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Supplemental Material

Prenatal Exposure to Mercury: Associations with Global DNA methylation and Hydroxymethylation in Cord Blood and in Childhood

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Supplementary Table S1. Multivariate linear models for the associations of estimated cell type composition in cord blood with 5-hydroxymethylcytosine content (%-5hmC), 5-methylcytosine content (%-5mC) and their ratio (N=473).

	%-5hmC		%-5mC		[‡]%-change in ratio of 5mC to 5hmC	
Estimated Cell type Proportion	β Coeff.	*P*	β Coeff.	*P*	%-change	*P*
CD8+	1.09	0.003	10.13	0.06	-94.4%	0.02
CD4+	0.68	0.04	6.36	0.17	-61.8%	0.36
NK-cells	0.59	0.28	8.71	0.27	25.3%	0.90
B-cells	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
Monocytes	0.57	0.15	-3.62	0.52	-94.8%	0.02
Granulocytes	0.62	0.02	7.06	0.06	-66.4%	0.20
nRBC	0.53	0.06	4.49	0.28	-65.1%	0.26

NA: B-cells left out as estimated cell types add to more than 100%

[†]Restricted to participants with cord blood epigenomic measurements and estimated cell-type composition from DNA methylation microarrays

Supplementary Table S2. [a] Adjusted association between global measures of DNA methylation (%-5mC) and DNA hydroxymethylation (%-5hmC) in cord blood and early childhood blood samples with cognitive function assessed during early childhood.

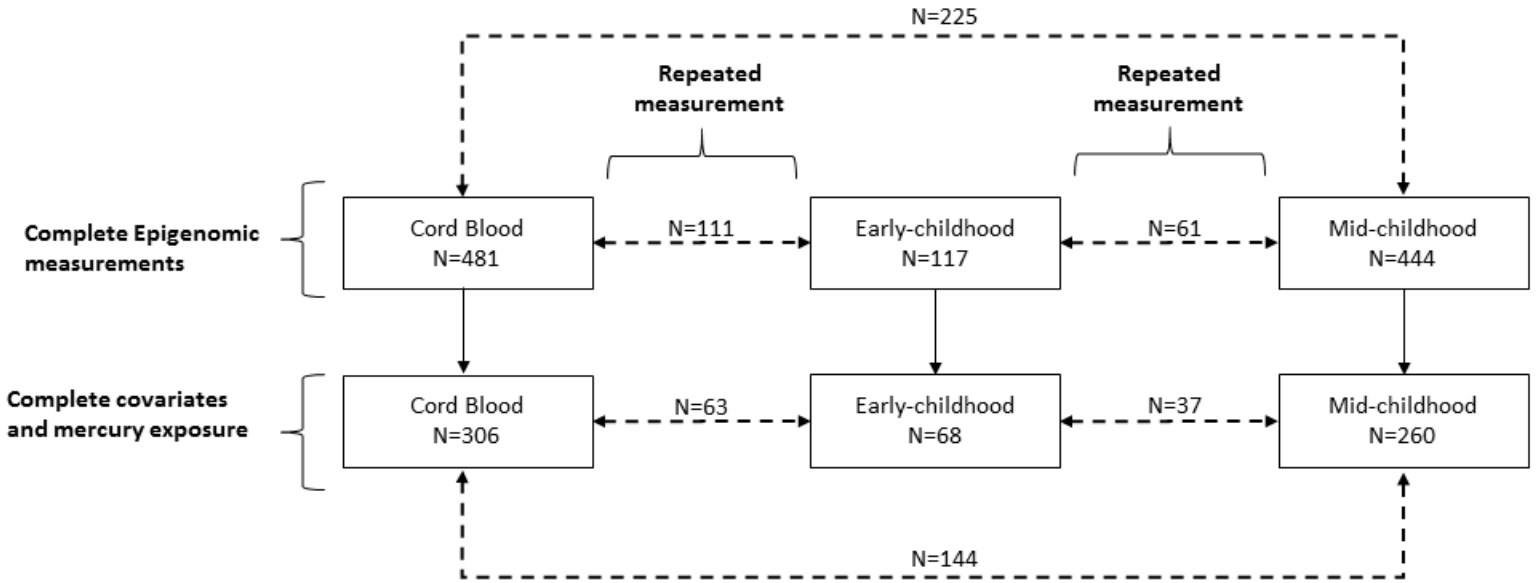
Epigenetic measure	PPVT scores in early childhood		WRAVMA scores in early childhood	
Cord blood (N=369)	**β-Coefficient (95% CI)**	***P***	**β-Coefficient (95% CI)**	***P***
Global %-5mC	0.19 (-2.67, 3.04)	0.89	0.63 (-1.62, 2.86)	0.58
Global %-5hmC	0.79 (-0.75, 2.33)	0.32	0.63 (-0.58, 1.84)	0.30
Ratio 5mC to 5hmC	-1.33 (-3.41, 0.75)	0.21	-0.84 (-2.4, 0.81)	0.32
Early childhood (N=88)	**β-Coefficient (95% CI)**	***P***	**β-Coefficient (95% CI)**	***P***
Global %-5mC	1.90 (-3.7, 7.5)	0.50	0.76 (-3.7, 5.2)	0.73
Global %-5hmC	2.1 (-1.4, 5.5)	0.24	1.23 (-1.5, 3.9)	0.37
Ratio 5mC to 5hmC	-3.0 (-8.1, 2.2)	0.25	-2.10 (-6.1, 1.9)	0.30

PPVT=Peabody Picture Vocabulary Test; WRAVMA=Wide Range Assessment of Visual Motor Abilities. For details on the methods see [a] Cardenas et al. 2017

[a] Estimates from linear regression models adjusted for maternal education at study enrollment, parity, maternal PPVT scores, self-reported alcohol use during pregnancy, fetal growth (sex-specific z-score of birth weight/gestational age), mean weekly fish intake during pregnancy, child age in days at the time of testing, sex, child race and any maternal smoking during pregnancy.

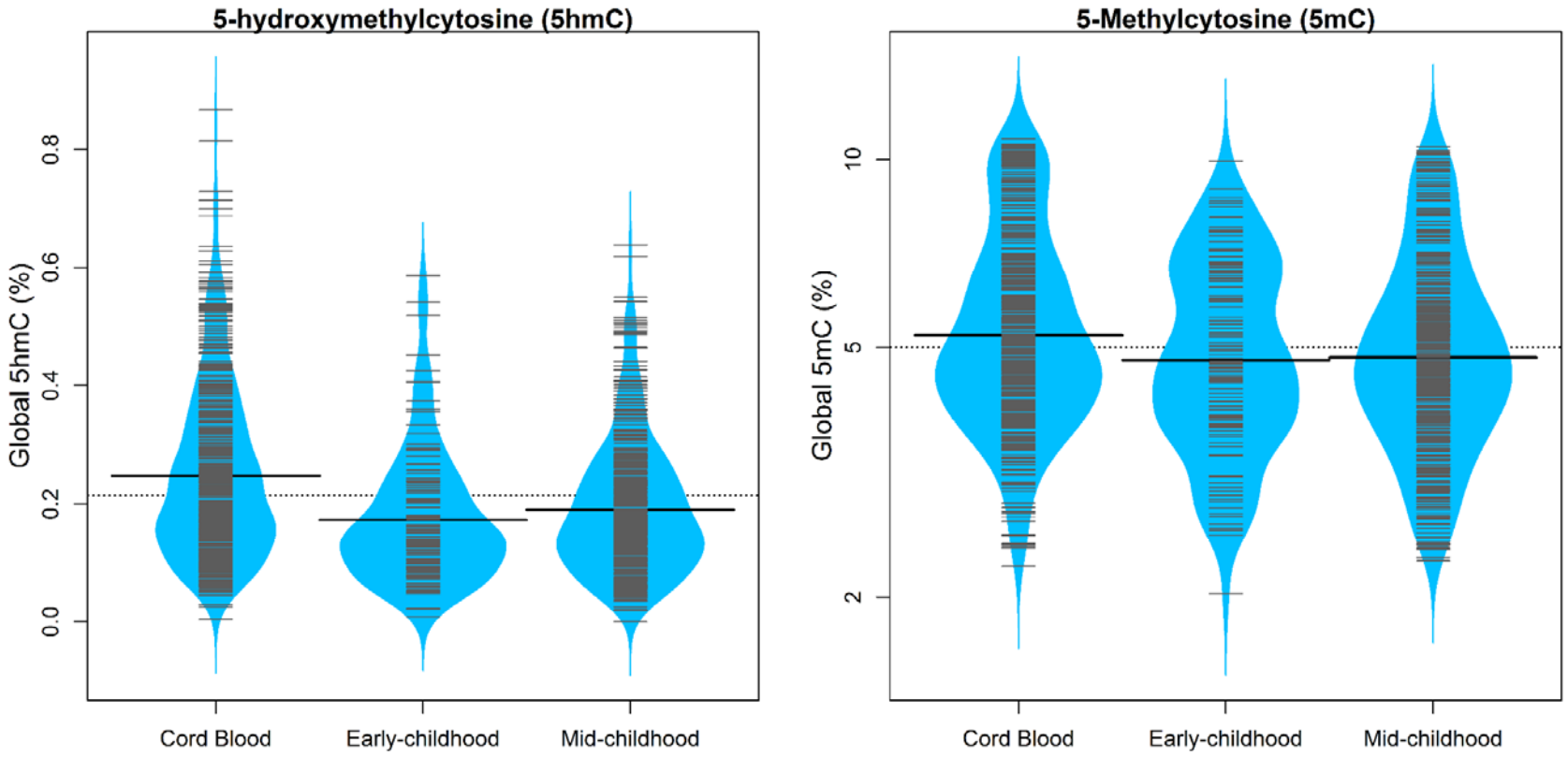
[a] Cardenas A, Rifas-Shiman SL, Agha G, Hivert M-F, Litonjua AA, DeMeo DL, et al. 2017. Persistent DNA methylation changes associated with prenatal mercury exposure and cognitive performance during childhood. *Scientific Reports* 7:28

Supplementary Figure S1. Diagram of the sample flow for the study: repeated measurements across different time-points for all epigenomic measurements and for the epigenomic measurements restricted to complete covariate and exposure information

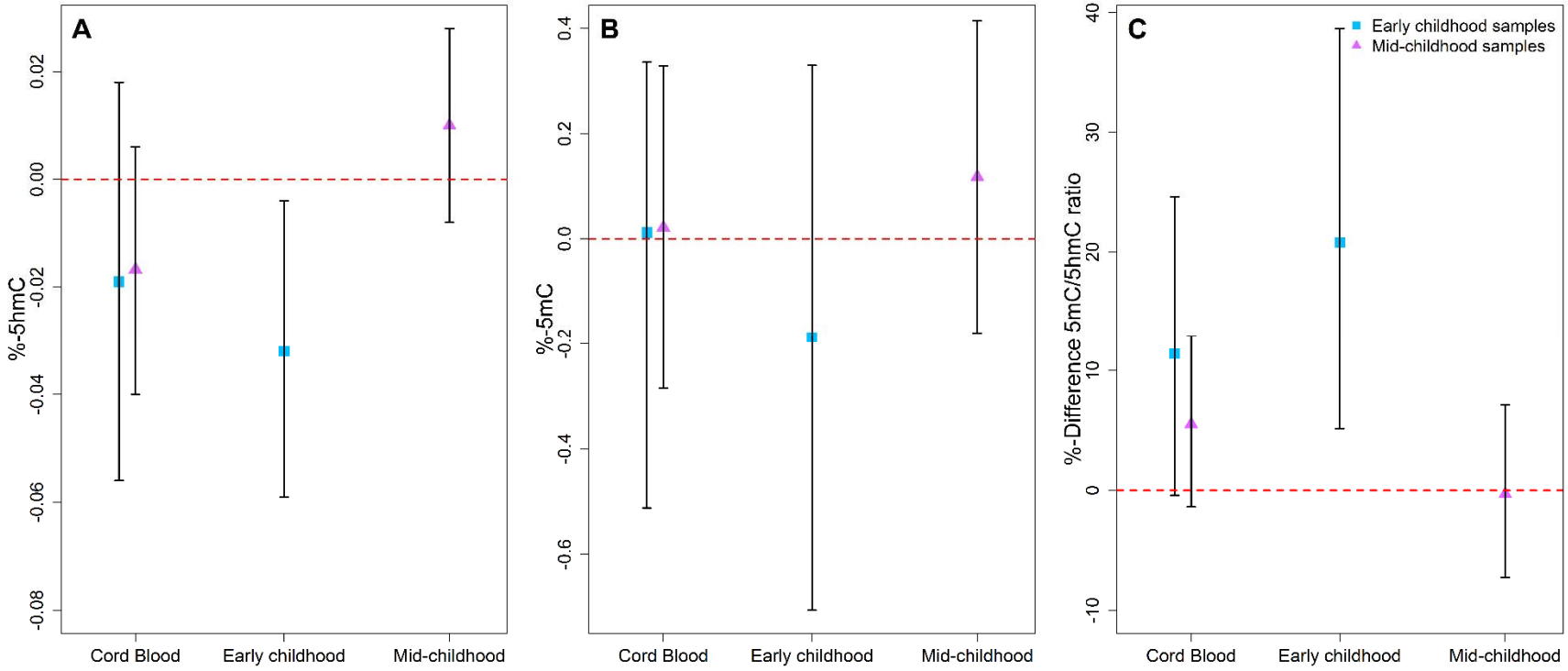


*32 participants had repeated epigenomic measurements (%-5hmC and %-5mC) in cord blood, early childhood and mid-childhood blood after restricting on covariates and

Supplementary Figure S2. Bean plots of the distributions of 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) in cord blood, early childhood and mid-childhood blood (black lines represent the mean levels of 5hmC and 5mC at sample collection and dotted line represents overall mean)

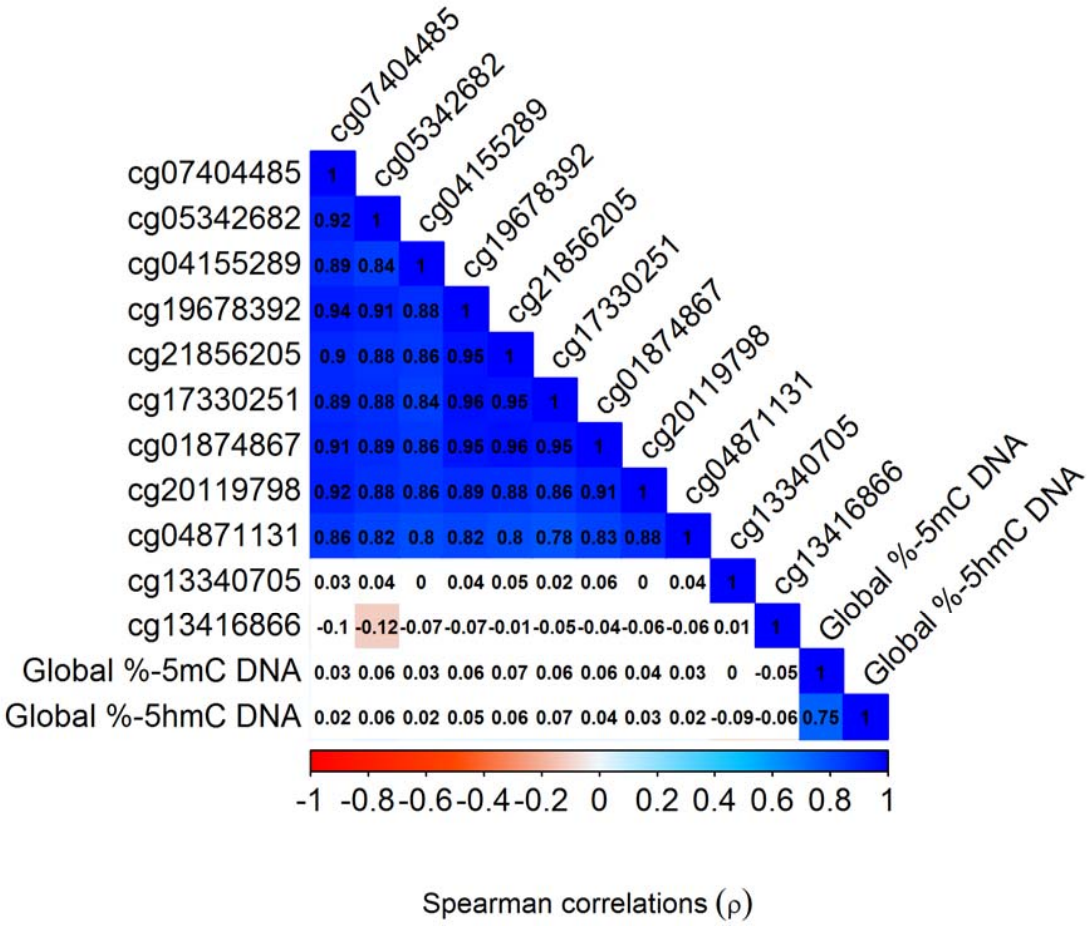


Supplementary Figure S3. [a] Fully adjusted association for A) %-5hmC B) %-5mC and C) %-difference in the ratio of 5mC to 5hmC per doubling in prenatal maternal mercury concentrations. Estimates for cord blood were restricted to either early (■) or mid-childhood (▲) samples for sensitivity analyses.

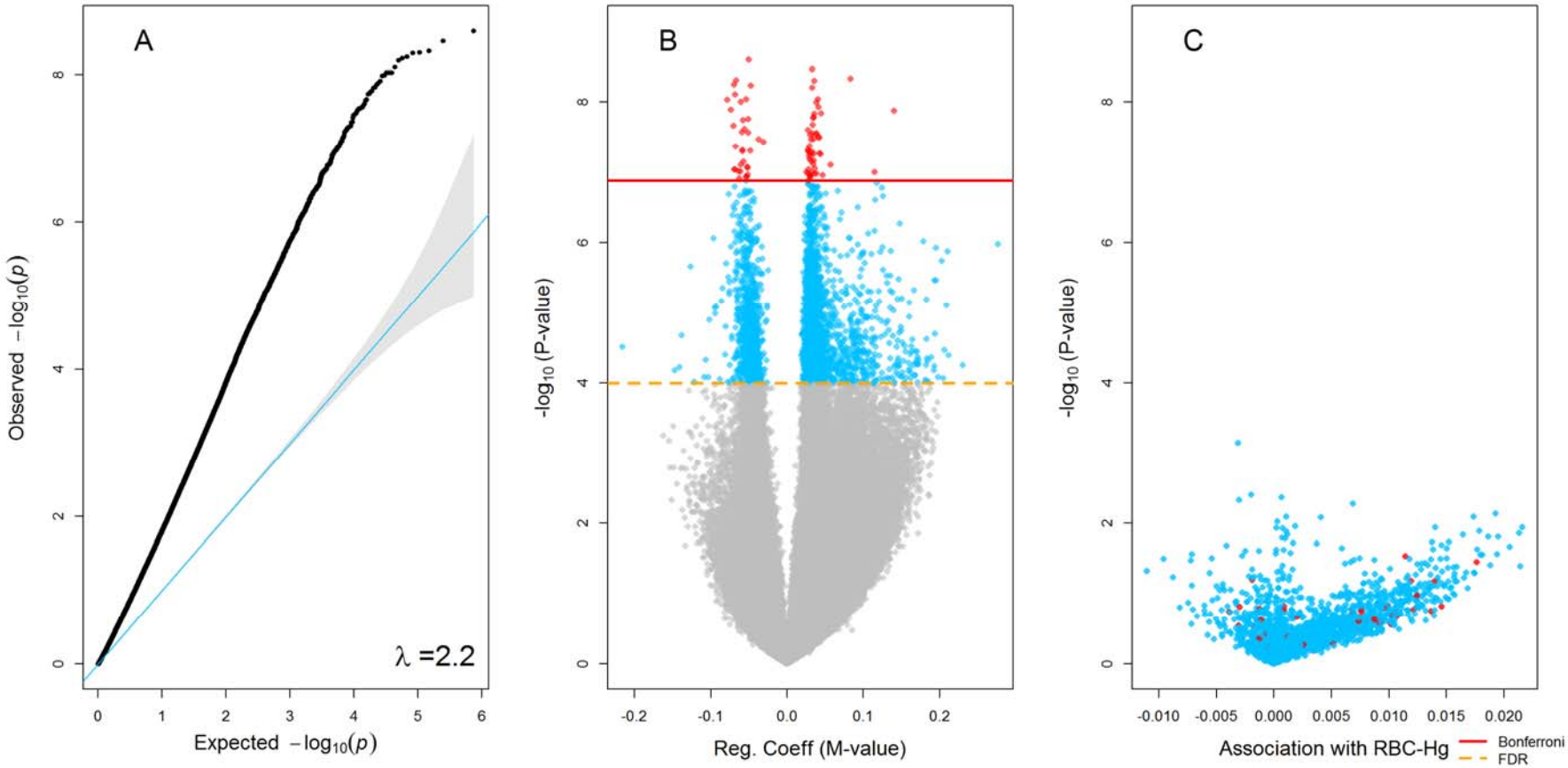


[a] Estimates from linear regression models adjusted for maternal education, age at enrollment, marital status, 1st trimester vitamin B-12 intake, 2nd trimester fish consumption, child race/ethnicity, sex, gestational age and birth weight for gestational age z-scores. Additionally, the %-5hmC model was further adjusted for 1st trimester betaine intake while the %-5mC model was adjusted for 1st trimester folate intake. The model for the ratio of 5mC to 5hmC was adjusted for both betaine and folate intake during the 1st trimester. All models were adjusted for estimated cell-type composition (the estimated nRBCs proportion was included in cord-blood models only)

Supplementary Figure S4. Spearman correlations coefficients for CpGs previously associated with prenatal maternal mercury exposure within this cohort and global measures of DNA methylation (%-5mC) and DNA hydroxymethylation (%-5hmC). Color saturation indicates strength of spearman correlation coefficient, white indicates non-significant correlations $P>0.05$.

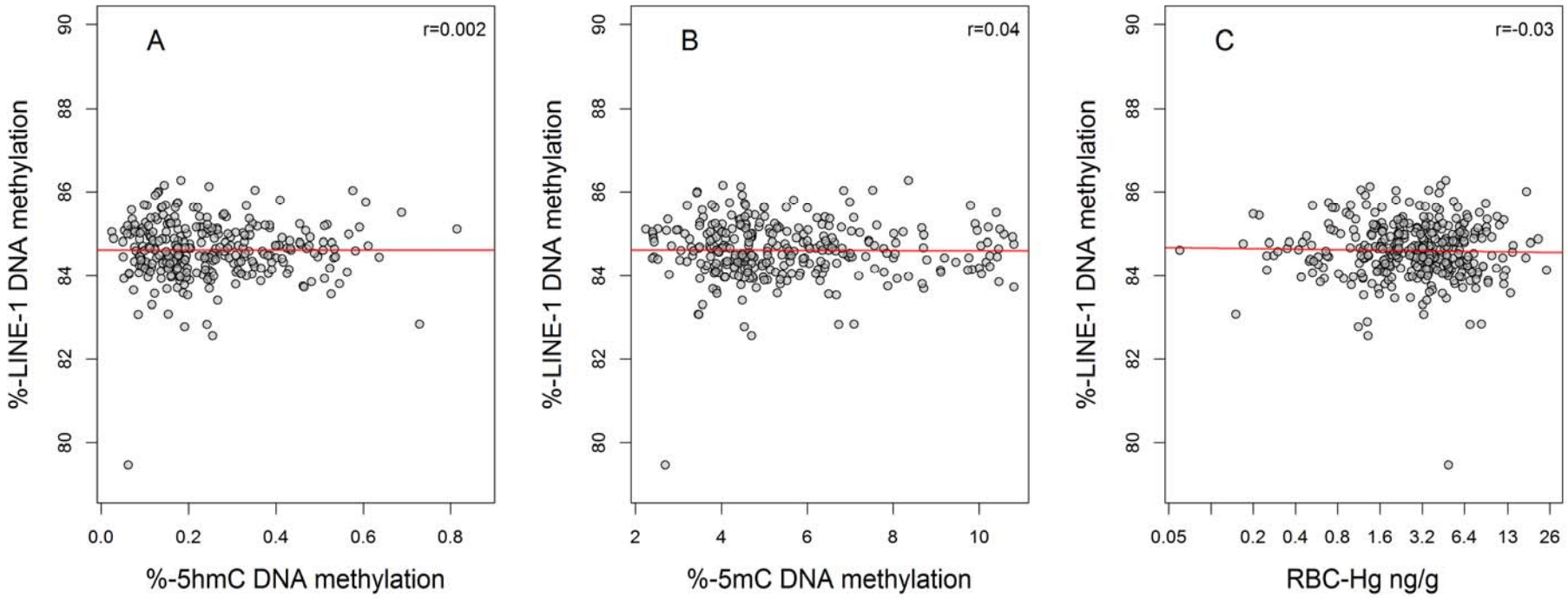


Supplementary Figure S5. [a]Adjusted EWAS of %-5mC and DNA methylation from the 450K in cord blood: (**A**) QQ-plot and genomic inflation (**B**) volcano-plot and multiple comparison adjustment and (**C**) volcano-plot for the association of prenatal maternal RBC-Hg and 2,415 CpGs significantly associated with %-5hmC (No CpG passed the FDR<0.05 for the 2,415 tests performed in C).



[a] EWAS adjusted for maternal age, parity, any smoking during pregnancy, college education, pre-pregnancy BMI, marital status, household income and estimated b12, folate and betaine intake during pregnancy. Child gestational age at birth, z-score of birthweight for gestational age, sex and race. Models were also adjusted for estimated cell type proportions from DNA methylation arrays (monocytes, granulocytes, CD8, CD4, NK-cells, B-cells and nucleated red-blood cells). Data were ComBat adjusted for sample plate. All CpG-by-CpG analyses were performed using robust linear regression models.

Supplementary Figure S6. Scatter plots and spearman correlations for the association of LINE-1 DNA methylation in cord blood and **A**) %-5hmC in cord blood **B**) %-5mC in cord blood and **C**) prenatal maternal RBC-Hg concentration (red: fitted regression line). *P*>0.30 for all correlations.



Supplementary Methods: Simultaneous quantification of %-5hmC and %-5mC by UPLC-MS/MS

Sample preparation:
Isolated DNA (1 µg) was enzymatically hydrolyzed to individual deoxyribonucleosides by a simple one-step DNA hydrolysis procedure (Godderis et al., 2014). A digest mix was prepared by adding phosphodiesterase I, alkaline phosphatase and benzonase® Nuclease to Tris-HCl buffer. Extracted DNA was spiked with internal standard mixture, dried and then hydrolyzed at 37°C for at least 8 h in presence of 10 µL digest mix. After hydrolysis, 490 µL ACN : H_2O (90:8, v/v) was added to each sample. Daylight has been avoided at maximum over the entire sample preparation procedure, in order to minimize potential deamination of the target compounds.

Chromatographic conditions (Waters® Acquity UPLC™):
A 20 µL aliquot was injected on a hydrophilic interaction liquid chromatography (HILIC) column (Phenomenex Kinetex 2.6 µm Hilic, 50 mm x 4.6 mm), held at 60°C. Chromatographic separation was achieved using a mixture of 20mM Ammonium Format Buffer pH3 (A) and acetonitrile (B) and the following gradient: the program starts at 13%A, was increasing linearly to 20%A from 0.1 to 2.2 min, then was hold from 2.2 to 2.4 min at 20%A, brought back to the initial status from 2.4 to 2.6 min and finally allowed to stabilized for another minute before the following injection. A flow rate of 0.4 mL/min was applied.

MS/MS parameters (Waters® Micromass Quattro Premier™ Mass Spectrometer):
The analyses were performed using electrospray ionization (ESI) in positive mode and the compounds were determined using multiple reactions monitoring (MRM), with argon as the collision gas (Supplementary Table S2).

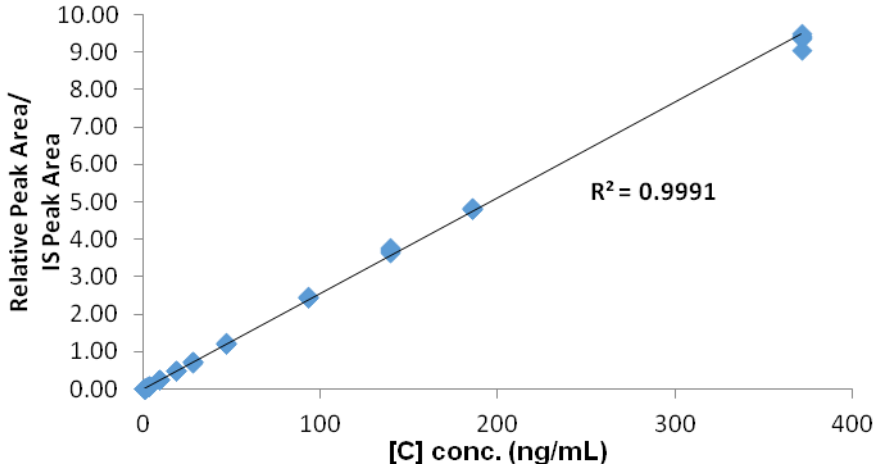
Supplementary Table S3. MS/MS parameters for specific detection by MRM for each target compound.

Compounds	Ionization mode	Transitions (m/z)	Collision energy (eV)	Cone (V)
5-methylcytosine (5mC)	ESI+	242 → 126	12	15
5-hydroxymethylcytosine (5hmC)	ESI+	258 → 142	10	15
Cytosine (C)	ESI+	228 → 112	15	12
[$^{15}N_3$]-2'-deoxycytidine (IS)	ESI+	231 → 115	15	12

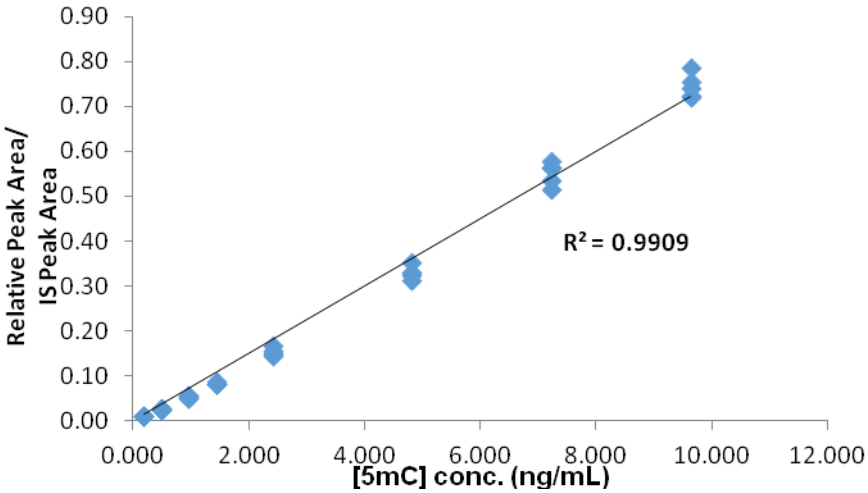
Method validation
The UPLC-MS/MS methods were validated according to the international rules (ICH Topic Q2B and Q2A; Riley, 1996; Causon, 1997). Stock solutions of Cytosine**,** 5-methylcytosine, and 5-hydroxymethylcytosine (Sigma-Aldrich), were prepared by dissolution of solid reference standards in water. The stock solutions were used to prepare the calibration standards. To compensate the matrix effects the validation was conducted using an artificial matrix simulating a mammalian DNA hydrolysate comprising three 2'-deoxyribonuleosides (2'-deoxiguanisine, 2'-deoxyadenosine, and thymidine from Sigma-Aldrich) (Brink et al., 2006). Stable-isotope-labeled derivative ([$^{15}N_3$]-2DC, 2'-deoxycytidine, $^{15}N_3$, 96-98%, Cambridge Isotope Laboratories) was used to overcome any potential losses. The correlation coefficients, R^2, of the regression equations exceeded the value of 0.99, demonstrating a good correlation between the measured response (peak area) and the concentration of the target compounds (Supplementary Figure S3). The limits of quantification were determined based on the lowest calibration levels analyzed in five replicates and corresponding to the following performance criteria: accuracy within the interval 85-115% of the target level and repeatability with a relative standard deviation lower than 15% (Supplementary table S3).

Supplementary Figure S7. Calibration curves for **A)** Cytosine [C], **B)** 5-methylcytosine [5mC], and **C)** 5-hydroxymethylcytosine [5hmC].

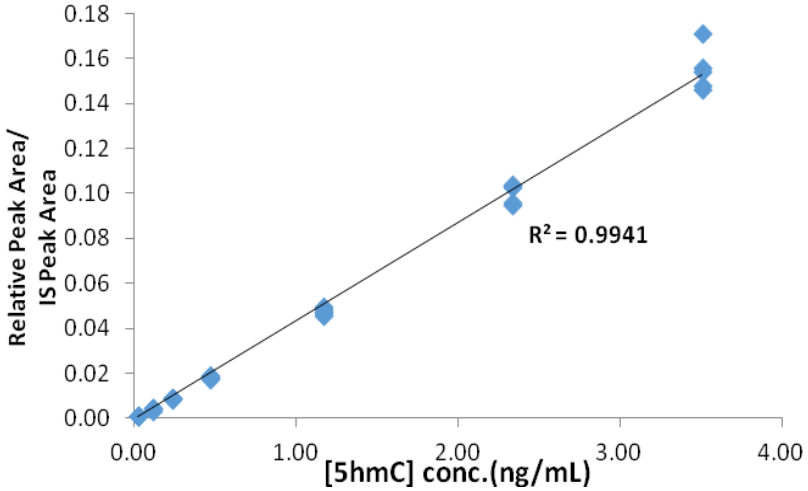
A)



B)



C)



Supplementary Table S4. Parameters of validation of the UPLC-MS/MS method for the determination of global DNA methylation and hydroxymethylation.

Compounds	5mC	5hmC	C
Linearity			
Domain (ng/mL)	0.482 - 9.64	0.023 - 3.51	1.856 - 371.2
R^2	0.9909	0.9942	0.9991
Lower limits of detection (LoD) and quantification (LoQ)			
LoD	0.096	0.008	0.619
LoQ	0.482	0.023	1.856
Accuracy (% of the target)			
Intra-batch (n=5)			
Level 1[a]	95.2	107.8	101.8
Level 2[b]	87.0	90.7	97.2
Level 3[c]	106.1	98.7	94.9
Inter-batch (n=15)			
Level 1[a]	90.6	97.3	101.6
Level 2[b]	87.1	89.4	97.6
Level 3[c]	114.8	95.7	96.3
Precision (RSD %)			
Intra-batch (n=5)			
Level 1[a]	4.5	12.1	0.8
Level 2[b]	3.7	6.6	1.8
Level 3[c]	5.7	3.3	1.2
Inter-batch (n=15)			
Level 1[a]	9.5	11.5	3.6
Level 2[b]	9.9	9.2	2.4
Level 3[c]	8.6	10.9	1.9

[a] – Level 1 corresponds to the following concentration: 0.482 ng 5mC/mL, 0.234 ng 5hmC/mL, and 9.280 ng C/mL; [b] – Level 2 corresponds to the following concentration: 4.820 ng 5mC/mL, 1.170 ng 5hmC/mL; and 27.840 ng C/mL; [c] – Level 3 corresponds to the following concentration: 9.640 ng 5mC/mL; 2.340 ng 5hmC/mL; and 139.20 ng C/mL.

References:

Godderis, L., Schouteden, C., Tabish, A., Poels, K., Hoet, P., Baccarelli, A., Van Landuyt, K. (2014). Global Methylation and Hydroxymethylation in DNA from Blood and Saliva in Healthy Volunteers. BioMed Research International, art.nr. 845041, 1-9.

ICH Topic Q2B: Validation of Analytical Procedures: Methodology, step 4 (CPMP/ICH/281/95).

ICH Topic Q2A: Validation of Analytical Methods: Definitions and Terminology, step 5 (CPMP/ICH/381/95)

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Brink A, Lutz U, Völkel W, Lutz WK. (2006) Simultaneous determination of O6-methyl-2'-deoxyguanosine, 8-oxo-7,8-dihydro-2'-deoxyguanosine, and 1,N6-etheno-2'-deoxyadenosine in DNA using on-line sample preparation by HPLC column switching coupled to ESI-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci.; 830(2):255-61.