-03	N_Shore	*FAM184B*	Body
cg27649764	15	44,719,030	0.001	<0.001	6.50E-08	0.7	6	2695	+-+-++	yes	4.5E-03	N_Shore	*CTDSPL2*	TSS1500
cg20588859	11	65,321,429	0.012	0.002	2.39E-07	0.3	6	2695	++++++	no	1.4E-02	Island	*LTBP3*	Body
cg11549143	9	133,805,361	0.006	0.001	7.22E-07	0.5	6	2695	++++++	no	3.5E-02	Island	*FIBCD1*	Body
cg10150824	4	187,855,570	0.009	0.002	7.59E-07	0.0	6	2695	++++++	no	3.5E-02	OpenSea	–	–
cg11166287	14	104,888,911	0.007	0.001	9.03E-07	0.2	6	2695	++++++	no	3.7E-02	OpenSea	–	–
cg20418529	6	166,260,012	−0.003	0.001	1.18E-06	0.3	6	2695	−+——	no	4.4E-02	OpenSea	*PDE10A*	Body

ALSPAC and HELIX cohorts are shown in Table S8, summary statistics of the sensitivity model unadjusted by MSDP are shown in Table S10. Notably, 20 out of the 24 FDR-significant CpGs of the postnatal SHS model unadjusted for MSDP had been reported previously by Joubert et al. as significantly associated with MSDP. This confirms that some of these findings are associated to MSDP in the adjusted model and highlights the importance of adjusting for MSDP to avoid biasing the main results.

3.5. Look up of postnatal second-hand smoke CpGs in previous smoking studies

We looked up whether the 11 FDR-significant CpGs associated with postnatal SHS in child blood in our study had been described before as significant and with the same direction of effect in EWAS of personal smoking in adults (Hoang, 2024; Joehanes, 2016; Sikdar, 2019) (named as Joehanes et al., Hoang et al., and Sikdar et al.), MSDP (Joubert et al., 2016) (Joubert et al.), or with adult SHS (Hulls, 2020; Hoang, 2024) (Hulls et al., and Hoang et al.). Nine of these 11 FDR-significant CpGs had been reported in at least one of these previous studies, with the same direction of effect. The 4 CpGs annotated to *MYO1G* (cg12803068, cg22132788, cg19089201, cg04180046) were significantly associated with personal smoking (Hoang, 2024; Joehanes, 2016; Sikdar, 2019) and with MSDP in cord and child blood (Joubert et al., 2016; Joehanes, 2016). Three additional CpGs (cg20418529 annotated to *PDE10A*, cg20588859 annotated to *LTBP3*, cg16449012 annotated to *FAM184B*) were also reported to be associated with MSDP in cord blood, and in the case of cg16449012 also in child blood (Joubert et al., 2016); and this latter CpG, cg16449012, and two other CpGs (cg10150824 and cg11166287, annotated to intergenic regions) were previously reported to be associated with personal smoking (Hoang, 2024; Joehanes, 2016; Sikdar, 2019). We examined the association statistics for the other two CpGs (cg11549143, annotated to *FIBCD1*, and cg27649764, annotated to *CTDSPL2*) in the comprehensive results from published meta-analyses on personal smoking (Hoang, 2024; Joehanes, 2016; Sikdar, 2019) and MSDP (Joubert et al., 2016). Although these CpGs followed the same direction of effect, their nominal p-values were far from the threshold of 0.05.

Notably, none of the 32 CpGs reported by Hulls et al. (Hulls, 2020) nor the 6 CpGs reported by Hoang et al. to be associated with adult exposure to SHS overlap with the FDR-significant or suggestive CpGs associated with childhood exposure to SHS identified in this meta-analysis (Upset plot in Figure S6). Of the 32 suggestive CpGs reported by Hulls et al., 17 are not included in the 450K array (only in the EPIC array), whereas other 3 did not pass our quality control. Of the remaining 14 CpGs, only one presented a nominal p-value of 0.03 and same direction of effect (cg14597637) in our results. Of the 6 significant CpGs reported by Hoang et al., 2 of them were not included in the 450K array. Of the remaining 4, only one (cg01678383) had a nominal p-value of 0.03 and same direction of effect in our results. Of the 11 FDR-significant CpGs identified in the current study, none of them showed a nominal p-value < 0.05 in Hoang et al. results for SHS in adults, with a mixture of similar and opposite effects to our study.

3.6. Potential functional impact of CpGs associated with postnatal second-hand smoke

To facilitate the biological interpretation of the main findings, we used the EWAS Catalog and EWAS Atlas to look up with which traits and exposures the 11 postnatal SHS CpGs have been previously associated (Table S11). As indicated before, nine out of the 11 CpGs have been previously reported with exposure to smoking and/or MSDP. DNAm at 3 CpGs was previously associated with cannabis and/or air pollution exposure in whole blood or lung cells with consistent direction of the effect in the case of cannabis. Moreover, DNAm at 6 CpGs was previously associated with conditions for which smoking constitutes a risk factor such as asthma, cancer, autoimmune diseases or behavioural traits (in whole blood or in specific tissues such as tumour cells), following the same direction of effect for most of the traits/diseases. Six CpGs have also been previously associated with chronological age, and 8 CpGs with sex. Furthermore, 4 CpGs have been previously associated with exposure to perinatal polychlorinated biphenyls and polychlorinated

dibenzofurans, 1 CpG with paracetamol exposure during pregnancy, and 4 CpGs with Down Syndrome and/or educational attainment, among others (Table S11).

Of the top 11 FDR-significant CpGs, all except for cg11549143, are partly regulated by mQTLs according to GoDMC database, while 2 of the CpGs annotated to *MYO1G* (cg12803068 and cg19089201) have been associated with expression of this gene in blood in children (Table S11).

4. Discussion

Here we conducted a large-scale epigenome-wide meta-analysis of postnatal SHS and child blood DNAm, including 2,695 children from six European cohorts of the PACE Consortium to identify associations. Our results showed that methylation at 11 CpGs was significantly associated with household postnatal SHS in childhood after adjusting for the *in utero* exposure. Nine of these 11 CpGs have been already associated with MSDP or personal smoking, while the other 2 (cg11549143, annotated to *FIBCD1*, and cg27649764 annotated to *CTDSPL2*) appear to be uniquely associated with exposure to SHS in childhood.

Four CpGs were annotated to the *Myosin immunoglobulin* gene (*MYO1G)* body and 3′UTR on chromosome 7. Association of smoking (former smoking, MSDP, and personal current adult smoking) with DNAm at *MYO1G* have been consistently reported (Joubert et al., 2016; Joehanes, 2016; Bakulski et al., 2019; Richmond et al., 2018; Rzehak, 2016; Wiklund, 2019). This type I unconventional myosin is specifically expressed in the plasma membrane of B and T lymphocytes and mast cells, regulating leukocyte adhesion, migration (to lymph nodes) and phagocytosis, which is required during immune response (Cruz-Zárate, 2021). Previous authors have suggested that this gene might be implicated in transmission of smoking effects on the cardiovascular system (Rzehak, 2016). Küpers et al, found that the effect of MSDP on birthweight of the offspring was mediated by smoking-induced changes to DNAm at 3 of these 4 *MYO1G* CpGs (cg22132788, cg12803068, cg19089201) in cord blood (Küpers, 2015). In fact, an increase in methylation at 2 of these 4 CpGs (cg04180046 and cg12803068), which are the two most significant CpGs in our study, is reported to be the most sensitive to early smoking exposures, while also highly associated with lung cancer (Bakulski et al., 2019). In line with this, an increase in DNAm in the other two significant CpGs annotated to *MYO1G* have also been associated to lung, prostate and renal carcinoma (Mb, 2013; Aref-Eshghi, 2018; Hillary, 2023). Finally, methylation changes in these 4 *MYO1G* CpGs have also been associated with systemic lupus erythematosus (Weeding, 2018), aggressive behaviour (van Dongen, 2021), educational attainment (Karlsson Linnér, 2017), chronic obstructive pulmonary disease (Hillary, 2023), ischemic heart disease an stroke (Hillary, 2023); chronic pain (Hillary, 2023); or psoriasis (Chandra et al., 2018). The fact that 2 of these CpGs have been previously characterized as eQTMs regulating *MYO1G* gene expression, further supports the functional implications of these DNAm differences. However, whether *MYO1G* DNAm in these tissues is simply a biomarker of smoke exposure or has a functional role in cancer or in any of these traits mentioned above remains unknown.

In addition to those identified within the *MYO1G* gene, our study revealed significant associations between postnatal exposure to SHS and methylation levels at five other CpGs that have also been previously associated with active smoking and/or prenatal smoke exposure (Joubert et al., 2016; Joehanes, 2016): cg16449012, annotated to the *Family with sequence similarity 184 member B* gene *(FAM184B)*, with functions that remain unclear; cg20588859, annotated to *Latent TGF-β binding protein3* gene (*LTBP3),* involved in regulation of cell differentiation and proliferation, and in cardiac maintenance (Nikpay, 2023); cg20418529, annotated to *Phosphodiesterase 10A gene* (*PDE10A*), a mediator of lung inflammation and associated to asthma and smoking-induced lung cancer (Hsu et al., 2021); and cg11166287 and cg10150824, annotated to open sea regions with no genes or enhancers nearby. Notably, alterations in methylation levels at these CpGs have been linked to various health outcomes in the literature, including chronic pain (Hillary, 2023), asthma (Nicodemus-Johnson, 2016), chronic obstructive pulmonary disease (Hillary, 2023), ischemic heart disease and stroke (Hillary, 2023), Down syndrome (Henneman, 2018), thyroid carcinoma (Bisarro Dos Reis, 2017), psoriasis (Chandra et al., 2018); type 2 diabetes (van Dijk, 2018), primary Sjogren syndrome (Luo, 2021), Kabuki syndrome (Aref-Eshghi, 2017); and inflammatory bowel disease (Agliata, 2020).

Concerning the remaining 2 significant CpGs, our study marks the first to identify an association between any form of smoke exposure and DNAm at these loci. Notably, cg11549143 is annotated to the *Fibrinogen C Domain Containing 1* gene body (*FIBCD1*), a conserved type II transmembrane endocytic receptor highly expressed in the lungs (Jepsen, 2018). Previous research has shown upregulation of *FIBCD1* in smoking-associated head and neck cancer patients compared to non-smokers (Irimie, 2018), indicating its potential involvement in smoke-related pathogenesis. Similarly, methylation at cg27649764, annotated to 200–1500 bases upstream of the transcription start site of *CTD Small Phosphatase Like 2* gene (*CTDSPL2*), which codes for a protein with phosphatase activity involved in lung function (de Vries, 2019). In addition, DNAm at this CpG has been previously associated with rheumatoid arthritis (Liu, 2013), following the same direction of effect as in our study. It is difficult to explain why methylation at these CpG may be affected by passive smoking in children but not by passive smoking in adults (Hulls, 2020; Hoang, 2024), active personal smoking (Hoang, 2024; Joehanes, 2016; Sikdar, 2019) or MSDP (Joubert et al., 2016). A possibility could be that this mark is sensitive to environmental factors such as cigarette smoke at particular ages, although further research is warranted. It also could be that postnatal SHS has a different structure of confounding factors compared with adults SHS, personal smoking and MSDP.

Overall, most of the 11 CpGs and their annotated genes are related to conditions or health problems known to be caused or aggravated by SHS (Flor, 2024; Bhat, 2018; Zhang, 2023; Elbeeh, 2023; Lashner et al., 1993; Zhou et al., 2020; Campi, 2023). Moreover, previous findings suggest that high SHS exposure may accelerate biological ageing (Lu, 2017), which is consistent with the fact that methylation changes at 7 out of 11 of the significant CpGs have been previously associated with age (Mulder, 2021; Xu, 2017). Since both active and passive tobacco smoke share the same route of exposure, differing only in dose, it is plausible that similar mechanisms could play a role in relation to DNAm. As reviewed by Caliri et al., heavy metals in tobacco smoke, such as cadmium, are known to replace zinc at critical binding sites, while other components induce various DNA lesions, potentially interfering with the activity of DNA methyltransferases (DNMTs) (Caliri et al., 2021). Additionally, metabolites of tobacco-related toxins may also compete for methyl donors, like S-adenosylmethionine (SAM), disrupting DNA methylation processes (Caliri et al., 2021). Finally, tobacco smoke can also generate reactive oxygen species (ROS), which promote oxidative stress and inflammation and influence the methylation of specific genes (Caliri et al., 2021; Seiler, 2020).

As mentioned above, there is substantial overlap between the CpGs identified for SHS in our study and CpGs described before for exposure to tobacco smoke: 7 CpGs were previously associated with personal smoking (Hoang, 2024; Joehanes, 2016; Sikdar, 2019), while 7 were previously associated with MSDP in cord blood and 5 in child blood (Joubert et al., 2016). This overlap with personal smoking effects is anticipated due to common physiological effects between exposure to first-hand smoke and SHS (Office on Smoking and Health (US), 2006; U. S. Department of Health and Human Services, 2014; Wang, 2015; de Borba, 2014; Flouris and Koutedakis, 2011). Hence, Hoang et al., consistently reported that most of the enriched pathways with a nominal p-value < 0.05 for genes annotated to CpGs significantly associated with MSDP and SHS in adults were enriched in personal smoking (Hoang, 2024). Nonetheless, in order to disentangle if the effects of postnatal SHS were purely postnatal and not due to residual confounding of MSDP, we

ran 2 sensitivity models in 2 of the largest cohorts. On one hand, the results of the model unadjusted for MSDP compared to the main model indicate a larger number of associations with postnatal SHS, and for most of them with larger effect sizes. On the other hand, the model restricted to non-smoking mothers in pregnancy showed similar effect sizes as in the main model. Assuming minimal misclassification of the *in utero* exposure, these results would suggest that effects could be purely postnatal. On the contrary, concurrence of CpGs associated with both postnatal SHS and MSDP may also be attributable to residual confounding as a consequence of some mothers underreporting their smoking status during pregnancy due to social pressures (Richmond et al., 2018). Similarly, some residual confounding could be attributed by the lack of precision in the MSDP variable, which does not consider the dose or duration of the exposure. It could also be that mothers did not smoke during pregnancy but were exposed to SHS themselves during pregnancy and this led to changes in DNAm similar to MSDP exposure (Fuemmeler, 2021). Finally, the meta-analysis focusing on MSDP adjusted for postnatal SHS revealed a greater number of associations and with larger magnitudes of the effects than the main meta-analysis on postnatal SHS, suggesting that prenatal exposure (or *in utero*) exerts more pronounced effects on DNAm.

Few studies have investigated the effects of SHS on DNAm. A previous study in children from the HELIX cohort, also participating in the current study, did not find any associations, likely due to its limited sample size (n = 1,203) (Vives-Usano, 2020). Regarding postnatal SHS exposure in adults, our study did not identify any shared associations with those reported by Hulls et al. (n = 769) (Hulls, 2020) or Hoang et al. (n = 2,884) (Hoang, 2024). This discrepancy may be explained by limited sample size in the case of Hulls et al., or at potential unique epigenetic responses to SHS during childhood, aligning with existing evidence of disparate effects on health of passive smoking between children and adults (Office on Smoking and Health, 2006). Of note, only two CpGs were commonly observed between Hulls et al. and Hoang et al, although they did not reach FDR-significance in both studies.

Strengths of this study include the large sample size and the use of standardized analysis plan and scripts in a multi-cohort setting, ensuring a consistent and rigorous analysis and increasing the generalizability of the findings. In addition, leave-one-out analyses for top CpGs showing consistency across cohorts, a secondary meta-analysis on MSDP that aligned with existing literature, and the consistent results observed in the two sensitivity analyses, further contribute to the robustness of the current findings.

It is important to acknowledge certain limitations in our study methodology. While SHS exposure rates can reach up to 40% globally, all cohorts in our study except for Helix showed significantly lower exposure rates (12.7–23.8%; Helix: 47.8%). Factors that influence both smoking behaviors and SHS exposure reporting include regional differences in smoking regulations, cultural practices and acceptability, socioeconomic factors, and generational variations across cohorts (some cohorts were established earlier than others). Although our aim was to capture smoking behavior close to the child, this information was not uniformly available across all cohorts. Consequently, we relied on data regarding smoking behavior of family members, assuming they smoked near the child. This together with the variations in the questionnaires administered in each cohort may have contributed to less precision in the exposure data. For instance, some cohorts only asked about smoking habits of the parents and others about all the household members. Moreover, while we aimed to assess exposure to SHS from birth to the time of DNAm assessment, this information was not available uniformly across cohorts. Consequently, we utilized exposure data collected at the time of DNAm measurement, which may not fully capture cumulative exposure over the entire period of interest. Importantly, if the SHS household exposure does not imply smoking in front of the child, this could lead to an overestimation of the SHS-exposed sample size and may contribute to an underestimation of the effect size of SHS on DNAm. Future studies with more precise measures of exposure (e.g., indoor versus outdoor smoking, duration, frequency, exposure dose) are needed to further refine these estimates. In addition, we did not explore whether the effect of postnatal SHS on child DNAm can be modified by the *in-utero* exposure. Thus, the existence of cell memory mechanisms and increased vulnerability to similar exposures later in life deserves further investigation. Moreover, we limited our analysis to DNAm between ages 7 to 10 years, but exploring other age ranges (e.g. adolescence) might show different vulnerabilities to the epigenetic effects of SHS. Finally, our study did not explore sex-specific effects due to power constraints, but notably, DNAm levels at eight out of ten of the top CpGs have been reported to differ by sex (Mulder, 2021; Singmann, 2015; Bozack, 2022; Shah, 2014; Yousefi, 2015) and previous research has suggested varying health effects of passive smoking by sex (Fischer and Kraemer, 2015). Thus, future studies would benefit from sex stratification to refine associations during critical developmental periods. Additionally, while we accounted for cell type proportions in our models, future research may further explore cell type-specific associations since previous literature has identified specific B cell smoking epigenomic signatures that could be relevant to disease risk (Wang, 2023). In line with this, our study accounted for key confounder and precision variables, including maternal smoking during pregnancy, child sex, child age at DNAm measurement, maternal educational level, blood cell type proportions, and, where applicable by cohort, additional technical covariates. However, data limitations restricted our ability to include other potentially relevant factors, such as diet and air pollutants. Moreover, exploring interactions between antioxidant dietary factors and SHS exposure on DNAm may provide further insights, as demonstrated in studies on air pollution (Guxens, 2012). Future research should incorporate these additional factors to better elucidate the epigenetic effects of SHS exposure. The present findings are based on a predominantly European population, thus limiting the generalizability of the results. Future studies with larger sample sizes and more diverse populations are needed to replicate and proof generalizability of our findings. Finally, it is important to note that DNAm is tissue-specific, and thus, our observations in peripheral blood may not necessarily reflect DNAm levels in other relevant tissues such as the lungs or heart.

Taken together, our results reveal evidence of associations between postnatal SHS exposure and DNAm in school-aged children at several loci. Most the loci have previously been related to personal smoking or MSDP, and although our sensitivity models suggest minimal residual confounding, further studies should be conducted to confirm the postnatal effects of SHS. Notably, most of the CpGs and their annotated genes are related to conditions or health problems known to be caused or aggravated by SHS. These findings may contribute to our understanding of how environmental exposures influence epigenetic mechanisms and contributes to the evidence base supporting public health policies aimed at reducing exposure to passive smoking, particularly in environments where children are present.

5. Data statement

Complete results of main meta-analyses are available at Zenodo platform (https://zenodo.org/records/12795069).

CRediT authorship contribution statement

Marta Cosin-Tomas: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Thanh Hoang:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Cancan Qi:** Writing – review & editing, Resources, Investigation, Formal analysis. **Giulietta S. Monasso:** Writing – review & editing, Resources, Investigation, Formal analysis. **Ryan Langdon:** Formal analysis, Writing – review & editing. **Simon Kebede Merid:**

Writing – review & editing, Resources, Investigation, Formal analysis. **Lucinda Calas:** Writing – review & editing, Resources, Project administration. **Paula de Prado-Bert:** Writing – review & editing, Resources, Methodology, Formal analysis, Conceptualization. **Rebecca Richmond:** Writing – review & editing, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Vincent VW Jaddoe:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Liesbeth Duijts:** Writing – review & editing, Resources. **John Wright:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Isabella Annesi-Maesano:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Regina Grazuleviciene:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Marianna Karachaliou:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Gerard H. Koppelman:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Erik Melén:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Olena Gruzieva:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Martine Vrijheid:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Paul Yousefi:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Janine F. Felix:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Stephanie J. London:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Mariona Bustamante:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2024.109204.

Data availability

Data will be made available on request.

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