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The early-life exposome and epigenetic age acceleration in children

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ABSTRACT

The early-life exposome influences future health and accelerated biological aging has been proposed as one of the underlying biological mechanisms. We investigated the association between more than 100 exposures assessed during pregnancy and in childhood (including indoor and outdoor air pollutants, built environment, green environments, tobacco smoking, lifestyle exposures, and biomarkers of chemical pollutants), and epigenetic age acceleration in 1,173 children aged 7 years old from the Human Early-Life Exposome project. Age acceleration

Abbreviations: [1]HNMR, Hydrogen Nuclear Magnetic Resonance; BC, Black Carbon; BiB, Born in Bradford; BMI, Body Mass Index; BMIQ, Beta-Mixture Quantile; BUPA, N-Butyl Paraben; CI, Confidence Interval; CpGs, Cytosine-phosphate-Guanine Sites; DBPs, Disinfection By-Product; DEP, Diethyl phosphate; DETP, Diethyl Thiophosphate; DMDTP, Dimethyl Dithiophosphate; DNA, Deoxyribonucleic acid; DNAm, DNA methylation; EC, Elemental Carbon; EDEN, *Étude des Déterminants Pré et Postnatals du Développement et de la Santé de l'Enfant*; eQTM, Expression Quantitative Trait Methylation; ExWAS, Exposome-Wide Association Study; GIS, Geographic Information System; HCB, Hexachlorobenzene; HELIX, Human Early-Life Exposome; INMA, *Infancia y Medio Ambiente*; IQR, Interquartile Range; KANC, Kaunas Cohort; KEGG, Kyoto Encyclopedia of Genes and Genomes; MoBA, Norwegian Mother, Father and Child Cohort Study; NO_2, Nitrogen Dioxide; OCs, Organochlorine Compounds; OPs, Organophosphate Pesticides; OXBE, Oxybenzone; PBDEs, Polybrominated Diphenyl Ethers; PCB-138, Polychlorinated Biphenyl-138; PCB-153, Polychlorinated Biphenyl-153; PCB-170, Polychlorinated Biphenyl-170; PCB-180, Polychlorinated Biphenyl-180; PCBs, Polychlorinated Biphenyls; PedBE, Paediatric-Buccal-Epigenetics; PFASs, Per- and Polyfluoroalkyl Substances; PFOS, Perfluorooctane Sulfonate; PM_{10}, Particulate Matter of <10μm in aerodynamical diameter; $PM_{2.5}$, Particulate Matter of <2.5μm in aerodynamical diameter; PM_{abs}, Absorbance of $PM_{2.5}$ filters, measurement of the blackness of $PM_{2.5}$ filters used as a proxy for elemental/black carbon; RHEA, Mother-Child Cohort in Crete; SHS, Second-hand smoke; SNPs, single nucleotide polymorphisms; TCs, Transcript Clusters; TEX, Toluene-Ethylbenzene-x-Xylene.

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was calculated based on Horvath's Skin and Blood clock using child blood DNA methylation measured by Infinium HumanMethylation450 BeadChips. We performed an exposure-wide association study between prenatal and childhood exposome and age acceleration. Maternal tobacco smoking during pregnancy was nominally associated with increased age acceleration. For childhood exposures, indoor particulate matter absorbance (PM_{abs}) and parental smoking were nominally associated with an increase in age acceleration. Exposure to the organic pesticide dimethyl dithiophosphate and the persistent pollutant polychlorinated biphenyl-138 (inversely associated with child body mass index) were protective for age acceleration. None of the associations remained significant after multiple-testing correction. Pregnancy and childhood exposure to tobacco smoke and childhood exposure to indoor PM_{abs} may accelerate epigenetic aging from an early age.

1. Introduction

Current evidence shows that early-life, including prenatal and early postnatal periods, could be considered as an important window of susceptibility to environmental exposures (Wright, 2017). Being exposed during these stages might permanently change the body's structure, metabolism, and physiology, and hence promote health or diseases in later stages of life (Barouki et al., 2012). Determining which exposures could be beneficial or detrimental for human health, and identifying the underlying biological mechanisms, could provide important evidence for reducing or enhancing exposure to them during early life (Buck Louis et al., 2017; Logan et al., 2018).

Aging is a gradual and multifactorial process, which is characterized by the physiological deterioration of the human body over time (López-Otín et al., 2013). At the molecular level, aging is described as the accumulation of cellular damage, which leads to structural and functional abnormalities and the decrease in the regenerative capacity of the cells (Carmona and Michán, 2016). Biological aging is reported to be a risk factor for the development of age-related diseases such as cancer, diabetes, cardiovascular, and neurodegenerative diseases as well as increased mortality (Kumar et al., 2017). In this context, aging could be considered as a continuous process already starting in early-life. Consequently, evaluating aging during this period might provide new evidence to slow down this process from the beginning and, prevent or delay the development of adverse health outcomes during adulthood and elderly (Benetos et al., 2013; Martens et al., 2019).

The evaluation of aging at the molecular level can be carried out using a series of biomarkers including epigenetic clocks (Horvath, 2013; Horvath et al., 2018; McEwen et al., 2019; Wu et al., 2019a), which predict DNA methylation age of an individual from its DNA methylation levels (Horvath and Raj, 2018). The property of DNA methylation to change with age is used by these clocks to identify a subset of cytosine-phosphate-guanine sites (CpGs) that can predict chronological age. There are a number of epigenetic clocks available with a few ones applicable to children (McEwen et al., 2019; Wu et al., 2019a). From these clocks, we can calculate epigenetic age acceleration, a measure of whether the individuals' are biologically younger or older than their chronological age (Gibson et al., 2019; Horvath and Raj, 2018; White et al., 2019). Although, DNA methylation is just one of the pathways by which epigenetics affect gene expression, besides histone modification or non-coding RNA, we will be referring to the rest of the text to DNA methylation age as epigenetic age. Epigenetic age acceleration has been linked to age-related conditions such as cancer (Ambatipudi et al., 2017; Dugué et al., 2018; Zheng et al., 2016), cellular senescence (Lowe et al., 2016), and mortality (Chen et al., 2016; Christiansen et al., 2016; Perna et al., 2016), among others (Horvath and Raj, 2018).

Recent evidence has shown that different environmental factors such as air pollution (Nwanaji-Enwerem et al., 2017; 2016; White et al., 2019), tobacco smoke (Yang et al., 2019) or cadmium exposure (Demanelis et al., 2017) can increase epigenetic age acceleration (Martin and Fry, 2018; Simpkin et al., 2016). However, the evidence available is still scarce and not consistent, and most of the studies evaluated the impact on adults (Gao et al., 2016; Wu et al., 2019b) and elderly populations (Ward-Caviness et al., 2016; Yang et al., 2019), with few studies available on such an impact on children (Javed et al., 2016; Simpkin et al., 2017). Moreover, most of the studies have investigated one environmental exposure, and there is a paucity of studies considering multiple exposures. This study aimed to investigate the association between the early life exposome (covering prenatal and childhood period) and epigenetic age acceleration in children from the Human Early-Life Exposome (HELIX) project.

2. Materials and methods

2.1. Study population

This study was conducted in the context of the HELIX project, which was based on six on-going longitudinal population-based birth cohorts established in six countries across different parts of Europe (Born in Bradford [BiB; UK], Étude des Déterminants Pré et Postnatals du Développement et de la Santé de l'Enfant [EDEN; France], Infancia y Medio Ambiente [INMA; Spain], Kaunas Cohort [KANC; Lithuania], Norwegian Mother, Father and Child Cohort Study [MoBa; Norway], and Mother-Child Cohort in Crete [RHEA; Greece]) (Magnus et al., 2016; Maitre et al., 2018; Vrijheid et al., 2014). Prior to the start of HELIX, all six cohorts had undergone the required evaluation by national ethics committees and obtained all the required permissions for their cohort recruitment and follow-up visits. Each cohort also confirmed that relevant informed consent and approval were in place for secondary use of data from pre-existing data. The work in HELIX was covered by new ethic approvals in each country and at enrolment in the new follow-up, participants were asked to sign a new informed consent form. For this study we sub-selected 1,173 children which had information on the exposome, and blood DNA methylation.

2.2. Exposome assessment during prenatal and early childhood

A broad range of environmental exposures was evaluated (Tamayo-Uria et al., 2019), including 83 prenatal and 103 childhood exposure variables (Table S1). Detailed exposure assessment was previously explained elsewhere (Tamayo-Uria et al., 2019). Briefly, geospatial models, monitoring stations, satellite data and land use databases were used to estimate the urban exposome and air pollution. An estimated exposure value was assigned to each study participant separately for their geocoded addresses of home through GIS platforms (Robinson et al., 2018). During pregnancy, at birth or during childhood, maternal and children blood and urine samples were collected by each cohort to assess chemical exposures: organochlorine compounds (OCs), organophosphate pesticides (OPs) metabolites, polybrominated diphenyl ethers (PBDEs), per- and polyfluoroalkyl substances (PFASs), essential minerals, non-essential minerals, phenols, phthalate metabolites, and cotinine (Robinson et al., 2018). During pregnancy, exposure to water disinfection by-product (DBPs) was also assessed based on models for the water supply according to their residency (Jeong et al., 2012). Indoor exposure to air pollutants including particulate matter absorbance (PM_{abs}), which is a proxy of elemental/black carbon as it measures the blackness of $PM_{2.5}$ filters, NO_2, benzene and TEX-toluene, ethylbenzene and xylene, was estimated through a prediction model trained in a

subgroup of children (n = 157) using home personal measurements and questionnaire data including parental smoking behaviour, among other variables (Tamayo-Uria et al., 2019). Questionnaires were used to gather information on active and/or passive tobacco smoking, socio-economic capital of the family, social participation, social contact, house crowding, and other lifestyle factors as maternal and children diet, physical activity and sleep duration.

An imputation process was performed for missing data of all exposures and covariates, resulting in 20 imputed databases (Tamayo-Uria et al., 2019). For comparability, continuous exposure variables were standardised by the interquartile range (IQR).

2.3. Methylation data collection

Blood samples were collected from HELIX subcohort children at a mean age of 8.1 years (Maitre et al., 2018). DNA was extracted from buffy coat using the Chemagen kit (Perkin Elmer). DNA concentration was determined in a NanoDrop 1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific) and with Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies). Blood leukocyte DNA methylation was measured using the Illumina Infinium HumanMethylation450 beadchip at the University of Santiago de Compostela – Spanish National Genotyping Center (CeGen-USC, Spain). Briefly, 700 ng of DNA were bisulfite-converted using the EZ 96-DNA methylation kit following the manufacturer's standard protocol, and DNA methylation measured using the Infinium protocol. Within each batch (slide), all the samples were randomized and balanced by cohort and sex. DNA methylation data was pre-processed using *minfi* R package (Aryee et al., 2014). Two samples were filtered due to overall quality: one had a call rate < 98% at detection p-value threshold < $1.0x10^{-16}$ (Lehne et al., 2015) and the other did not pass general quality control parameters (Van Iterson et al., 2014). Then, data was normalized with the functional normalization method with Noob background subtraction and dye-bias correction (J.-P. Fortin et al., 2014). Then, we checked sex consistency (J. P. Fortin et al., 2014), genetic consistency of technical/biological duplicates and other samples making use of the genotype probes of the array and the genome-wide genotyping data when available. In total four samples were excluded, two with discordant sex and two with discordant genotypes. Batch effect (slide) was corrected using the *ComBat* R package (Johnson et al., 2007). Control probes, probes in sexual chromosomes, probes designed to detect single nucleotide polymorphisms (SNPs) and probes to measure methylation levels at non-CpG sites were removed, giving a final number of 386,518 probes. Finally, CpGs were annotated using the IlluminaHumanMethylation450kanno.ilmn12.hg19 R package (Hansen and Aryee, 2012).

2.4. Calculation of epigenetic age with different clocks

Child epigenetic age based on different clocks (Horvath's All Tissue clock (Horvath, 2013), Horvath's Skin and Blood clock (Horvath et al., 2018), the Paediatric-Buccal-Epigenetics' (PedBE) clock (McEwen et al., 2019)) and Wu's methylation-based age prediction model (Wu et al., 2019a) was calculated using the *methylclock* R package (Gonzalez and Pelegí-Sisó, 2021; Pelegí-Sisó et al., 2020). Briefly, from normalized and batched corrected methylation data, the package extracts methylation levels of CpGs included in each clock (*Table S2*). Subsequently, the coefficients obtained through elastic net in the prediction models of each of the clocks in the original papers are used to calculate DNA methylation age and epigenetic age acceleration. Therefore, for each clock we obtained: i) DNA methylation predicted age (DNAm age) in years, ii) ageAcc, difference between DNAm and chronological age in years; iii) ageAcc2, residuals obtained after regressing chronological age on DNAm age, and iv) ageAcc3, residuals obtained after regressing chronological age and blood cell type proportions on DNAm age. We estimated blood cell type proportion (CD4T, CD8T, Mono, Bcell, NK, Neu and Eos) using the Reinius et al. (2012) reference panel as implemented in *meffil* package (Min et al., 2018).

Additionally, we retrieved the same variables for the Horvath All Tissue clock through the Horvath's online calculator (Horvath, n.d.), which starts from raw data (IDAT files) and implements a normalization based on re-purposing the beta-mixture quantile (BMIQ) method (Teschendorff et al., 2013).

2.5. Gene expression data collection

RNA was extracted from whole blood collected in Tempus tubes with the MagMAX for Stabilized Blood Tubes RNA Isolation Kit (TermoFisher). The quality of RNA was evaluated with a 2100 Bioanalyzer (Agilent) and the concentration with a NanoDrop 1000 UV–Vis Spectrophotometer. Gene expression was assessed with the Affymetrix Human Transcriptome Array 2.0 ST arrays (HTA 2.0) at the University of Santiago de Compostela (USC, Spain), following manufacturer's recommendations. Samples were processed in two different rounds. In each round, several batches of 24–48 samples were processed. Samples were randomized per batch taking into account sex and cohort. Raw data were extracted with the AGCC software (Affymetrix) and stored into CEL files. The GCCN (SST-RMA) algorithm was applied to normalize data at gene level. After normalization four samples with discordant sex were excluded. The HTA-2_0 Transcript Cluster Annotations Release na36 (hg19) was employed to annotate transcript clusters (TCs) to genes. A TC is defined as a group of one or more probes covering a region of the genome reflecting all the exonic transcription evidence known for the region and corresponding to a known or putative gene. Control probes and probes in sexual chromosomes or probes without chromosome information were excluded. Probes with a DABG (Detected Above Background) p-value < 0.05 were considered to have an expression level different from the background, and they were defined as detected. Probes with a call rate < 1% were excluded from the analysis. The final dataset consisted of 58,254 TCs. Gene expression values were log2 transformed and batch effect was controlled by residualizing the effect of surrogate variables calculated with the sva method (Leek and Storey, 2007) while protecting for main variables in the study (cohort, age, sex, and blood cellular composition).

2.6. Covariates

During pregnancy and in the childhood follow-up examination information on the following key covariates was collected: cohort (BIB, EDEN, INMA, MOBA, KANC and RHEA), self-reported maternal education (primary school, secondary school and university degree or higher), maternal age at conception (continuous in years), self-reported ancestry (European, Asian and Pakistani, or other), self-reported maternal pre-pregnancy body mass index (BMI) (continuous in kg/m^2), birth weight (<2500 g, >=2500–3500 g, >=3500–4000 g, or >=4000 g), gestational age at delivery (continuous in weeks) and child's BMI z-score (continuous in kg/m^2) (De Onis et al., 2007; "WHO | BMI-for-age (5–19 years)," n.d.). A bivariate analysis was conducted through linear regression models to determine the crude relationship between the covariates and the outcome of our study (Table S3),

2.7. Statistical analyses

2.7.1. Descriptive analyses and correlations

For continuous variables, we calculated median and interquartile range (IQR) and for categorical variables, frequency and percentage. Pearson's correlation was used to test the correlation between DNA methylation age, calculated with different epigenetic clocks, and chronological age.

2.7.2. Exposome-wide association study of epigenetic age acceleration

To assess the association between the prenatal and childhood exposome and age acceleration we performed an exposome-wide association

study (ExWAS) using the *rexposome* R package (Hernandez-Ferrer and Gonzalez, 2019). The ExWAS approach consists of an exposure-by-exposure estimation of the association between each exposure and the outcome adjusting for potential confounders through independent linear regression models (Patel et al., 2010). It was performed separately for the prenatal and childhood exposome. Results from the 20 imputed datasets were aggregated as described before (Hernandez-Ferrer and Gonzalez, 2019). Results of the ExWAS analyses were expressed as β coefficients and 95% confidence intervals (CIs), that were reported for each IQR increase for continuous exposures or relative to the reference category for binary and categorical exposures. Nominal significance was established at nominal p-value < 0.05. For multiple hypothesis testing correction, we adapted the Bonferroni procedure to handle correlated exposures: we estimated the number of truly independent tests observed according to the correlation structure of the prenatal and childhood exposome (ENT), as $ENT = \sum_{i=1}^{M}[I(\lambda i > 1)(\lambda i - 1)]$, where I(x) is an indicator function and λ_i are the eigenvalues of the matrix of correlations between M exposures (adapted from Li et al. 2012 and Li, 2005 (Li and Ji, 2005; Li et al., 2012)) and then we divided the nominal significance by these calculated effective number of tests. This gave the following p-value thresholds (TEF): $1.01x10^{-3}$ and $8.39x10^{-4}$ for the prenatal and childhood exposome, respectively).

In the main analyses we evaluated the association between prenatal or childhood exposome and age acceleration adjusted for blood cell type proportions. Models for both periods were adjusted for a common set of confounders identified *a priori* based on literature and covariate selection through the DAGitty tool (Textor and Hardt, 2011) (Figs. S1 and S2): (i) child's sex, (ii) cohort, (iii) self-reported maternal education, (iv) self-reported ancestry and (v) maternal age at conception. We also fitted models further adjusted for maternal pre-pregnancy BMI, birthweight (grams) and gestational age at delivery (weeks) in pregnancy exposome models, and maternal pre-pregnancy BMI, birthweight (grams) and child's BMI z-score (De Onis et al., 2007; "WHO | BMI-for-age (5–19 years)," n.d.) in childhood models.

2.7.3. Additional insights on main exposure – epigenetic age acceleration associations

For nominally significant exposure - epigenetic age acceleration associations we did further analyses. Firstly, we investigated dose and duration of the maternal smoking exposure during pregnancy and parental smoking behaviour in childhood. Second, to assess the potential window of susceptibility for smoking effect, we ran additional mutually adjusted models: maternal tobacco smoke during pregnancy adjusted for parental smoking in childhood, and vice-versa. The correlation between exposure to tobacco smoke in both exposure periods was calculated using the polychoric correlation test (Revelle, 2017). Third, we evaluated the association between childhood exposure to indoor PM_{abs} and age acceleration adjusted for maternal tobacco smoke during pregnancy or for parental smoking in childhood. Forth, we tested the association between log2 concentration levels of urinary hippurate obtained by ^{1}HNMR spectroscopy, which is a metabolite marker of fruits and vegetables consumption (Lau et al., 2018), and epigenetic age acceleration to determine whether the potential association found between OP pesticides and epigenetic age acceleration could be explained by a high fresh fruit and vegetables consumption (Papadopoulou et al., 2019). Furthermore, we ran additionally adjusted models: OP pesticides (undetected/detected) adjusted for fruit intake, vegetable intake or hippurate concentrations, besides the common set of confounders identified a priori.

2.7.4. Sensitivity analyses

We repeated the main and further adjusted models of the ExWAS evaluating the association between the prenatal and childhood exposures and age acceleration non-adjusted for blood cell type proportions. Moreover, we repeated the analysis restricted to European ancestry children (n = 1,048) to determine if the ancestry had any influence. We also conducted a cohort-by-cohort analysis for each association with a nominal p-value < 0.05, in the main model, to check the pattern of association within each cohort. The *meta* R package (Schwarzer, 2007) was used to conduct the fixed effect meta-analyses based on the estimates and standard errors of the associations. We looked at the I^2 statistics to describe the percentage of variation across the different cohorts that is due to heterogeneity.

2.7.5. Expression quantitative trait methylation (eQTM)

To provide further biological insight into the "Horvath's Skin and Blood clock", we performed pathway enrichment analyses with the genes associated with the methylation levels of the CpGs used to construct the clock. We conducted a cis eQTM analysis using data from 874 HELIX children of European ancestry (https://helixomics.isglobal.org/) (Ruiz-Arenas et al., 2020). First, we linked each one of the 391 CpGs in the "Horvath's Skin and Blood clock" to the nearby genes (1 Mb window from the CpG and the transcription start site). Then, we tested the association between DNA methylation and gene expression levels of the 12,208 identified CpG-gene pairs through linear regression models adjusted for cohort, child's age and sex. After, multiple-testing correction through a permutation processes, we identified 129 unique genes associated with the methylation of 72 of the 391 CpGs in the "Horvath's Skin and Blood clock". Finally, we performed a pathway enrichment analysis of these 129 genes using the over-representation method of the *ConsensusPath* tool (Kamburov et al., 2011) and three different databases (Kyoto Encyclopedia of Genes and Genomes (*KEGG*), *Reactome* and *BioCarta*). We accepted as significant those pathways with a minimal overlap of 2 genes and a cut-off at q-value of 0.025.

The statistical framework R (version 3.6.0) was used to perform all the analyses (R Core Team, n.d.).

3. Results

3.1. Study population

The study included 1,173 children from the HELIX project, aged between 6 and 11 years old that had information on DNA methylation and on the exposome. Of these children 89.3% were of European ancestry, 54.9% were males, a 20.9% were overweight or obese, and 50.7% were born from mothers with a university degree or higher education level (Table 1).

3.2. Selection of the best epigenetic clock for children

We calculated epigenetic age using different clocks: "Horvath's All Tissue clock" (with *methylclock* R package and Horvath's online calculator), "Horvath's Skin and Blood clock", "PedBe's clock" and Wu's methylation-based age prediction model (with *methylclock* R package) (Table S2). The correlations of the epigenetic age (DNAmAge) calculated from each clock vs. chronological age measured in years are presented in Fig. 1. We found that "Horvath's Skin and Blood clock" showed the strongest correlation with chronological age ($R = 0.85$, $p < 2.2x10^{-16}$). The methylation-based age prediction model by Wu et al., and "Horvath's All Tissue clock", obtained from *methylclock* R package showed a slightly weaker correlation ($R = 0.75$, $p < 2.2x10^{-16}$; $R = 0.72$, $p < 2.2x10^{-16}$, respectively). However, slightly stronger than the one obtained from "Horvath's online calculator" ($R = 0.61$, $p < 2.2x10^{-16}$). The "PedBE's clock", although trained in DNA methylation from buccal cells in children (from 0 years to 20 years), showed the lowest correlation ($R = 0.53$, $p < 2.2x10^{-16}$).

Considering that DNAm data was obtained from blood and observing Pearson's correlation results between epigenetic age and chronological age in our study population, we decided to continue the analyses with the "Horvath's Skin and Blood epigenetic clock", using age acceleration adjusted for blood cell type proportion as the main outcome. This clock

Table 1
Characteristics of the study population (n = 1,173).

Variable	n (%) or median (IQR)
Cohort	
BIB	203 (17.3)
EDEN	146 (12.4)
INMA	215 (18.3)
KANC	198 (16.9)
MOBA	212 (18.1)
RHEA	199 (17)
Self-reported ancestry	
Asian and Pakistani	98 (8.4)
European	1047 (89.3)
Other	27 (2.3)
Maternal age (years)	31 ± 6.8
Maternal education	
Primary school	176 (15)
Secondary school	402 (34.3)
University degree or higher	595 (50.7)
Maternal pre-pregnancy BMI (kg/m^2)	24.1 ± 5.9
Sex of the child	
Female	529 (45.1)
Male	644 (54.9)
Birthweight	
<2500 g	40 (3.4)
>=2500–3500 g	662 (56.4)
>=3500–4000 g	357 (30.4)
>=4000 g	114 (9.7)
Gestational age (weeks)	40 ± 2
Child z-score BMI (kg/m^2)	0.3 ± 1.5
Child BMI (WHO categorization)	
Grade 1/2/3 thinness and Normal weight	927 (79.1)
Overweight or Obese	246 (20.9)
Age at blood collection (years)	7.2 ± 2.4

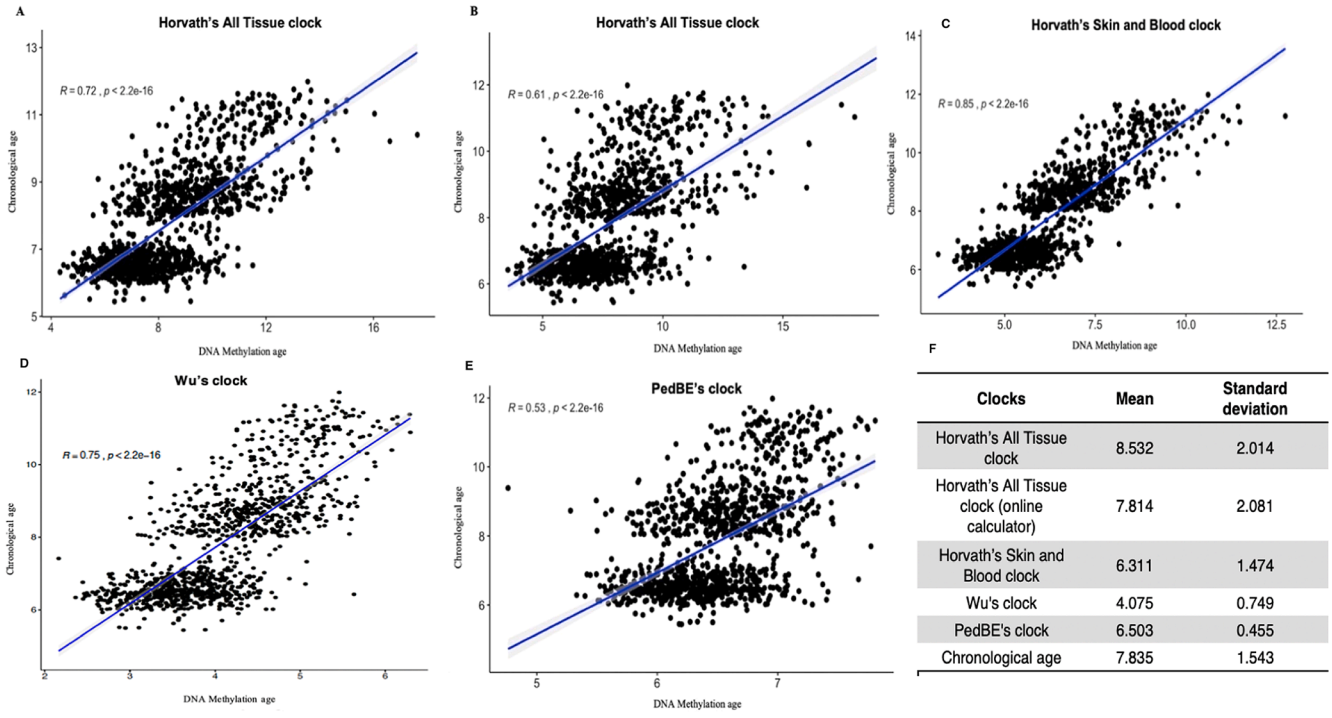
Note: BIB = Born in Bradford; EDEN = Étude des Déterminants Pré et Postnatals du Développement et de la Santé de l'Enfant; INMA = Infancia y Medio Ambiente; KANC = Kaunas Cohort; MoBa = Norwegian Mother, Father and Child Cohort Study; RHEA = Mother-child Cohort in Crete; BMI = Body Mass Index.

was created and trained on a sample size of 1206 individuals from 0 to 75 years old and it is widely used in the aging field as marker of biological aging.

3.3. Exposome-wide association study (ExWAS)

For the prenatal exposome, the ExWAS identified that maternal tobacco smoke during pregnancy was associated with increased epigenetic age acceleration (β = 0.14, 95% CI = 0.02 to 0.26) although it did not pass multiple testing correction ($p < 1.01x10^{-3}$ for the pregnancy exposome) (Table 2, Fig. S4A). Moreover, this association remained similar when the model was further adjusted for maternal pre-pregnancy BMI, birthweight, and gestational age at delivery (no vs. yes; β = 0.13, 95% CI = 0.01 to 0.25) (Table S4 and Fig. S5A; Supplementary Material 2 for the full set of results: Excel Tables S1-S2).

With regard to the childhood exposome, the ExWAS identified two exposure variables that were associated with an increase in age acceleration ($p < 0.05$): indoor particulate matter absorbance (PM_{abs}) (β = 0.07, 95% CI = 0.02 to 0.12) and parental smoking (neither vs. both parents; β = 0.15, 95% CI = 0.01 to 0.29) (Table 2, Fig. S4B). Moreover, two other variables were inversely associated with age acceleration ($p < 0.05$): the organic pesticide (OP) dimethyl dithiophosphate (DMDTP) (undetected vs. detected; β = −0.13, 95% CI = −0.24 to −0.02) and of the persistent pollutant polychlorinated biphenyl-138 (PCB-138) (β = −0.07, 95% CI = −0.14 to −0.01) (Table 2, Fig. S4B). None of these associations passed multiple testing correction ($p < 8.39x10^{-4}$ for the childhood exposome). After further adjustment of the models for maternal pre-pregnancy BMI, birthweight and child's z-score BMI, indoor PM_{abs} (β = 0.06, 95% CI = 0.01 to 0.11) and DMDTP (undetected vs. detected; β = −0.12, 95% CI = −0.24 to −0.02) were the only two exposures that remained significant ($p < 0.05$) (Table S4 and Fig. S5B). Furthermore, in the fully adjusted model, the N-butyl paraben (BUPA) was also nominally associated with age acceleration ($p < 0.05$) (β = 0.04, 95% CI = 0.01 to 0.08) (Table S4 and Fig. S5B; Supplementary



Clocks	Mean	Standard deviation
Horvath's All Tissue clock	8.532	2.014
Horvath's All Tissue clock (online calculator)	7.814	2.081
Horvath's Skin and Blood clock	6.311	1.474
Wu's clock	4.075	0.749
PedBE's clock	6.503	0.455
Chronological age	7.835	1.543

Fig. 1. Pearson's correlations between DNA methylation age, calculated with different clocks, and chronological age. Each graph shows a different epigenetic clock: (A) Horvath's All Tissue clock (*methylclock*. R package); (B) Horvath All Tissue clock (Horvath's online calculator); (C) Horvath's Skin and Blood clock (*methylclock* R package); (D) Wu's clock (*methylclock* R package); (E) PedBE's clock (*methylclock* R package); (F) Summary table of the mean and standard deviation (sd) of the clocks evaluated.

Table 2
ExWAS* of prenatal and childhood exposures vs. age acceleration adjusted for blood cell type proportions (main model).

				ExWAS*	
	Exposure	**Exposure family**	**Units**	**Estimate (95% CI) [a]**	**P-value**
Prenatal	Maternal tobacco smoking	Tobacco smoke	No vs. Yes	0.14 (0.02, 0.26)	0.025
Childhood	Indoor PM_{abs}	Indoor air	ug/m^3	0.07 (0.02, 0.12)	0.003
	Parental smoking	Tobacco smoke	Neither vs. Both	0.15 (0.01, 0.29)	0.036
	Dimethyl dithiophosphate (DMDTP)	OP Pesticides	Undetected vs. Detected (adjusted for creatinine)	−0.13 (−0.24, −0.02)	0.017
	Polychlorinated biphenyl-138 (PCB-138)	OCs	ng/g (adjusted for lipids)	−0.07 (−0.14, 0.01)	0.037

Note: ExWAS = exposome-wide association study; PM_{abs} = Particulate Matter Absorbance, DMDTP = Dimethyl dithiophosphate; OP Pesticides = Organophosphate Pesticides; PCB-138 = Polychlorinated biphenyl-138; OCs = Organochlorine compounds; IQR = Interquartile range. *Results are presented only for the exposures with nominal significance (p value < 0.05) in the ExWAS adjusted for: child's sex, cohort, self-reported maternal education, self-reported ancestry and maternal age in years. The analyses were conducted in 1,173 children from the HELIX subcohort. [a]Coefficient estimates are given in age acceleration effect change for each IQR increase in continuous exposure variables, or relative to the reference category in binary and categorical variables.

Material 2 file for the full set of results: Excel Tables S1 and S2).

3.4. Additional insights on main exposure – epigenetic age acceleration associations

Firstly, we tested the effect of dose and duration of the maternal exposure to tobacco smoking during pregnancy, through linear regression models (Table S5), that suggested a slightly positive trend. Childhood exposure to second-hand tobacco smoke due to parental smoking (classified as: none, one parent, or both parents) also showed a linear trend (Table S5). Secondly, we assessed the effect of the exposure window to tobacco smoke, by running mutually adjusted models. The effect estimates of maternal tobacco smoke during pregnancy adjusted for childhood parental smoking were smaller (28.57% β reduction) (Table S6). A similar pattern was observed when adjusting parental smoking in childhood for maternal tobacco smoke during pregnancy (40% β reduction) (Table S6). To determine if we could disentangle prenatal and childhood smoking exposure, we calculated their correlation. Indeed, a strong positive correlation was shown between exposure to tobacco smoke in both exposure periods (polychoric coefficient = 0.72, prenatal vs. childhood exposure).

In regards to indoor PM_{abs}, we evaluated the influence of parental smoking behaviour and also maternal smoking during pregnancy on the association between indoor PM_{abs} and epigenetic age acceleration as indoor PM_{abs} was estimated through a prediction model trained in a subgroup of children (n = 157) using home personal measurements and questionnaire data including parental smoking behaviour, among other variables (Tamayo-Uria et al., 2019). After adjustment for prenatal or childhood exposure to tobacco smoke, no differences in the estimate coefficients of PM_{abs} were observed (0% β change), suggesting independent effects (Table S7).

Furthermore, given that OP pesticides are present in fruit and vegetables, we speculated that they could be a proxy of fresh fruit and vegetable intake in the study. However, fruit and vegetable intake, were not associated with age acceleration (Supplementary Material 2 file for the full set of results: Excel Table S2) and the effect size of the models of DMDTP additionally adjusted for vegetable or fruit intake did not change substantially (7.14% β increase for vegetables and 0% β change for fruit) (Table S9). Given that food frequency questionnaire data has some misclassification issues, we also adjusted the model of DMDTP for urinary hippurate, which is a metabolite marker of fruits and vegetables consumption (Lau et al., 2018; Pallister et al., 2017). Again, the effect size did not change (0% β change) (Table S9), although hippurate was higher in those children with DMDTP over the limit of detection (mean difference = 0.211, p-value < $2x10^{-16}$).

Finally, when evaluating the childhood model further adjusted for birthweight, maternal pregnancy body mass index and child z-score body mass index, the effect size of the association between PCB-138 and epigenetic age acceleration was drastically attenuated (53.16% β reduction) (Supplementary Material 2 file for the full set of results: Excel Table S2). In our data, as in other studies (Huang et al., 2019), we observed a positive association between child z-score BMI with age acceleration (β = 0.08, 95% CI = -0.05 to 0.12, not adjusted for cell type proportions; β = 0.07, 95% CI = -0.03 to 0.10, adjusted for cell type proportions). Thus, the association of child's BMI with PCB-138 and with epigenetic age acceleration could explain this reduction.

3.5. Sensitivity analyses

When assessing the association between the prenatal and childhood exposures and epigenetic age acceleration non-adjusted for blood cell type proportions we detected all the associations described above for the main model (Fig. S7). However, additional childhood exposures of the same exposure families identified in the main model reached nominal significance (PCB-153, PCB-170, PCB-180, Diethyl thiophosphate (DETP)), as well as of new exposure families (Perfluorooctane sulfonate (PFOS), Hexachlorobenzene (HCB) and Oxybenzone (OXBE)) (Fig. S7B). None of these associations passed multiple testing correction (Supplementary Material 2 file for the full set of results: Excel Tables S1 and S2).

We repeated the prenatal and childhood ExWAS restricting the analyses to European ancestry children and similar results were obtained (Supplementary Material 2 file for the full set of results: Excel Tables S3 and S4). For the significant exposure – epigenetic age acceleration associations described above, the absolute percent change in the coefficients (β) between the whole study sample and the subset of European ancestry sample was < 12%.

Finally, we conducted fixed effects inverse variance weighted meta-analysis of results by cohort of the top exposure – epigenetic age acceleration associations (Fig. S8). For maternal tobacco smoking during pregnancy and childhood indoor PM_{abs} estimated effects were consistent across cohorts (Figs. S8A, S8D). For the other associations the pattern was slightly more heterogeneous with some cohorts going in the opposite direction (Figs. S8B, S8C, and S8E). The statistic I^2 was lower than was 41% for all exposures.

3.6. eQTM analyses

To interpret the biological meaning of epigenetic age, we searched the genes whose expression was associated with the methylation levels of the CpGs included in the "Horvath's Skin and Blood clock" in HELIX. 72 CpGs out of the 391 (18.41%) in "Horvath's Skin and Blood clock" were associated with the expression of 151 unique transcript clusters (TCs, or genes), which were annotated to 129 unique gene symbols (Supplementary Material 2 file: Excel Table S5). 122 out of 129 genes were mapped in ConsensusPathDB and were enriched for the following biological pathways (q-value < 0.025): i) Adaptive and innate immune system, ii) Apoptosis, cell cycle and cancer, and iii) Detoxification of xenoestrogens (Supplementary Material 2 file: Excel Tables S6 and S7).

4. Discussion

This is the first study to evaluate associations between a wide range of prenatal and childhood environmental exposures (the early-life exposome) and the epigenetic age acceleration in children.

We observed a positive association between maternal tobacco smoke during pregnancy and exposure to parental smoke through childhood and age acceleration in childhood, in line with previous studies (Javed et al., 2016; Simpkin et al., 2016; Wu et al., 2019b; Yang et al., 2019). For instance, in adult and elderly populations, active smoking has been correlated with increased epigenetic age acceleration (Gao et al., 2016). Others, using the "Horvath's All tissue clock" clock, have found that maternal smoking increases epigenetic age acceleration as early as at birth (Javed et al., 2016) and that effect is persistent at least until childhood (Simpkin et al., 2016). We also observed that childhood exposure to second-hand smoke (SHS) was associated with increased age acceleration in children. Adjustment for maternal smoking during pregnancy attenuated the association, and considering that pregnancy active smoking implies a higher dose than childhood passive smoking and that smoking effects on blood DNA methylation seem to be persistent (Vives-Usano et al., 2020), these results suggest that the SHS association may have been partly confounded by the exposure during the pregnancy period. Prior research described that maternal tobacco smoking was associated with cord blood DNA methylation at >6,000 CpG sites (Joubert et al., 2016). However, none of these CpGs overlapped with the CpGs used in the Horvath's Skin and Blood clock, thus suggesting different mechanisms. Moreover, we observed a dose dependent effect regarding exposure to tobacco smoke during pregnancy and childhood, in which a higher dose or a longer duration of the exposure was related with increased estimates. What we observed is biologically plausible and is consistent with previous evidence related to other health outcomes (Banderali et al., 2015; Vives-Usano et al., 2020; Zhuge et al., 2020).

In relation to air pollution, we found a positive association between epigenetic age acceleration and childhood indoor PM_{abs}, which is used as a proxy of elemental/black carbon (EC or BC) in the HELIX project. Both pollutants are particles coming from the incomplete combustion of fossil fuels, biofuels, and biomass (Briggs and Long, 2016). Adjustment for childhood SHS exposure did not change this association suggesting that the association observed was led by other sources of PM. Two longitudinal cohort studies, one in adult women and the other in adult men, found that exposure to outdoor BC and ambient $PM_{2.5}$ were associated with increased age acceleration (Nwanaji-Enwerem et al., 2016; Ward-Caviness et al., 2016). Also, a recent study observed that different clusters defined by outdoor $PM_{2.5}$ component profiles were related to accelerated aging in women (White et al., 2019). As far as we know there are no studies of the air pollution effects on epigenetic age in children, neither for indoor nor outdoor levels. Thus, further exploration in children is needed.

In this study, we observed an association between higher DMDTP exposure and decreased age accelerations, which is contrary to what we would expect as DMDTP exposure could be considered as a risk factor for age-related diseases (mainly neurodegenerative (Hayden et al., 2010)). We tried to explain the results that we obtained by looking at the possible association reported before between DMDTP exposure and fruit/vegetable intake (Pallister et al., 2017), in which a higher consumption of fruit/vegetable intake was associated with higher concentrations of DMDTP. As our data on fruit and vegetable intake was obtained by a food frequency questionnaire in which we might be facing misclassification issues, we evaluated a urinary metabolite called hippurate, which is accepted as a metabolite marker of fruits and vegetables consumption. Thus, here hippurate is a biomarker of fruit and vegetables intake, and it might be associated with OPs. However, the role of DMDTP reflecting the effects of fruit and vegetable intake needs further investigation in other studies.

PCBs are widely present in the environment, although whose production was banned in 2001 due to their toxicity and persistence in health (Sun et al., 2005). In our study, we observed a protective effect of PCB-138 on epigenetic age acceleration. When we additionally adjusted the model for child BMI z-score, the association was largely attenuated, suggesting that z-score BMI could explain a notable part of the association. It has been previously reported an inverse association between PCB-138 and child z-score BMI or BMI as these chemicals are highly lipophilic and are stored in fat tissues (Agudo et al., 2009; Dirinck et al., 2011; Domazet et al., 2020; Vrijheid et al., 2020). We thus hypothesize that the association we observed for PCBs might be capturing the relationship between epigenetic age acceleration and body mass index, instead of PCBs exposure. Future studies should address this issue by considering adipose tissue and BMI distinctly, and incorporating toxicokinetic models of PCBs during childhood (Cadiou et al., 2020; Jackson et al., 2017; Vrijheid et al., 2020; Wood et al., 2016).

When evaluating epigenetic age acceleration non-adjusted for blood cell type proportions we detected the same associations as in the main model adjusted for cellular composition plus additional associations with lipophilic chemical compounds. These compounds tend to accumulate in lipid-rich tissues, and their serum levels depend on child's adipose tissue content. At the same time child's adipose tissue content (i.e., obesity) leads to an inflammatory state and an imbalance of the blood cellular percentages. Thus, we hypothesize that when not considering blood cell type proportions we might be capturing the effects of BMI on epigenetic age acceleration, and in turn confounded associations with the lipophilic chemical compounds. In this sense, most of these associations disappeared in models further adjusted for child's BMI.

Aging is a multi-factorial process which involves multiple and complex interactions between biological mechanisms (Borup et al., 2008; Weinert and Timiras, 2003). Aging is related to increased oxidative stress and inflammation, increased DNA damage due to reduced DNA repair, and decreased immune response to external agents and tumorigenic cells (Franzke et al., 2015; Lovell and Markesbery, 2007; Sadighi Akha, 2018). We found that part of the CpGs of the Horvath's Skin and Blood clock were related to the expression of genes involved in pathways relevant for aging processes such as immune response, cell cycle and apoptosis, and detoxification, suggesting that they might mediate the effects of exposure to tobacco smoke, indoor PM_{abs}, and BMI (Camous et al., 2012; Horstman et al., 2012; Kuba and Raida, 2018; Leandro et al., 2015; Weng, 2006).

Major strengths of the present study encompass detailed and comprehensive assessment of the early-life exposome in six populations across Europe with different cultures and settings. We were able to characterize a broad range of environmental exposures for a relatively large sample size in two separate periods of time, pregnancy and childhood, which can be considered as critical periods of vulnerability for children's development (Wright, 2017). Moreover, the environmental exposures that shown an association with epigenetic age acceleration were not correlated among them revealing a non-linear relationship. We have conducted a screening analyses of single exposures using the ExWAS approach, which was characterized by its high sensitivity, but also high false positive rate (Holme et al., 2016). In addition, we have published all the estimates obtained for each exposure-epigenetic age acceleration association to avoid selective reporting bias (Reid et al., 2015). Finally, we have conducted several sensitivity analyses, which did not result in a notable change in our findings.

Nevertheless, our results should be interpreted in the context of its limitations. First, the "Horvath's Skin and Blood clock" used in this study was trained considering all ages and it is not specific for children, which could lead to less precision in assessing epigenetic age. However, one of the known epigenetic clocks based on children population used buccal cells, which gave lower correlations with chronological age likely due to the major differences between tissues than among age ranges. A more robust and improved epigenetic clock within the range of age of children based on blood cells is needed as the one evaluated in this study (Wu

et al., 2019a) was as based on a smaller sample size and it was not widely validated. Second, we acknowledge that we cannot directly contrast the effect size and significance levels between exposures as the exposures evaluated in this study were measured with different measurement errors. Third, when evaluating the childhood exposome our study had a cross-sectional design, and we could not establish a causal link between the environmental exposures and the epigenetic age acceleration. Moreover, although we have adjusted the models for several variables, there can still be residual confounding. In our analyses we evaluated a wide range of prenatal and childhood environmental exposures, however we could not include all factors affecting age acceleration and cover a complete exposome. Therefore, we encourage future studies to further investigate the factors not considered in our analyses. Fourth, the statistical power of our study was relatively limited, because of our sample size.

5. Conclusions

In summary, our study found that prenatal and childhood exposure to tobacco smoke and childhood indoor PM_{abs} are associated with accelerated epigenetic aging. Epigenetic modifications in pathways involved in inflammation, detoxification and cell cycle control may be mechanisms by which these environmental exposures can impact human health from early life onward. As aging is considered a public health issue worldwide, new evidence in child populations might drive new policies to reduce environmental exposures and promote a "healthy aging" from early stages of life.

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.

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Author's contributions

PdP-B, MB and MV designed the study. MV is the coordinator of the HELIX project, with the help of LM and OR. SA, SC, MC, LC, RG, KBG, JL, RMc, RS, MV and JS are the PIs of the cohorts, recruited participants or obtained biological samples. LSH, MN and PD were involved in the measurements of environmental or chemical pollutants or gave advise about them. AC and MB produced DNA methylation data; MV-U and MB obtained gene expression data. HCK and LM obtained urinary metabolomics data. CR-A and CH-F performed the quality control of the methylation and expression data. JRG, CH-F, and DP developed the *rexposome* and *methylcock* R packages. PdP-B, under the supervision of MB, MV and JS, performed the statistical and bioinformatics analyses. PdP-B and MB wrote the manuscript and all others approved it.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106683.

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