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Using methylome data to inform exposome-health association studies: An application to the identification of environmental drivers of child body mass index

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ABSTRACT

Background: The exposome is defined as encompassing all environmental exposures one undergoes from conception onwards. Challenges of the application of this concept to environmental-health association studies include a possibly high false-positive rate.
Objectives: We aimed to reduce the dimension of the exposome using information from DNA methylation as a way to more efficiently characterize the relation between exposome and child body mass index (BMI).
Methods: Among 1,173 mother–child pairs from HELIX cohort, 216 exposures ("whole exposome") were characterized. BMI and DNA methylation from immune cells of peripheral blood were assessed in children at age 6–10 years. A priori reduction of the methylome to preselect BMI-relevant CpGs was performed using biological pathways. We then implemented a tailored Meet-in-the-Middle approach to identify from these CpGs candidate mediators in the exposome-BMI association, using univariate linear regression models corrected for multiple testing: this allowed to point out exposures most likely to be associated with BMI ("reduced exposome"). Associations of this reduced exposome with BMI were finally tested. The approach was compared to an agnostic exposome-wide association study (ExWAS) ignoring the methylome.
Results: Among the 2284 preselected CpGs (0.6% of the assessed CpGs), 62 were associated with BMI. Four factors (3 postnatal and 1 prenatal) of the exposome were associated with at least one of these CpGs, among

Abbreviations: BMI, body mass index; BPA, bisphenol A; DDE, 4,4′dichlorodiphenyl dichloroethylene; DDT, 4,4′ dichlorodiphenyltrichloroethane; DNA, Deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; ExWAS, exposome-wide association study; FDP, false discovery proportion; FDR, false discovery rate; HCB, hexachlorobenzene; MWAS, methylome-wide association study; PBDE, polybrominated diphenyl ether; PCB, polychlorinated biphenyl; PFNA, perfluorononanoate; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate; PFUNDA, perfluoroundecanoate; PM, particulate matter; POP, persistent organic pollutants; zBMI, z-score of body mass index

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which postnatal blood level of copper and PFOS were directly associated with BMI, with respectively positive and negative estimated effects. The agnostic ExWAS identified 18 additional postnatal exposures, including many persistent pollutants, generally unexpectedly associated with decreased BMI.
Discussion: Our approach incorporating a priori information identified fewer significant associations than an agnostic approach. We hypothesize that this smaller number corresponds to a higher specificity (and possibly lower sensitivity), compared to the agnostic approach. Indeed, the latter cannot distinguish causal relations from reverse causation, e.g. for persistent compounds stored in fat, whose circulating level is influenced by BMI.

1. Introduction

The exposome concept recognizes that individuals are simultaneously exposed to a multitude of environmental factors from conception onwards (Wild, 2005). The exposome might explain an important, yet currently not accurately quantified, part of the variability in chronic diseases risk (Manrai et al., 2017). Since the 2010s, environmental epidemiology has progressively embraced the exposome concept and complemented common "single exposure studies" with studies relying on simultaneous measurements of several environmental factors (Agier et al., 2019; Buck Louis et al., 2011; Lenters et al., 2016), which, although not including all possible environmental factors, can be seen as examples of exposome studies. Amongst the many challenges faced by these exposome studies (Agier et al., 2016; Siroux et al., 2016) is a possibly high false discovery rate (Agier et al. 2016). Specifically, a simulation study considering a realistic exposome of 237 exposures assessed in 1200 individuals, with a proportion of the health outcome variability explained by the exposome varying between 3% and 70%, demonstrated that regression-based methods had a suboptimal sensitivity and high false discovery proportion (FDP) (Agier et al. 2016). All of the approaches tested displayed a FDP well above 5% when there was correlation within the exposome. The widely-used ExWAS (Exposome wide association study) approach, consisting in applying independent linear regression models and correcting association p-values for multiple testing, provided the highest sensitivity, at the cost of a very high FDP.

Dimension reduction could be a way to overcome this issue related to false positive findings. Dimension reduction can be performed agnostically, i.e. without relying on external information, with purely statistical techniques, such as variable selection via penalized regression (Lenters et al., 2018; Zou and Hastie, 2005) or Partial-Least-Square (PLS) regressions (Chun & Keles, 2010). However, as Agier et al. (2016) showed, these methods, even if they tend to perform better than ExWAS, are still expected to yield relatively high FDP.

Dimension reduction can also be biologically-driven. Relying on a priori information, one may integrate into statistical models relevant information from, for example, the toxicology and fundamental biology fields. Typically, this could be done by restraining analyses to exposures having biological plausibility, based on existing knowledge on associations with biological layers or on pathways linking exposures and the health outcome of interest. This logic has similarities with the concepts of *Mode of Action* and *Adverse Outcome Pathways* used in toxicology (OECD- Organisation for Economic Co-operation and Development, 2012; Vinken, 2013). DNA methylation can be regarded as such an intermediate informative layer, as it is expected to be under environmental (in addition to genetic) influences (Baccarelli et al., 2009; Feil and Fraga, 2012; Joubert et al., 2016; Marioni et al., 2018) and as these epigenetic alterations can result in modifications of disease risk (Ho et al. 2012, Fasanelli et al., 2015). Epigenetic mechanisms, defined as changes in a chromosome which result in heritable phenotype without alterations in the DNA sequence (Berger et al., 2009), have a key role in regulating transcription and thus cell differentiation, cell functioning, and they can ultimately influence the phenotype.

An option to identify biomarkers associated with both exposures and the health outcome from a single intermediate DNA methylation layer is the Meet-in-the-Middle approach. It has been developed to point out intermediate biomarkers by considering as putative mediators the overlap between omics signals associated with an exposure and omics signals associated with the outcome (Chadeau-Hyam et al., 2011; Vineis and Perera, 2007).

In the case of an intermediate layer with a high dimension, one would have to test the associations of the intermediate putative biomarkers with exposures and health, which might entail a high false positive rate, in particular in the context of correlated exposures or biomarkers. It would appear relevant here to reduce the dimension of the intermediate layer, focusing on biological pathways (or intermediate biomarkers) a priori relevant for the outcome of interest.

In this study, we aimed to identify, in an exposome context, environmental factors associated with child BMI, by using information from child methylome layer to reduce the exposome dimension. Childhood obesity and overweight, whose prevalence has rapidly increased over the last three decades (Finucane et al., 2011), are multifactorial conditions, and the most important risk factors, genetic predispositions and energy imbalance, may not suffice to fully explain the magnitude and rapidity of their recent prevalence increase (Park et al., 2017). The effects on BMI of some environmental factors, such as maternal smoking during pregnancy (Oken et al., 2008) or endocrine and metabolic disruptors exposures in early life (Thayer et al., 2012), have already been identified (Agay-Shay et al., 2015; Holtcamp, 2012). The environmental obesogenic hypothesis states that these early exposures play a key role in future obesity risk by altering metabolic programming (Janesick and Blumberg, 2011; Park et al., 2017). Only a few large multi-exposures (Braun, 2017; Fan et al., 2017; Lauritzen et al., 2018) or methylome-wide (Fradin et al., 2017; Rzehak et al., 2017) approaches to the study of child postnatal growth have been conducted.

2. Materials and methods

2.1. Overall strategy

We relied on the HELIX project, in which the exposome (pregnancy and childhood), the DNA methylome (from peripheral blood in childhood) and BMI were assessed in 1173 mother–child pairs (Haug et al., 2018; Tamayo-Uria et al., 2019). Biological information from genetic databases was used to a priori reduce the methylome dimension. We implemented a « Meet-in-the-Middle » approach to identify exposures sharing differentially methylated CpGs (i.e. methylation sites) with BMI, as a way to build a *reduced exposome*. The association of this reduced exposome with BMI was then tested.

More precisely, the approach consisted in 5 steps:

a) preselection of CpGs located in genes relevant for BMI, using external databases;
b) test of the associations between the methylation levels of these CpGs and BMI;
c) test of the association between the methylation levels of the CpGs found to be associated with BMI in *b)* and each exposure, using child BMI as an adjustment factor, allowing to obtain a *reduced exposome*;
d) test of the association between BMI and the *reduced exposome*;
e) comparison with a purely agnostic ExWAS approach ignoring the methylome (i.e. without steps *a)* to *c)* allowing exposome dimension reduction, sensitivity analysis I).

We also implemented mediation analyses for the significant associations of the main approaches, sensitivity analyses testing the same approach without initial a priori selection of BMI-relevant CpGs, and additionally correcting for cell-type, as well as a sensitivity analysis considering the cell-types as the intermediate layer.

2.2. Study population and outcome

The study was part of the Human Early Life Exposome (HELIX) project (Maitre et al., 2018; Vrijheid et al., 2014), which aimed to describe the early-life exposome and its relations with child development and health.

In HELIX, six population-based European birth cohorts were pooled: BiB (Born in Bradford; United Kingdom) (Wright et al., 2013), EDEN (Étude des Déterminants pré et postnatals du développement et de la santé de l'ENfant; France) (Heude et al., 2016), INMA (INfancia y Medio Ambiente; Spain) (Guxens et al., 2012), KANC (Kaunas Cohort; Lithuania) (Grazuleviciene et al., 2015), MoBa (Norwegian Mother, Father and Child Cohort Study; Norway) (Magnus et al., 2016) and Rhea (Greece) (Chatzi et al., 2017), summing up to 1,301 mother–child pairs from singleton pregnancies for whom external exposures (Tamayo-Uria et al., 2019), health outcomes and confounders were measured and harmonized.

Height and weight were measured according to standardized procedures between 6 and 10 years of age (Maitre et al., 2018). BMI was calculated as the mass in kilograms divided by the squared height in meters. We used age- and sex-standardized z-scores (named hereafter zBMI) according to the international World Health Organization reference curves (de Onis et al., 2007) in order to allow comparison with other studies on child BMI and to take into account the age-related shift in BMI in childhood. Various lifestyle, social and anthropometric factors were additionally assessed (Table 1).

2.3. Exposome assessment

Details of the exposome assessment have been published elsewhere (Haug et al., 2018; Tamayo et al., 2018). Among the 234 exposures assessed in HELIX (Tamayo et al., 2018), we excluded exposures with a time window of one day or one week, which were a priori considered unlikely to influence BMI. This led to 216 prenatal and postnatal exposures (list available in Supplementary Material 1). Metals, organochlorines, organophosphate pesticides, polybrominated diphenyl ethers (PBDE), perfluorinated alkylated substances (PFAS), phenols and phthalates were assessed by biomarkers in mothers during pregnancy from one urine or blood sample and in children at the time of the clinical examination, from a pool of two urine samples or one blood sample (Casas et al., 2018; Haug et al., 2018). Built environment exposures, indoor air exposures, lifestyle factors, meteorological data, natural spaces quantification, noise, traffic, socio-economic capital and concentrations of disinfection by-products in drinking water were assessed during pregnancy and during the year before child examination by environmental models and questionnaires.

Exposures were transformed to approach normality, using a Box-Cox power transformation approach that chooses among log-transforming or raising the data to the powers -2, -1, $-0{:}5$, $1/3$, 0.5, 1, or 2. Transformation chosen for each variable is detailed in Supplementary Material 1. Exposures were standardized using their interquartile range after imputing missing data for all exposures using mice R package (Buuren and Groothuis-Oudshoorn, 2011).

2.4. DNA methylation

Peripheral blood was collected in EDTA tubes during the clinical examination that took place when children were between 6 and 10 years old. DNA was extracted from buffy coat; DNA methylation was assessed with the Infinium Human Methylation 450 beadchip (Illumina), following the manufacturer's protocol. Sample locations on chips were drawn at random balancing chips for cohort and infant sex. Some samples were analysed in duplicate and a control HapMap sample was added in each 96-well plate.

DNA methylation data were pre-processed using the minfi R package (Aryee et al., 2014). A first quality control of the data was done with MethylAid package (van Iterson et al., 2014); probes with low call rate were then filtered following guidelines of Lehne et al. (2015). The functional normalization method was further applied, including Noob background subtraction and dye-bias correction (Triche et al., 2013). Several quality control checks were performed: sex consistency using the shinyMethyl package (Fortin et al., 2014); consistency of duplicates; genetic consistency for the samples that had genome-wide genotypic data. Finally, duplicated samples and control samples were removed as well as probes to measure methylation levels at non-CpG sites (Jang

Table 1
Characteristics of the 1,173 mother–child pairs from HELIX cohort.

Characteristic	Mean (SD)	n (%)
Child BMI (kg/m^2)	16.9 (2.7)	
Child sex		
Female		529 (45)
Male		644 (55)
Child age (years)	7.9 (1.5)	
Cohort		
BiB		203 (17)
EDEN		146 (12)
INMA		215 (18)
KANC		198 (17)
MoBa		212 (18)
RHEA		199 (17)
Maternal education		
Low		176 (15)
Middle		402 (34)
High		595 (51)
Maternal pre-pregnancy BMI (kg/m^2)	25.0 (5.0)	
Parity before index pregnancy		
0		530 (45)
1		430 (37)
2 or more		213 (18)
Trimester of conception		
January-March		368 (31)
April-June		234 (20)
July-September		260 (22)
October-December		311 (27)
Maternal tobacco smoke pregnancy exposure		
None		624 (53)
Only passive exposure		374 (32)
Smoker		175 (15)
Child postnatal tobacco smoke exposure		
Not exposed		745 (64)
Exposed		428 (36)
Maternal age (years)	30.7 (4.9)	
Birthweight		
less than 2500 g		40 (4)
2500 to 3500 g		662 (56)
3500 to 4000 g		357 (30)
≥ 4000 g		114 (10)
Breastfeeding duration		
less than 10.8 weeks		361 (31)
10.8 to 34.9 weeks		419 (36)
greater than 34.9 weeks		393 (34)
Parents born in the country of inclusion		
None		134 (11)
Only one		58 (5)
Both		981 (84)
Ethnicity		
African		7 (1)
Asian		19 (2)
European ancestry		1048 (89)
Native American		2 (0)
Pakistani		79 (7)
Other		18 (2)

et al., 2017). A final filtering was performed to eliminate probes with a single-nucleotide polymorphism (SNP), probes that cross-hybridize and probes on sex chromosomes, restricting to 386,518 CpG probes available for 1,192 subjects. The study was performed on the 1173 subjects among them who also had valid exposures data.

We then used Combat procedure to remove the batch effects supported by the slide. Methylation levels were expressed as Beta values (average methylation levels for an individual, between 0 for a never methylated CpG site and 1 for an always-methylated CpG site).

Cell types were computed according to Houseman et al. (2012) algorithm and Reinius reference panel (Reinius et al., 2012); tests of associations including methylation levels were corrected for cell types only in a sensitivity analysis.

Correlation within the methylome was estimated by averaging the Pearson's correlation within 10 sets of 2284 randomly selected CpGs (same size as the restricted methylome, see next paragraph), to avoid computing all pairwise correlations between the 386,518 CpGs.

2.5. A priori preselection of BMI-relevant CpGs

Biological pathways a priori relevant for BMI were selected using the KEGG database (Tanabe and Kanehisa, 2012), searching with the key words "growth" "obesity" and "fat" in the following categories: "Human energy metabolism", "Human lipid metabolism", "Human endocrine system", "Human digestive system", "Human Excretory system", "Human endocrine and metabolic diseases", "Human genetic Information Processing: 'Transcription – Translation - Folding, sorting and degradation - Replication and repair' ". We thus identified a list of 16 pathways (Supplementary Material 2) and the corresponding list of genes, which were restricted to the CpGs identified as enhancers, leading to a final dataset of 2284 CpGs belonging to 387 genes and 16 different biological pathways (Supplementary Material 2), which we further refer to as the "restricted methylome". Correspondence between genes and CpGs as well as enhancer annotation and CpGs was based on Illumina annotation (Hansen, 2016). A sensitivity analysis not restricted to enhancers and these 16 pathways was performed.

2.6. Meet-in-the-Middle and ExWAS approaches – Statistical analyses

Our Meet-in-the-Middle design itself consisted in three successive steps, as described in 2.1 (steps *b*), *c)* and *d)*): in step *b)*, we tested the association of the methylation levels of the preselected CpGs with BMI considered as the outcome; in step *c)*, we tested the associations (adjusted for child BMI) of each exposure with the CpGs found to be associated with BMI in *b)*, leading to the identification of a "reduced exposome"; step *d)* is the test of the association of this reduced exposome (the exposures found to be associated to some CpGs in step *c)*) with the outcome.

Univariate linear regressions models were applied, and p-values were corrected for multiple testing using a FDR (False-Discovery Rate) control procedure (Benjamini & Hochberg, 1995) at all steps involving regression modelling. Adjustment factors coded linearly in all our regression analyses were maternal pre-pregnancy BMI (additionally coded with a quadratic term in the exposome-outcome associations test), maternal education, maternal age, parental country of birth, maternal smoking during pregnancy, cohort (fixed effect), parity, trimester of conception, child age and child sex (see Table 1 for the categories). We additionally adjusted analyses of postnatal exposures effects for birth weight, breastfeeding duration and passive smoking during childhood (see Table 1) and models including methylation data

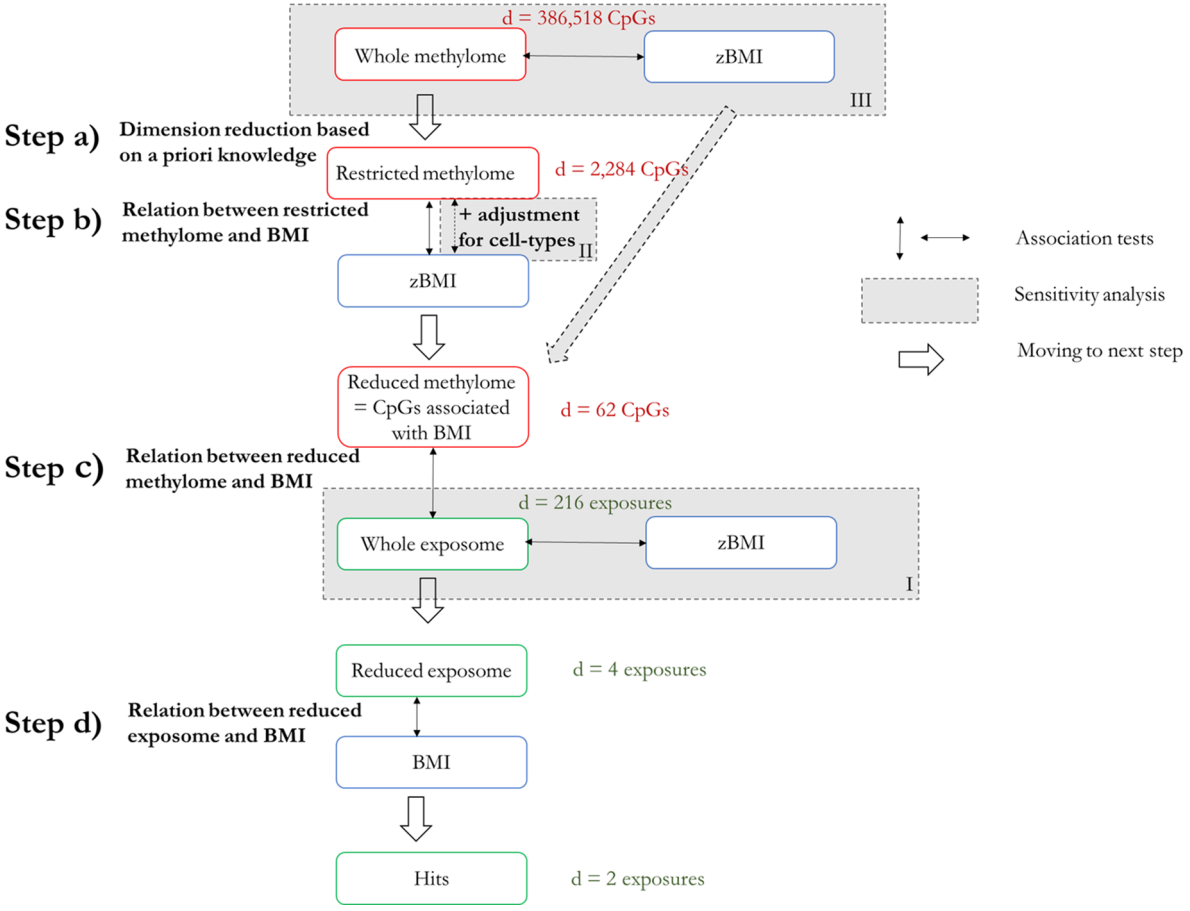

Fig. 1. Workflow of the main statistical analyses. (in color; 1-column fitting image).

for ethnicity (self-reported by parents, with different questions across the cohorts). At step *c)*, correction for multiple testing was done considering together all associations tested between exposures and CpGs, i.e. a number of test equal to the product of the number of exposures with the number of CpGs associated with zBMI. A mediation analysis using package MMA (Yu and Li, 2017) was performed for exposures found associated with the outcome in step *d)*, considering the CpGs both associated with the exposure and the outcome.

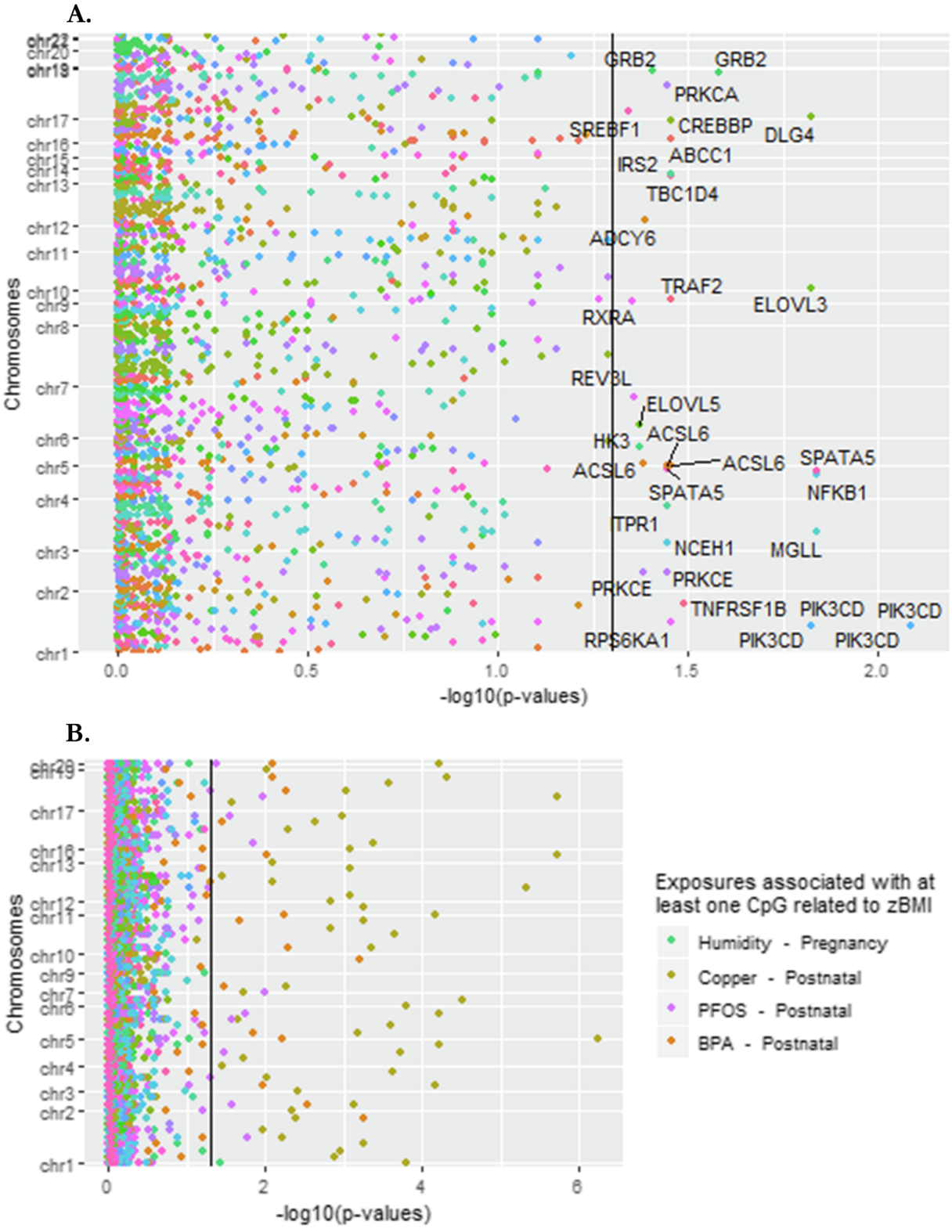

Fig. 2. Manhattan plots of the FDR corrected p-values of adjusted associations obtained with the Meet-in-the-middle approach applied on the reduced methylome at steps *b)* and *c)*. **A:** Associations between preselected CpG and zBMI. Each colour corresponds to a gene. The black vertical line shows the (FDR-corrected) 0.05 significance level. Lowest p-value: 3.20×10^{-3}. **B.** Associations between exposures and CpGs associated with childhood zBMI. Each color corresponds to a different exposure. The black vertical line shows the (FDR-corrected) 0.05 significance level. Lowest p-value: 5.66×10^{-7}. BPA: Bisphenol A; PFOS: Perfluorooctanesulfonic acid.

2.7. Sensitivity analyses and test of selection relevance

We compared our results to those obtained with a *totally agnostic* approach, consisting in an ExWAS between the exposome and zBMI, ignoring the methylome, with exactly the same statistical methods as in our step *d)* (sensitivity analysis I). Our agnostic ExWAS has some differences with that performed by Vrijheid et al. (2020) in Helix data (submitted manuscript, available upon request). In particular, we restricted the population of 1301 children used by Vrijheid et al. (2020) to 1173 children with methylome data (see paragraph 2.4.), and we chose to additionally adjust for trimester of conception, ethnicity and pre- and postnatal smoking, which could influence the methylome. We additionally ran two agnostic (prenatal and postnatal) multivariate linear regression models simultaneously adjusted for the whole exposome and potential confounders and corrected for multiple testing.

We performed three other sensitivity analyses. First, we repeated our approach with an additional adjustment on cell-type heterogeneity for all association tests involving the methylome (sensitivity analysis II). Second, we repeated our approach on the unrestricted methylome, i.e. without the first step of a priori selection of CpGs using information from biological pathways database and annotation (i.e. removing step *a)* and starting from step *b)* with 386,518 CpGs). This modified step *b)* corresponds to a methylome-wide analysis, or MWAS (methylome-wide association study) in which we tested the association between methylation levels of the whole unrestricted methylome and zBMI (sensitivity analysis III).

To further inform the relevance of the a priori CpG selection of step *a)*, the proportion of CpGs belonging to our candidate list of a priori BMI-relevant CpGs (i.e. our restricted methylome) among CpGs whose methylation levels was found associated with zBMI by MWAS was compared to the corresponding proportion in the whole methylome.

A workflow of the statistical analyses is shown Fig. 1.

We additionally performed a fourth sensitivity analysis (sensitivity analysis IV) by repeating the whole Meet-in-the-Middle approach considering the cell-types instead of methylation data as the intermediate layer.

3. Results

3.1. Population characteristics

At the time of BMI measurement, mean age was 7.9 years. Mean child BMI was 16.9 kg/m^2 (5th and 95th percentiles: 13.8; 22.4), with substantial differences between cohorts (Supplementary Material 3), INMA and RHEA showing higher zBMI compared to the other cohorts. The other characteristics of the study population are given Table 1 and by cohorts in Supplementary Material 4.

The mean levels of the 216 exposures considered are displayed in Supplementary Material 1. Mean absolute correlation between quantitative exposures was 0.11 (5th and 95th percentiles: 0.00, 0.35); the distribution of the coefficients of correlation is given in Supplementary Material 5.

Within the whole methylome, the estimated mean correlation was 0.09 while it was 0.12 for the 2284 CpGs of the reduced methylome (5th and 95th percentiles: 0.00; 0.37).

3.2. Meet-in-the-Middle approach

The analysis testing the association between the restricted methylome and zBMI (step *b)*) identified 62 CpGs belonging to 43 different genes (FDR adjusted p-values ≤ 0.05; Supplementary Material 6). The mean correlation among these CpGs was 0.59. Fig. 2A shows a Manhattan plot of the FDR-adjusted p-values.

The test of association of these 62 CpGs with each of the 216 environmental factors adjusted for child BMI (step *c)*) identified 4 exposures associated with at least one CpG (Fig. 2B, Table 2, Supplementary Material 7): copper (postnatal level), BPA (Bisphenol A, postnatal level), PFOS (Perfluorooctanesulfonic acid, postnatal level) and one meteorological variable (humidity, pregnancy average); this constituted our *reduced exposome*. In total, 53 CpGs were associated with at least one exposure.

The last step (step *d)*) identified that within the reduced exposome, postnatal blood copper and PFOS levels were directly associated with zBMI. The corresponding estimated parameters were respectively 0.22 (95% CI: 0.14; 0.30; adjusted p-value, 1.43×10^{-6}) and −0.13 (95% CI: −0.23; −0.04; adjusted p-value, 0.02) (see Table 3 for the other components of the reduced exposome). A mediation analysis quantified that for copper, the 52 CpGs mediated 29% of the total effect of postnatal blood copper level on zBMI, while for PFOS, the 12 selected CpGs mediated 28% of the total effect of postnatal blood PFOS level.

3.3. Agnostic exposome-wide approach

An agnostic ExWAS using FDR correction for multiple testing between the whole (not reduced) exposome and zBMI identified 20 postnatal exposures significantly associated with zBMI (Sensitivity analysis I, Table 4). These included postnatal copper and PFOS level (also identified at step *d)* of the main approach). In addition to metals and perfluorinated alkylated substances (PFAS), these exposures belonged to the organochlorines, polybrominated diphenyl ethers (PBDE), lifestyle and indoor air pollution families. Organochlorines, PBDE and PFAS compounds, as well as postnatal cobalt levels showed negative regression coefficients, corresponding to a decreased zBMI with increasing exposure levels. The most significant associations were observed for 5 of the postnatal PCB levels, which formed a group with higher correlation (mean absolute correlation, 0.50) than the rest of the quantitative exposome (mean absolute correlation, 0.11). When applying a multiple linear regression model simultaneously adjusted for the whole exposome and potential confounders, 2 (postnatal) variables were selected after multiple testing correction: copper (positive parameter) and HCB (negative parameter, Supplementary material 8).

3.4. Other sensitivity analyses

In order to determine if our a priori CpGs selection led to a concentration of information, we quantified the overrepresentation of our preselected BMI-relevant CpGs among the discoveries of a methylome-wide analysis linking the whole methylome to zBMI. As expected, the

Table 2
Number of CpGs associated with both exposures and zBMI in the adjusted associations between the exposome and CpGs associated with zBMI in 1,173 children from the HELIX cohort (ExWAS model adjusted on zBMI, step *c)* of the Meet-in-the-Middle approach applied on the reduced methylome). Results are presented only for exposures associated with a (stringently corrected for multiple hypothesis testing) p-value of less than 0.05 in exposure-CpGs ExWAS-type analyses, with CpGs being previously selected in a CpGs-zBMI ExWAS-type analysis. *Details of the CpGs and genes are given in Supplementary Material 6.

Exposure	Number of CpGs associated both with the exposure and zBMI	Number of corresponding genes*
Copper (postnatal)	52	37
Bisphenol A (BPA) (postnatal)	15	14
Perfluorooctanesulfonic acid (PFOS) (postnatal)	12	12
Humidity average (pregnancy)	1	1

Table 3
Adjusted associations between the reduced exposome and zBMI in 1,173 children from HELIX cohort (ExWAS model, step *d)* of the Meet-in-the-Middle approach applied to the reduced methylome). "Significant" associations are indicated in bold.

Group	Label	Unit	Transformation	Effect estimate*	95% CI	Uncor-rected p-Value	FDR-corrected p –Value
Meteorological	Humidity (pregnancy)	%	None	0.05	−0.34; 0.44	0.81	0.81
Phenols	BPA (postnatal)	µg/g	Log2	−0.07	−0.14; $2.8x10^{-4}$	0.05	0.07
Metals	**Copper (postnatal)**	**µg/L**	**Log2**	**0.22**	**0.14; 0.30**	**$3.57x10^{-7}$**	**$1.43x10^{-6}$**
Perfluorinated alkylated substances (PFAS)	**PFOS (postnatal)**	**µg/L**	**Log2**	**−0.13**	**−0.23; −0.04**	**$7.69x10^{-3}$**	**0.02**

* Adjusted change in mean zBMI for each unit increase in transformed exposure level. Models were adjusted for maternal BMI, maternal education, maternal smoking during pregnancy, parental country, cohort, parity, trimester of conception, ethnicity, child age and child sex and additionally only for postnatal exposures birth weight, passive smoking during childhood and breastfeeding duration.

CpGs associated with zBMI in an MWAS considering the whole exposome were enriched in enhancers CpGs selected as being relevant for BMI from KEGG database (1.22%) compared to the other CpGs (0.46%, a ratio of 2.6 to 1). However, most significant associations found by the MWAS were not part of the a priori selected list of CpGs (1760 out of 1788, Supplementary Material 9).

When the whole Meet-in-the-Middle was repeated without the step of CpGs preselection using external biological information (sensitivity analysis III), final results differed from those obtained using the restricted methylome: additionally to blood postnatal copper and PFOS levels (which were also found significant in this analysis), blood postnatal hexachlorobenzene (HCB), Pentabromodiphenyl ether (PBDE) 153 and dichlorodiphenyltrichloroethane (DDT) levels (with negative associations with zBMI) and blood postnatal caesium level (with a positive association with zBMI) were identified in step *d)* (Table 5). These 5 exposures were also associated with zBMI in the agnostic ExWAS approach (sensitivity analysis I). To give more details, 1788 out of 386,518 CpGs were associated with zBMI in step *b)* (Fig. 3A and Supplementary Material 10). In step *c)* of the analysis, 28 exposures were significantly associated with at least one of these 1788 CpGs. Among them, postnatal blood levels of copper, BPA and PFOS (Supplementary Material 11 and Fig. 3B) and prenatal humidity exposure, which had all been previously found in the main analysis, were associated with respectively 110, 449, 180 and 47 CpGs. All the other exposures were associated with less than 10 CpGs.

When we repeated our analysis adding a correction for blood cell-types (sensitivity analysis II), no association was significant at step *b)* (lowest p-value with Benjamini-Hochberg correction: 0.72). When we repeated the analysis corrected for blood cell-types without the preselection step, we found one association between the whole methylome and zBMI, but the corresponding CpG (cg02032125) was not associated with any exposure at step *c)* so that no exposure was eventually selected as associated with BMI.

When we considered the cell-types instead of the methylation data as our intermediate layer, (sensitivity analysis IV), results were very similar to those of the main analysis: the reduced exposome consisted in three exposures, postnatal blood copper and BPA levels and average pregnancy humidity exposure. In the last step, only copper level was associated with zBMI. The three cell-types associated with both copper and zBMI mediated 13% of the effect of copper on zBMI. Detailed results of sensitivity analysis IV are available in Supplementary Material 12.

4. Discussion

We implemented a modified Meet-in-the-Middle approach among 1,173 mother–child pairs to identify components of the exposome influencing child BMI through DNA methylation changes. The analysis highlighted postnatal copper blood level as being positively associated with zBMI, an association supported by changes with copper levels in the methylation levels of 52 CpGs from genes that are relevant for BMI based on a priori knowledge. Blood perfluorooctanesulfonic acid postnatal level was also found related to zBMI in our Meet-in-the-Middle approach, an association likely due to reverse causality.

Our work is one of the first studying the link of an exposome including both chemical and nonchemical stressors during the prenatal and postnatal time windows with child BMI. Beside Helix studies, the largest studies considering multiple chemical exposures and child BMI considered up to 27 components (Agay-Shay et al., 2015; Fan et al., 2017; Lauritzen et al., 2018)).

The efficiency of our Meet-in-the-Middle approach to detect true predictors of zBMI within the exposome relies on three main assumptions: 1) that part of the effects of the exposome on child BMI are mediated by changes in methylation levels that can be observed from peripheral blood; 2) that these methylation changes are strong enough to be detectable and that they can be used to select plausible exposures and thus reduce the exposome dimension; 3) that existing databases of biological pathways and regulatory regions (enhancers) allow to relevantly reduce the dimension of the methylome a priori to study its association with BMI. We discuss here the relevance of these three assumptions, as well as our choice not to correct for cell-types heterogeneity.

4.1. Are some effects of the exposome on child BMI likely to be mediated by the methylome?

Methylation has been reported to mediate part of the effect of specific exposures on health. This has been suggested for example for smoking effects on health: Fasanelli et al. (2015) pointed methylation mediation for smoking effects on lung cancer and Wahl et al. (2018) showed that site-specific methylation can mediate the effect of smoking on the expression of inflammatory proteins. For BMI, evidence of mediation arises from animal toxicological studies, which showed that long-term obesity risk can result from effects of early overfeeding/underfeeding mediated by methylation changes on specific regulatory CpG sites in different tissues (Carone et al., 2010; Lillycrop et al., 2008, 2005; Plagemann et al., 2009). In humans, studies based on a subjects who experienced famine during intra-uterine life suggested that blood cells methylation could mediate effects of prenatal undernutrition on later overweight: early-life exposures to famine had an effect on CpGs regulating growth and metabolic mechanisms involved in obesity (Heijmans et al., 2008; Tobi et al., 2009). In addition, such a mediation between prenatal exposure to famine and adult metabolic traits has been statistically demonstrated; indeed, studies (Tobi et al., 2018a;2018b) pointed out that even if, from a biological point of view, DNA methylation measured in peripheral blood was not likely to be a causal mediator of BMI change, it could be a proxy of epigenetic regulation changes in specific tissues, and thus allow to detect mediation. For early exposures other than nutrition, little evidence is currently available regarding an effect on BMI or growth mediated by epigenetic changes (Richmond et al., 2015): however, Cao-Lei et al. (2015) suggested that some gene-specific methylation could mediate part of the

Table 4
Sensitivity analysis I: adjusted associations between the whole exposome and zBMI in 1,173 children from the HELIX cohort (ExWAS agnostic approach, ignoring the methylome). Results are presented only for exposures with a (FDR-corrected for multiple hypothesis testing) p-value lower than 0.05.

Exposure group	Exposure variable	Unit	Transfor-mation	Effect estimate*	95%CI		Uncorrected p -value	FDR-corrected p –value
Organochlorines	PCB 180 - Postnatal	ng/g lipids	Log2	−0.92	−1.05	−0.78	$2.14x10^{-38}$	$4.62x10^{-36}$
Organochlorines	HCB - Postnatal	ng/g lipids	Log2	−0.66	−0.76	−0.57	$5.08x10^{-38}$	$5.48x10^{-36}$
Organochlorines	PCB 170 - Postnatal	ng/g lipids	Log2	−0.82	−0.95	−0.70	$9.56x10^{-37}$	$6.88x10^{-35}$
Organochlorines	Sum of PCBs - Postnatal	ng/g lipids	Log2	−0.80	−0.92	−0.67	$7.15x10^{-34}$	$3.86x10^{-32}$
Organochlorines	PCB 138 - Postnatal	ng/g lipids	Log2	−0.67	−0.78	−0.55	$5.56x10^{-28}$	$2.40x10^{-26}$
Organochlorines	PCB 153 - Postnatal	ng/g lipids	Log2	−0.70	−0.82	−0.58	$1.29x10^{-27}$	$4.66x10^{-26}$
Organochlorines	DDE - Postnatal	ng/g lipids	Log2	−0.54	−0.64	−0.43	$3.42x10^{-22}$	$1.06x10^{-20}$
Polybrominated diphenyl ethers	PBDE 153 - Postnatal	ng/g lipids	Log2	−0.40	−0.52	−0.28	$8.70x10^{-11}$	$2.35x10^{-9}$
Organochlorines	PCB 118 - Postnatal	ng/g lipids	Log2	−0.28	−0.38	−0.19	$1.39x10^{-9}$	$3.33x10^{-8}$
Metals	Copper - Postnatal	µg/L	Log2	0.21	0.13	0.30	$5.67x10^{-7}$	$1.11x10^{-5}$
Perfluorinated alkylated substances	PFOA - Postnatal	µg/L	Log2	−0.24	−0.33	−0.15	$5.29x10^{-7}$	$1.11x10^{-5}$
Organochlorines	DDT - Postnatal	ng/g lipids	Log2	−0.27	−0.38	−0.16	$1.11x10^{-6}$	$2.01x10^{-5}$
Perfluorinated alkylated substances	PFNA - Postnatal	µg/L	Log2	−0.19	−0.28	−0.10	$3.65x10^{-5}$	$6.07x10^{-4}$
Perfluorinated alkylated substances	PFUNDA - Postnatal	µg/L	Log2	−0.21	−0.32	−0.09	$4.06x10^{-4}$	$6.27x10^{-3}$
Metals	Cesium - Postnatal	µg/L	Log2	0.20	0.09	0.31	$4.89x10^{-4}$	$7.04x10^{-3}$
Metals	Cobalt - Postnatal	µg/L	Log2	−0.12	−0.19	−0.05	$7.52x10^{-4}$	0.01
Tobacco Smoke	Active smoking - Pregnancy	–	–	0.34	0.13	0.55	$1.45x10^{-3}$	0.02
Indoor air	Indoor PM absorbance - Postnatal	10^{-5} m^{-1}	Log	0.13	0.04	0.21	$2.95x10^{-3}$	0.03
Indoor air	Indoor $PM_{2.5}$ - Postnatal	µg / m^3	Log	0.11	0.04	0.19	$2.94x10^{-3}$	0.03
Perfluorinated alkylated substances	PFOS - Postnatal	µg/L	Log2	−0.14	−0.24	−0.04	$4.59x10^{-3}$	0.05

* Adjusted change in mean zBMI for each unit increase in transformed exposure level. Models were adjusted for maternal BMI, maternal education, maternal smoking during pregnancy, parental country, cohort, parity, trimester of conception, ethnicity, child age and child sex and additionally only for postnatal exposures birth weight, passive smoking during childhood and breastfeeding duration.

effect of prenatal maternal stress child BMI and central adiposity. Less directly, several exposures were identified as possibly influencing epigenetic marks, and some of these alterations may occur on genes involved in signaling pathways controlling growth and adipose tissue development (Richmond et al., 2015). Importantly, effects in the opposite causal directions are also likely, in that changes in BMI could influence methylation levels on specific loci, as suggested by Dekkers et al. (2016) and Richmond et al. (2016).

Our approach, as well as the agnostic ExWAS, identified postnatal blood copper level as positively associated with zBMI and additionally with changes in BMI-relevant CpGs. The mediating effect that we estimated was of 29% of the estimated total copper effect. Copper is an essential trace element involved via oxidoreduction reactions in a broad range of processes, including energy expenditure, mitochondrial respiration, antioxidant defences and inflammation (Tisato et al., 2010). Human copper intake is most often due to presence of copper in drinking water, food or vitamin supplement (Brewer, 2010; Pal et al., 2014) and is known to influence blood copper level (Silverio Amancio et al., 2003; Uauy et al., 1998). Elevated copper concentrations have been observed in many diseases, including cancer, Alzheimer and metabolic diseases (Brewer, 2010; Salustri et al., 2010; Squitti et al., 2009; Tisato et al., 2010). Specifically, a positive link between copper level and high BMI or obesity in children has been previously described in the same data from HELIX (Vrijheid et al., 2020) and elsewhere (Fan et al., 2017; Lima et al., 2006; Yakinci et al., 1997). These studies in children are cross-sectional and an important question relates to the direction of any causal link between copper level and obesity. Overweight might disrupt copper level, for example due to a higher food intake linked to an increased appetite, as hypothesized by Yakinci et al. (1997), or due to metabolic changes. Other arguments exist in favour of copper being a proximal cause of overweight. Nutrition studies in human showed that changes in copper intake (depletion or supplementation) can have adverse health effects such as metabolic and cardiovascular abnormalities (Klevay, 2018; Milne and Weswig, 2018). The toxicity of copper (Brewer, 2010) and its ability to induce oxidative stress are well-known in humans (Brewer, 2010; Uriu-Adams and Keen, 2005) and from animal models (Galhardi et al., 2004; Pereira et al., 2016). Part of this process can occur via methylation changes, as shown in zebrafish, in which stress-related gene expression can be modified by early-life copper exposure (Dorts et al., 2016). Although we cannot formally exclude a situation in which copper levels are influenced by the child's overweight status (e.g. as in Fig. 4D), in particular due to the cross-sectional design of our study of DNA methylation-BMI links, the above-mentioned experimental and prospective studies make copper a plausible causal biomarker or predictor of BMI , with clues for effects possibly mediated by methylation changes (as in Fig. 4A).

On the contrary, the negative and less strong association of PFOS level with zBMI may correspond to reverse causality (see below). An influence of PFOS levels on blood or serum methylation change has some plausibility (Ruiz-Hernandez et al., 2015; Watkins et al., 2014).

4.2. Can information be borrowed from the blood methylome to reduce the dimension of the exposome?

Dimension reduction is one of the possible cures of the curse of dimensionality (Jimenez and Landgrebe, 1998). Assuming that part of the effects of the exposome on BMI are mediated by the methylome, and that blood methylome constitutes a proxy of methylation levels in other target organs, identifying exposures associated with methylation changes on CpGs relevant for BMI is a way to restrict the analysis to a subgroup of exposures with higher likelihood of having an effect on BMI. If the a priori CpG selection is accurate, one can expect the reduced exposome to contain a higher proportion of true predictors of BMI than the full exposome. In a situation of expected limited power, testing only the association of this reduced exposome with BMI could lead to a better specificity (fewer false positives) than an agnostic

Table 5
Sensitivity analysis III - Meet-in-the-Middle without CpGs preselection based on KEGG database: adjusted association between the reduced exposome and zBMI in 1,173 children from the HELIX cohort (ExWAS model, step *d)* of the Meet-in-the-Middle approach applied on the whole methylome). Bold lines indicate significant associations.

Exposure group	Exposure variable	Unit	Transfor-mation	Effect estimate*	95%CI	Unadjusted p-value	FDR adjusted p –value
Organochlorines	**HCB - Postnatal**	**ng/g lipids**	**Log2**	**−0.66**	**−0.76; −0.56**	**$7.87x10^{-38}$**	**$2.20x10^{-36}$**
Polybrominated diphenyl ethers	**PBDE 153 - Postnatal**	**ng/g lipids**	**Log2**	**−0.40**	**−0.52; −0.28**	**$1.62x10^{-10}$**	**$2.26x10^{-9}$**
Metals	**Copper - Postnatal**	**μg/L**	**Log2**	**0.22**	**0.14; 0.30**	**$3.57x10^{-7}$**	**$3.33x10^{-6}$**
Organochlorines	**DDT - Postnatal**	**ng/g lipids**	**Log2**	**−0.28**	**−0.39; −0.17**	**$6.06x10^{-7}$**	**$4.24x10^{-6}$**
Metals	**Caesium - Postnatal**	**μg/L**	**Log2**	**0.19**	**0.08; 0.30**	**$8.50x10^{-4}$**	**$4.76x10^{-3}$**
Perfluorinated alkylated substances	**PFOS - Postnatal**	**μg/L**	**Log2**	**−0.13**	**−0.23; −0.04**	**$7.69x10^{-3}$**	**0.04**
Phenols	BPA - Postnatal	μg/g	Log2	−0.07	−0.14; $2.83x10^{-4}$	0.05	0.20
Phthalates	OH-MiNP - Pregnancy	μg/g	Log2	−0.07	−0.14; $4.04x10^{-3}$	0.06	0.23
Phthalates	MEHP - Postnatal	μg/g	Log2	−0.07	−0.16; 0.01	0.10	0.31
Perfluorinated alkylated substances	PFHXS - Postnatal	μg/L	Log2	−0.10	−0.22; 0.02	0.12	0.33
Built Environment	Population density - Postnatal	people / km^2	Square root	0.06	−0.02; 0.15	0.15	0.38
Lifestyle	Soda intake - Postnatal	Times/ week	Tertiles	−0.10	−0.26; 0.05	0.18	0.42
Socio-eco capital	Social participation - Postnatal	–	None	−0.08	−0.24; 0.07	0.28	0.60
Lifestyle	Fastfood intake - Pregnancy	Times/ week	Tertiles	0.13	−0.14; 0.39	0.34	0.66
Phthalates	MiBP - Postnatal	μg/g	Log2	−0.05	−0.16; 0.06	0.35	0.66
Phthalates	MBzP - Postnatal	μg/g	Log2	−0.03	−0.11; 0.04	0.40	0.70
Organochlorines	PCB 138 - Pregnancy	ng/g lipids	Log2	−0.05	−0.18; 0.08	0.48	0.78
Organochlorines	Sum of PCBs - Pregnancy	ng/g lipids	Log2	−0.05	−0.20; 0.11	0.55	0.80
Air Pollution	PM2.5 - Pregnancy	μg / m^3	None	−0.04	−0.17; 0.10	0.57	0.80
Noise	Traffic noise (24 h) - Postnatal	dB(A)	None	−0.06	−0.26; 0.13	0.54	0.80
Meteorological	Humidity - Pregnancy	%	None	0.05	−0.34; 0.44	0.81	0.85
Organochlorines	PCB 170 - Pregnancy	ng/g lipids	Log2	−0.03	−0.16; 0.10	0.69	0.85
OP Pesticides	DETP - Pregnancy	μg/g	Log2	0.02	−0.09; 0.12	0.77	0.85
Lifestyle	Vegetables intake - Pregnancy	Times/ week	Tertiles	−0.02	−0.18; 0.15	0.85	0.85
Natural Spaces	Green spaces (300 m) - Pregnancy	–	None	−0.02	−0.17; 0.14	0.84	0.85
Phenols	PRPA - Pregnancy	μg/g	Log2	0.01	−0.09; 0.12	0.82	0.85
Metals	Thallium - Postnatal		None	−0.04	−0.29; 0.21	0.78	0.85
Lifestyle	Yogurt intake - Postnatal	Times/ week	Tertiles	0.04	−0.14; 0.21	0.69	0.85

* Adjusted change in mean zBMI for each increase by 1 in transformed exposure level. Models were adjusted for maternal BMI, maternal education, maternal smoking during pregnancy, parental country, cohort, parity, trimester of conception, ethnicity, child age and child sex and additionally only for postnatal exposures birth weight, passive smoking during childhood, breastfeeding duration.

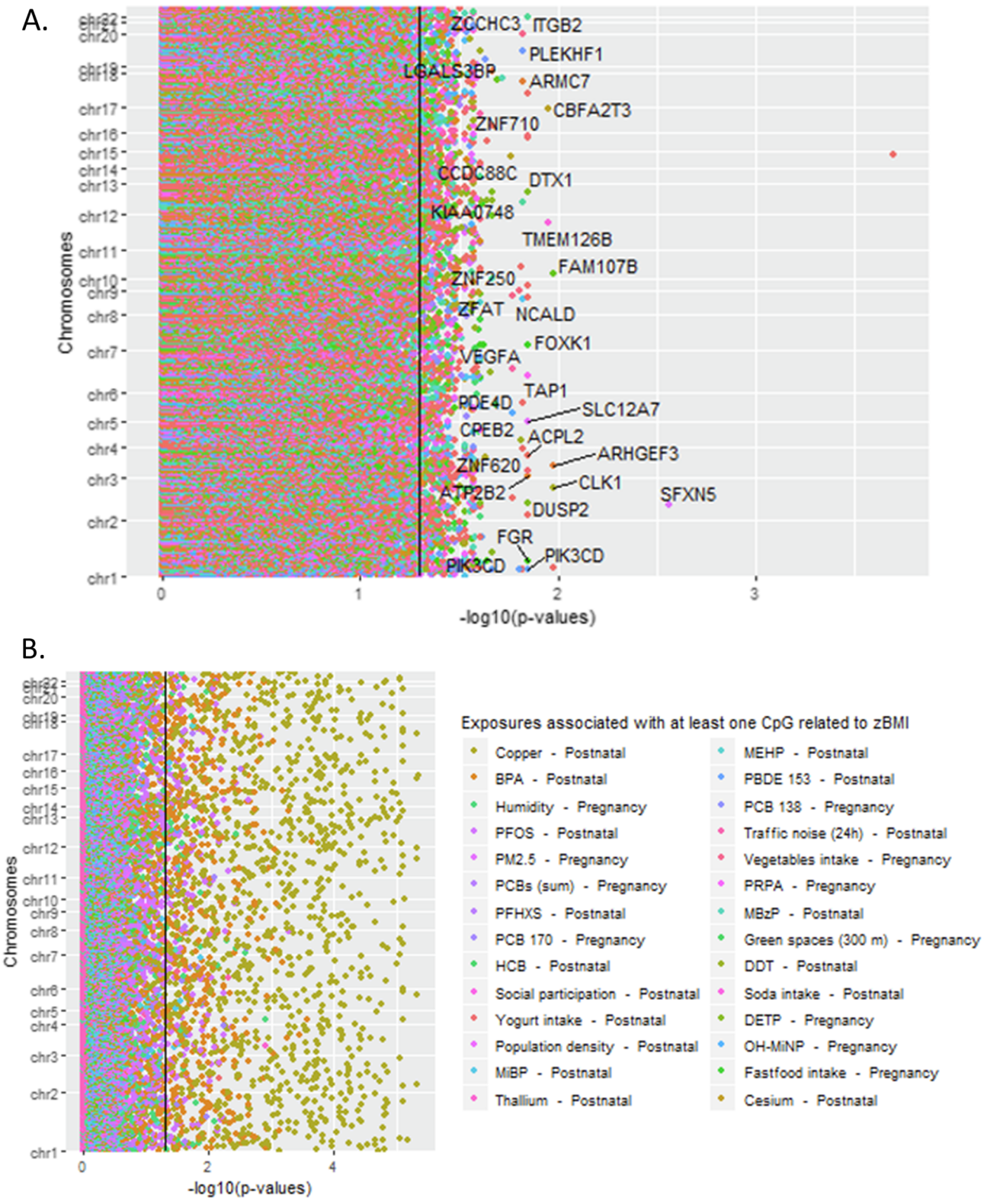

Fig. 3. Sensitivity analysis III - Meet-in-the-Middle approach without a priori preselection of CpGs: Manhattan plots of the FDR corrected p-values of adjusted associations obtained with the Meet-in-the-middle approach applied on the whole methylome at steps *b*) and *c*). **A**: Associations between all CpG and zBMI. Each colour corresponds to genes. The black line is the (FDR-corrected) 0.05 significance threshold. Lowest p-value: 2.04×10^{-4}. **B.** Associations between exposures and CpGs associated with childhood zBMI. Each colour corresponds to a different exposure. Lowest p-value: 4.51×10^{-6}.

ExWAS performed on the whole exposome, which may suffer from high FDP because of the correlation within the exposome (Agier et al., 2016). This approach however comes at the cost of possibly excluding true BMI predictors within the exposome whose effect on BMI are not identifiable from the blood methylome. Yet, we considered a high FDP to be of greater concern for exposome studies than low sensitivity.

Compared to the classical Meet-in-the-Middle framework (Vineis et al., 2013), we additionally adjusted for the outcome when testing for

associations between exposures and CpGs. This adjustment was meant to exclude some cases of spurious association between the exposome and the methylome. Thus, we used this tailored Meet-in-the-Middle approach with a different goal than mediators identification, to focus on a subset of the exposome relevant for the considered outcome, with the ultimate goal to increase specificity.

We used ExWAS-type methods to identify our reduced exposome, which has a possibly high sensitivity and an expected high false positive rate (Agier et al. 2016). This could make our reduced exposome possibly inaccurate, containing exposures selected by chance even if the test is adapted to the underlying causal structure. However, our reduced exposome was considerably smaller than the full exposome (4 exposures *vs.* 216) and our results provided far fewer discoveries (2 *vs.* 20) than an agnostic ExWAS ignoring the methylome data. This lower number of discoveries is not consistent with our approach having a higher rate of spuriously mediated exposures. Rather, it could be explained by our approach having a lower FDP and/or a lower sensitivity. To discuss these hypotheses, we compare the plausibility of the results of the Meet-in-the-Middle approach and of the agnostic ExWAS obtained in the same population.

As discussed before, copper, identified by both approaches, is a plausible causal predictor of BMI mediated by methylation. Among the 19 other exposures significantly associated with zBMI in the agnostic approach, four associations corresponded to positive slopes: postnatal blood caesium level, prenatal maternal active smoking and postnatal indoor particulate matter ($PM_{2.5}$) concentration and absorbance. The last three associations may be (at least partly) due to the well-known effect of prenatal and postnatal smoking on the obesity (Oken et al., 2008; Vázquez Nava et al., 2006): indeed, postnatal smoking variables were used to compute indoor particulate matter levels. An odd ratio greater than 1 was also reported for the influence of high urinary caesium levels on diabetes, an obesity-related outcome (Menke et al., 2016). The remaining 15 associations corresponded to negative slopes (that is, a lower BMI with increasing exposure levels assessed in child blood): it was the case for postnatal blood levels of perfluorinated compounds, cobalt and of persistent organochlorine compounds (some PCBs, DDT (an insecticide), its metabolite DDE, and HCB). For some of these exposures, associations of prenatal levels with overweight or obesity have been reported in the literature. PFAS, including PFOS, prenatal exposures have been associated with *higher* BMI in childhood and adulthood (Braun, 2017; Lauritzen et al., 2018; Saikat et al., 2013); obesogenic effects of early-life PCBs levels have also been reported (Heindel and vom Saal, 2009; Thayer et al., 2012). However, for childhood exposure, several studies found *negative* associations of PCBs (Rönn et al., 2011) and PFAS (Nelson et al., 2010) with BMI, similarly to our results. These negative associations may be indicative of reverse causality. This is supported by the facts that 1) the postnatal exposome and outcome were assessed simultaneously; 2) lipophilic compounds such as PFAS, PCBs and DDT are stored in fat, which makes the blood level a possibly inaccurate marker of exposure: studies on seals showed that, for identical levels of exposures, higher persistent organic pollutants (POP) levels in blood are found in thin compared to fatter animals (Debier et al., 2006; Lydersen et al., 2002). In humans, Rönn et al. (2011) found positive associations of fat mass with blood levels of lightly chlorinated PCBs with fat mass and negative association for highly chlorinated PCBs, which are more lipophilic and therefore more stored in fat. This is in favour of the negative associations between POPs (persistent organic pollutants) levels and BMI being explained by fat levels influencing the blood POPs levels (causal models C to K, Fig. 4), rather than by POPs influencing adiposity (causal models A or B). Unfortunately, we could not access fat tissue POP concentrations, which may have been a better exposure biomarker.

The Meet-in-the-Middle approach did not select PCBs, DDT, DDE, HCB and other POPs highlighted by the agnostic ExWAS. This may be due to our approach coping more efficiently with reverse causality. A recent simulation study showed that Sobel's test of mediation, under specific hypotheses, has far better detection rates for true mediation effects than in a reverse causality situation (Tobi et al., 2018). Our

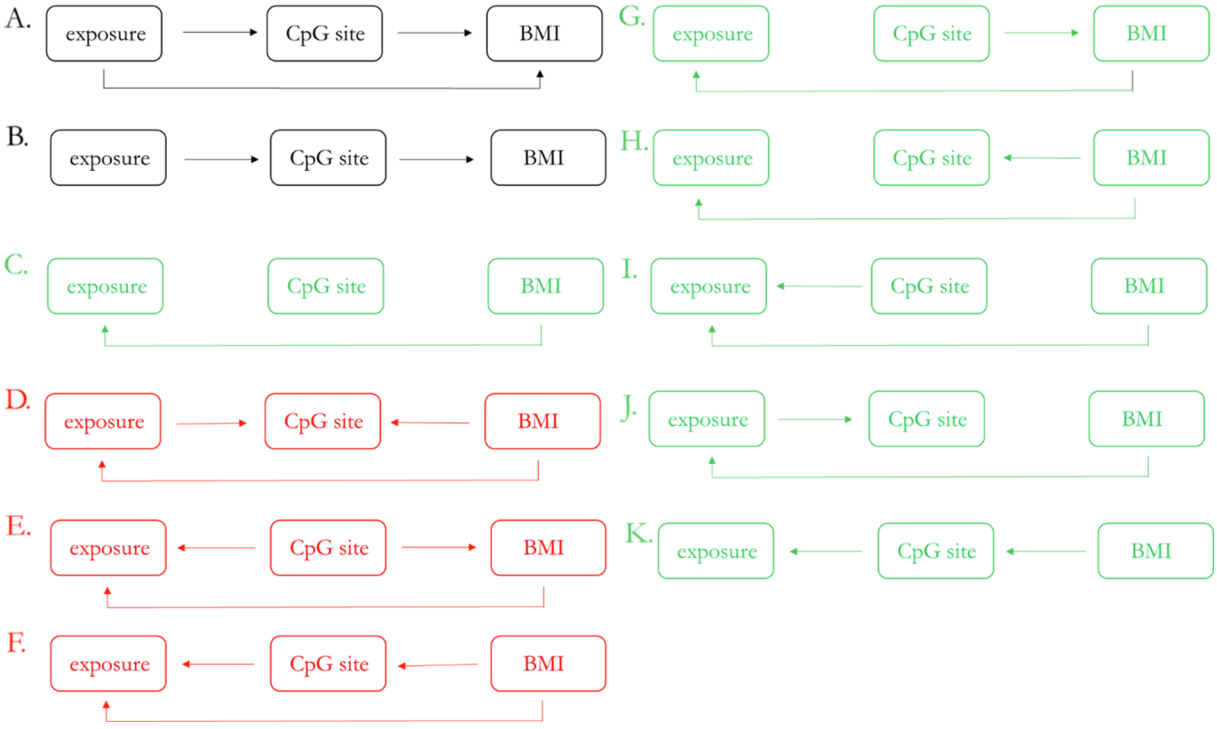

Fig. 4. Different causal models involving one exposure, one CpG site and the outcome (BMI). Among the causal models corresponding to the exposure-BMI link corresponding to reverse causality (C to K), the models in which the Meet-in-the-Middle approach is expected to be able to provide a truly negative result are displayed in green, and causal models in which our Meet-in-the-Middle approach can be expected to provide a false-positive result are displayed in red. (in color; 1.5-column fitting image). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

approach, which has similarities to a mediation test, is moreover specifically designed to efficiently handle some situations of reverse causality.

Fig. 4C to K shows all possible causal situations corresponding to reverse causality between an exposure (E) and zBMI. An ExWAS approach, which cannot distinguish between E influencing zBMI and zBMI influencing E, is expected to detect all of them (assuming perfect power), whereas our Meet-in-the-Middle approach is not expected to conclude that E and zBMI are linked in cases C and I. Moreover, since we adjusted our second test (the exposome-methylome test) for zBMI, cases G and H, which seem very plausible from a biological point of view, should not be erroneously detected by our approach either (indeed, there is no exposure-CpG association conditionally on BMI in models G and H). Finally, as methylation is not likely to influence exposure levels in another way than via its link with the level of fat and as we preselected CpGs in pathways likely to link exposures and BMI, cases E, F and K may not be very frequent situations. Case D however cannot be excluded and may be erroneously detected by our approach, which may be the case for PFOS in our study. We can thus hypothesize that our Meet-in-the-Middle approach is likely to (erroneously) identify a link between E and Y in fewer causal models corresponding to reverse causation than the ExWAS approach. This may limit the number of false-positive findings compared to the agnostic ExWAS.

Regarding now the sensitivity of our approach, it strongly depends on the proportion of exposures truly affecting BMI for which one of the underlying mechanisms relates to changes in blood DNA methylation. If the effect of most exposures is (at least partly) reflected in the blood methylome, then we can expect a high sensitivity, empowered by the smaller dimension of the reduced exposome compared to the whole exposome, which makes the correction for multiple testing less penalizing than in the agnostic ExWAS. If on the contrary most exposures affecting BMI do so by pathways unrelated to the methylome, then our approach is expected to have a low sensitivity. We did not find any association with prenatal exposures to endocrine disruptors (some of which are possible obesogens (Braun, 2017)) or with dietary factors such as soda intake, whose effects on BMI are well documented (Heo et al., 2017; Hu and Malik, 2010; Murakami and Livingstone, 2016) but may not be visible from the blood methylome. Overall, the sensitivity of our approach will depend on the (unknown) proportion of exposures whose effect is mediated by the methylome. Whether the likely lower sensitivity is considered to be compensated by the expected decrease in FDP would depend on the specific nature of the exposome study, with e.g. confirmatory studies putting the emphasis on the limitation of false positive signals.

4.3. Is a preliminary dimension reduction of the methylome necessary to efficiently borrow information from it?

We tried to identify a relevantly reduced exposome with ExWAS-type methods applied to methylome data. ExWAS is known to have a high-false positive rate, particularly if correlation exists among predictors (Agier et al. 2016). To cope with this problem, we modified the original Meet-in-the-Middle framework by testing the exposome-CpGs associations only for CpGs which we had found to be associated with zBMI. This drastically reduced the number of tests done at step *c)* (10,152 tests versus 83,487,888, a division by 8200) and thus increased power for this step. Moreover, we also performed a preliminary reduction of the size of the methylome, relying on external biological information from the KEGG pathways database (Tanabe and Kanehisa, 2012). Our preselection of CpGs was, again, drastic, as it reduced the methylome from 386,518 to 2284 CpGs, possibly allowing a gain of power to test associations between methylation levels and zBMI, under strong assumptions. These assumptions relate to the quality and completeness of the KEGG database (and of our query) to identify BMI-relevant genes and to the quality of the ILLUMINA annotation about enhancers CpGs and on the link between genes and CpGs. These can be questioned: in particular the KEGG database is based on publications of various quality, and pathways selected may not be relevant for 6–10-year-old children. Moreover, ILLUMINA annotation is not tissue-specific whereas enhancer characteristic is; it is consequently unclear whether the ILLUMINA enhancer tag is relevant for blood immune cells, on which methylation was assessed. Finally, changes in the methylation level of the enhancer CpGs of one gene may not be linked with the protein level of this same gene, but of a remote gene (Jang et al., 2017).

To try to quantify the impact of our CpGs preselection, we used two approaches. First, we performed a sensitivity analysis, in which the Meet-in-the-Middle approach was performed without step *a)* of CpGs preselection. This led to quite different results. Although, in the last step, postnatal levels of copper and PFOS were again found to be associated with zBMI, 4 other exposures were additionally significantly negatively associated with zBMI: postnatal blood levels of HCB, PBDE 153, DDT and caesium. Contrarily to copper and PFOS levels, which were associated with respectively 1110 and 180 CpGs at step *c)*, these four additional exposures were each associated with not more than 2 CpGs. Moreover, as discussed above, for the organochlorine compounds, their association with BMI may be due to reverse causality. Thus, the discoveries of this analysis without CpGs preselection contained more compounds little likely to be causative predictors of the zBMI than the discoveries of the main analysis. This might be explained by the high number of false positives expected from an ExWAS-type method in high dimension, at steps *b)* and *c)*. The dimension reduction of the methylome in our case seemed to help to cope with this problem. Further studies are needed to determine if this impact of CpGs preselection is generally expected or not.

To further test the quality of our a priori CpGs preselection, we tried to establish if our a priori reduction led or not to concentration of information, by quantifying the overrepresentation of our preselected CpGs in the significant associations found by a methylome-wide analysis relating the whole methylome to zBMI. The information was indeed concentrated (apparently higher specificity of the selected CpGs), but at the cost of a considerable loss of information: giving up at least 1760 CpGs associated with zBMI implies a risk not to identify some exposures truly associated with CpGs which are themselves associated with zBMI, and consequently may decrease the sensitivity of our approach (Supplementary Material 9). This illustrates, again, that our approach, which was built to gain in specificity, may in principle have a cost in terms of loss in sensitivity compared to the agnostic approach.

4.4. Correction for cell-type heterogeneity

Correction for the proportion of the cell-types in which DNA methylation is assessed is now applied in most methylome studies, although its relevance is debated (Holbrook et al., 2017). Between-subject differences in DNA methylation may or may not depend on cellular heterogeneity. Generally, it is assumed that differences in DNA methylation not due to cell-types mixture are more likely to be causative of disease, while methylation differences caused by differences in cell-types proportion are considered a likely consequence of disease or at least of the disease process (i.e. to correspond to reverse causality, for example in the case of obesity-induced inflammation leading to changes in leukocytes proportion (Zeyda and Stulnig, 2009)), or to be a confounder whose effect needs to be controlled for. However, diseases are also often associated with the distribution of cell types, and cell type proportion can also in some situations be a cause of disease or a marker for e.g. inflammatory or immune-related diseases (Holbrook et al., 2017). This is particularly relevant for obesity development, which is known to involve inflammatory pathways (Hotamisligil, 2003). The differential methylation driven by cell-type heterogeneity could therefore mediate exposure effects rather than be a consequence of the overweight. Thus, cell types proportion could be 1) a consequence or very close marker of our outcome; in such case, adjusting for it in the model linking methylation and zBMI is irrelevant, as adjustment for

consequences of the diseases, which are by definition not confounders, could have harmful consequences (Barton et al., 2019); 2) a cause of the health outcome. In this case, if (option 2a) it is a mediator of an effect of an exposure on zBMI or a proxy of such a mediator, we should not adjust for it: as we want to identify exposures whose effect on zBMI is biologically mediated by the methylome layer, correcting for cell-types could prevent us from selecting potential exposures of interest associated with zBMI and whose effect is mediated by cell-type-dependent methylation. If (2b) it is not a mediator, i.e. if it is a cause of outcome but not a consequence of exposure, it is a potential confounder and as such should be corrected for in the test of association between methylation and zBMI. We chose not to adjust the DNA methylation-BMI model of the main analysis for cell-type heterogeneity because we a priori consider hypotheses 1) and 2a) as more likely than 2b), and because the consequence of erroneously adjusting may, in our study in which identifying the intermediate causal factors was not the main aim, be more harmful (Barton et al., 2019) than adjusting.

In our sensitivity analysis IV, we repeated the whole Meet-in-the-Middle design considering the cell-types instead of the methylation data as the intermediate layer. Final results were very similar to those of the main analysis, making it possible that differential methylation driven by cell-type heterogeneity explained the association between methylome layer and both copper and zBMI, and which therefore mediate copper effects (case 2a). However, the reduced exposome was slightly smaller when considering cell-types and the proportion mediated was lower (13% *vs.* 29%). This may mean that the cell-type did not convey as much information on the biological effect of exposures than DNA methylation on the path between selected exposures and zBMI.

We acknowledge several limitations to this original work; first, our results are dependent on the quality of a priori information; second, the methylome and the outcome (and a part of the exposome) were assessed simultaneously, a design which makes difficult to exclude reverse causation with an agnostic test of association. Measurement error is also expected for both the methylome and the exposome; finally, only monotonous associations were tested to limit the number of tests. The strengths of our study include a multilayer analysis of the exposome, methylome and BMI in a large and well-characterized population, the consideration of multiple testing in the composite tests and of a priori information on the methylome-BMI relation to restrict the number of tests done. This composite design may allow improving the specificity of exposome-health studies and limit associations due to reverse causality, with a possible cost on sensitivity.

5. Conclusion

This work is to our knowledge the first epidemiological study relying on an intermediate blood methylome layer to try to better characterize the exposome-health association. Purely agnostic exposome studies are expected to suffer from a high false positives rate, and possibly a low sensitivity (due to the correlation within the exposome) (Agier et al. 2016). Our approach may allow reducing the false positive rate by using a modified Meet-in-the-Middle design that permits a biologically driven reduction of the exposome, which may in particular allow discarding some of the associations of the outcome with the exposome due to reverse causality. It comes at a cost of being insensitive to exposures acting on the outcome via pathways not causing changes in the methylome of peripheral blood. Extensions of this approach to other biologically relevant layers might allow avoiding this limitation in the future.

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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.

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Contributions

SC and RS designed the analytical and statistical methods. SC analysed the data. SC, MB, LA, MV and RS interpreted the results and wrote the paper. MV, MB and LM coordinated the HELIX data collection. All authors contributed to the data collection and to the manuscript, and approved the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105622.

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