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Epigenetic Changes Due to Prenatal Environment

Prenatal maternal antidepressants, anxiety, and depression and offspring DNA methylation: epigenome-wide associations at birth and persistence into early childhood

Abstract

Background: Maternal mood disorders and their treatment during pregnancy may have effects on the offspring epigenome. We aim to evaluate associations of maternal prenatal antidepressant use, anxiety, and depression with cord blood DNA methylation across the genome at birth and test for persistence of associations in early and mid-childhood blood DNA.

Methods: A discovery phase was conducted in *Project Viva*, a prospective pre-birth cohort study with external replication in an independent cohort, the *Generation R Study*. In Project Viva, pregnant women were recruited between 1999 and 2002 in Eastern Massachusetts, USA. In the Generation R Study, pregnant women were recruited between 2002 and 2006 in Rotterdam, the Netherlands. In Project Viva, 479 infants had data on maternal antidepressant use, anxiety, depression, and cord blood DNA methylation, 120 children had DNA methylation measured in early childhood (~ 3 years), and 460 in mid-childhood (~ 7 years). In the Generation R Study, 999 infants had data on maternal antidepressants and cord blood DNA methylation. The prenatal antidepressant prescription was obtained from medical records. At-mid pregnancy, symptoms of anxiety and depression were assessed with the Pregnancy-Related Anxiety Scale and the Edinburgh Postnatal Depression Scale in Project Viva and with the Brief Symptom Inventory in the Generation R Study. Genome-wide DNA methylation was measured using the Infinium HumanMethylation450 BeadChip in both cohorts.

Results: In Project Viva, 2.9% (14/479) pregnant women were prescribed antidepressants, 9.0% (40/445) experienced high pregnancy-related anxiety, and 8.2% (33/402) reported symptoms consistent with depression. Newborns exposed to antidepressants in pregnancy had 7.2% lower DNA methylation (95% CI, − 10.4, − 4.1; $P = 1.03 \times 10^{-8}$) at cg22159528 located in the gene body of *ZNF575*, and this association replicated in the Generation R Study ($\beta = -2.5\%$; 95% CI − 4.2, − 0.7; $P = 0.006$). In Project Viva, the association persisted in early ($\beta = -6.2\%$; 95% CI − 10.7, − 1.6) but not mid-childhood. We observed cohort-specific associations for maternal anxiety and depression in Project Viva that did not replicate.

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Conclusions: The *ZNF575* gene is involved in transcriptional regulation but specific functions are largely unknown. Given the widespread use of antidepressants in pregnancy, as well as the effects of exposure to anxiety and depression, implications of potential fetal epigenetic programming by these risk factors and their impacts on development merit further investigation.

Keywords: Maternal depression, Maternal anxiety, Antidepressants, DNA methylation, Fetal programming

Background

Anxiety and depression are common during pregnancy, affecting up to 8% and 12% of pregnant women, respectively [1–3]. Prenatal anxiety and depression are associated with poor perinatal outcomes including suboptimal fetal growth [4, 5] and preterm birth [6]. While generally thought to be safe, medications to treat mood disorders in pregnancy have been associated with risks of adverse long-term consequences for children including impaired neuromotor development [7] as well as behavioral and emotional problems [8–11].

Antidepressants such as selective serotonin reuptake inhibitors (SSRIs) are used to reduce symptoms of anxiety and depression in approximately 8% of US pregnant women [12]. Two recent reviews of the literature found that fetuses exposed to antidepressants such as SSRIs may have abnormal motor and heart rate activity during fetal development [13, 14]. It is well established that early-life environments may influence fetal and later child development [15]. Epigenetic processes during fetal development are one pathway by which environmental factors may affect phenotype later in life [16]. Whether antidepressants or the underlying psychopathology in pregnancy affects fetal programming through epigenetic processes such as DNA methylation remains unknown.

Epigenome-wide association studies (EWAS) can be a powerful tool to discover biomarkers of disease and to understand biologic processes [17]. Using an epigenome-wide approach, we aimed to identify differences in DNA methylation in neonates associated with prenatal maternal antidepressant use, anxiety, and depression. We hypothesized that prenatal maternal exposure to antidepressants, anxiety, and depression would lead to differences in DNA methylation in cord blood that would persist into childhood.

Results

Overall, in Project Viva, 2.9% (14/479) of women were prescribed antidepressants during pregnancy, 9.0% (40/445) experienced high pregnancy-related anxiety, and 8.2% (33/402) reported symptoms consistent with depression in pregnancy. In the Generation R Study, there were 999 mother-infant pairs eligible for analyses, 1.4% (14/999) were prescribed antidepressants, 5.8% (56/969) experienced high anxiety, and 3.2% (31/969) reported symptoms consistent with clinical depression in pregnancy. Demographic characteristics of participants in both cohorts are presented in Table 1.

In Project Viva, exposure to antidepressants was associated with DNA methylation differences at 130 CpG sites that passed FDR < 0.05, among which 16 sites also passed Bonferroni significance ($P < 1.34 \times 10^{-7}$) in models adjusted for maternal, parity, self-reported race, smoking during pregnancy, body mass index (BMI), mode of delivery, education and infant sex, gestational age at birth, and nucleated cell-type proportions (Table 2). In replication analyses in the Generation R Study, among Bonferroni significant sites discovered in Project Viva, we confirmed that DNA methylation of one of these sites, cg22159528, was significantly lower among infants whose mothers were prescribed antidepressants during pregnancy. This CpG site is located in the body of the Zinc Finger Protein 575 gene (*ZNF575*) on chromosome 19 and annotated to a CpG island. Specifically, in Project Viva, we observed that infants born to mothers prescribed antidepressant in pregnancy had 7.2% lower DNA methylation (95% CI − 10.4, − 4.1; $P = 1 \times 10^{-8}$) at this site and in the Generation R Study, exposed infants had 2.5% lower DNA methylation (95% CI − 4.2, − 0.7; $P = 0.006$) at the same site in adjusted models. In the discovery cohort, we also observed an additional four CpG sites (cg01080902, cg04798919, cg10571104, and cg17970176) near cg22159528 in the *ZNF575* gene that were nominally associated with antidepressant use ($P < 0.05$) in the replication cohort but did not reach a Bonferroni adjusted (0.05/16) levels of significance (Fig. 1). One other CpG site in the replication cohort (cg00367463; *BEST4* gene) passed the $P < 0.05$ criteria for replication but its effect estimate was in the opposite direction.

In Project Viva, we observed 13 individual CpG sites differentially methylated relative to high maternal pregnancy-related anxiety and three individual sites associated with prenatal maternal depression (FDR < 0.05) but these associations were not robust to external replication in the Generation R Study (Additional file 1: Table S1). For single CpG analyses, the genomic inflation factor (λ) was 0.87 for prenatal antidepressants, 1.17 for high anxiety, and 0.94 for depression indicating a reasonable fit (Additional file 2: Figure S1). As a secondary

Table 1 Characteristics of the discovery cohort, Project Viva, and the independent replication cohort, Generation R Study

Characteristics	Discovery cohort Project Viva $N = 479$		Replication cohort Generation R Study $N = 999$	
Maternal	Mean (SD) or *n* (%)			
Age, years	32.1 (5.4)		32.2 (4.3)	
Pre-pregnancy BMI, kg/m^2	24.7 (5.2)		23.4 (3.9)	
Antidepressant use	14 (2.9%)		14 (1.4%)	
High anxiety	40 (9.0%)[a]		56 (5.8%)[b]	
Depression mid-pregnancy	33 (8.2%)[a]		31 (3.2%)[b]	
Race/ethnicity				
	Non-Hispanic White	341 (71.2%)	Dutch	931 (93.2%)
	Non-Hispanic Black	56 (11.7%)	Non-Dutch Western	63 (6.3%)
	Hispanic	37 (7.7%)	Non-western	5 (0.5%)
	Other	45 (9.4%)	–	
College graduate or more education	317 (66.2%)		669 (67.0%)	
Smoking status				
	Never	327 (68.3%)	Never during pregnancy	772 (77.3%)
	Former	100 (20.9%)	Quit when pregnancy was known	93 (9.3%)
	During pregnancy	52 (10.9%)	Continued during pregnancy	134 (13.4%)
Perinatal/infant	Mean (SD) or *n* (%)			
Cesarean delivery	79 (16.5%)		103 (10.3%)	
Gestational age at delivery, weeks	39.8 (1.4)		40.2 (1.4)	
Birth weight-for-gestational age, *z*-score	0.27 (1.0)		0.26 (0.87)[c]	
Female infant	229 (47.8%)		484 (48.4%)	

[a]34 missing data on maternal anxiety and 77 missing data on maternal depression in Project Viva
[b]86 missing data on maternal depression and anxiety in the Generation R Study
[c]1 missing data for birthweight-for-gestational age in Generation R Study

approach, we conducted regional analyses using DMRcate: we did not find any differentially methylated regions relative to prenatal antidepressant prescription, anxiety, or depression in the discovery cohort.

We evaluated the persistence of the observed association at cg22159528 in the *ZNF575* gene for antidepressants and DNA methylation in Project Viva, in blood collected in early and mid-childhood. In adjusted models, prenatally exposed children ($n = 4$ out of 120) had 6.2% lower DNA methylation (95% CI − 10.7 to − 1.6; $P = 6.70 \times 10^{-3}$) compared to non-exposed children in early childhood. This association was in the same direction but attenuated and non-significant in mid-childhood ($\beta = -3.7$, 95% CI − 8.8 to 1.4; $P = 0.16$) ($n = 12$ exposed out of 460). Unadjusted differences in DNA methylation were similar to adjusted differences for exposed and unexposed infants at birth, early, and mid-childhood (Fig. 2).

To evaluate the potential neurological implications of our findings, we tested correlations between blood and brain DNA methylation using external reference data. DNA methylation at cg22159528 in the *ZNF575* gene from over 70 adults showed positive correlations between blood and brain tissue of the prefrontal cortex ($r = 0.54$, $P = 6.45 \times 10^{-7}$), entorhinal cortex ($r = 0.41$, $P = 2.33 \times 10^{-4}$), superior temporal gyrus ($r = 0.49$, $P = 7.87 \times 10^{-8}$) but not the cerebellum ($r = -0.01$, $P = 0.97$) (Fig. 3). These results must be interpreted with caution given that reference blood and brain samples were collected from adults and might not accurately reflect variation in cord blood or early childhood blood samples with brain DNA methylation.

Discussion

Using an agnostic epigenome-wide approach, we observed differences in DNA methylation across multiple CpG sites for infants prenatally exposed to maternal antidepressants and replicated this observation at one CpG site. While 13 CpG sites were associated with high maternal prenatal anxiety and three with prenatal maternal depression in Project Viva, we did not confirm these associations in the Generation R Study. In both Project Viva and Generation R Study, antidepressant prescription during pregnancy was associated with lower DNA methylation at a CpG site located within the *ZNF575* gene body. Exposure to antidepressants during pregnancy was also associated with lower DNA methylation at this site in early childhood blood with a similar

Table 2 Differentially methylated CpGs in umbilical cord blood DNA associated with prenatal maternal antidepressants in pregnancy

CpG	Chr	Genomic Position	Gene	Discovery cohort Project Viva ($n = 479$)			Replication cohort Generation R Study ($n = 999$[c])		
				Mean (SD) %-DNA methylation	Adjusted % change in DNA methylation (95% CI)[a]	*P*	Mean (SD) %-DNA methylation	Adjusted % change in DNA methylation (95% CI)[b]	*P*
cg00367463	1	45,249,899	*BEST4*	1.9 (0.4)	0.26 (0.16, 0.37)	2.01×10^{-8}	10.3 (2.7)	− 0.53 (− 1.02, − 0.03)	0.04
cg27566858	1	208,084,099	*CD34*	2.0 (0.3)	0.27 (0.15, 0.39)	3.13×10^{-9}	12.6 (3.3)	0.26 (− 0.53, 1.06)	0.52
cg03536711	1	221,509,067	*LOC400804*	53.3 (11.4)	− 11.63 (− 15.80, −7.45)	3.24×10^{-8}	54.9 (9.3)	2.25 (− 1.53, 6.02)	0.24
cg07729367	3	128,479,008	*RAB7A*	98.2 (0.5)	− 0.49 (− 0.70, − 0.27)	1.08×10^{-7}	90.6 (2.1)	− 0.31 (− 1.36, 0.75)	0.57
cg22065513	3	144,241,532		97.1 (1.4)	− 1.22 (− 1.73, − 0.72)	4.11×10^{-9}	90.7 (2.0)	− 0.36 (− 1.33, 0.61)	0.47
cg27299660	3	171,527,797	*PLD1*	1.7 (0.4)	0.77 (0.49, 1.04)	1.19×10^{-7}	8.3 (2.3)	0.11 (− 0.55, 0.77)	0.75
cg14499053	7	19,158,954		2.2 (0.5)	− 0.33 (− 0.45, − 0.22)	5.44×10^{-8}	11.5 (2.1)	0.35 (− 0.48, 1.19)	0.41
cg15881597	7	73,085,754	*VPS37D*	40.1 (4.9)	− 3.64 (− 5.09, − 2.19)	5.66×10^{-9}	47.3 (4.7)	− 1.82 (− 4.06, 0.42)	0.11
cg06645921	12	8,025,394	*SLC2A14*	6.6 (3.7)	− 2.38 (− 2.95, − 1.81)	9.11×10^{-16}	*NA*	*NA*	*NA*
cg27161197	12	47,224,649		71.5 (5.9)	− 5.17 (− 7.12, − 3.23)	1.77×10^{-9}	68.9 (5.5)	− 2.01 (− 5.23, 1.21)	0.22
cg25121621	15	45,926,780	*SQRDL*	19.6 (2.9)	2.74 (1.82, 3.67)	2.71×10^{-12}	28.4 (4.2)	− 0.80 (− 4.37, 2.78)	0.66
cg06358612	17	28,619,293	*BLMH*	1.4 (0.3)	0.22 (0.14, 0.31)	6.07×10^{-8}	8.1 (1.4)	− 0.18 (− 0.71, 0.34)	0.49
cg22159528	*19*	*44,039,727*	*ZNF575*	*51.0 (6.8)*	*− 7.23 (− 10.36, − 4.10)*	1.03×10^{-8}	*54.7 (5.9)*	*− 2.46 (− 4.23, − 0.69)*	*0.006*
cg12489353	19	48,231,499	*EHD2*	79.3 (14.5)	− 10 (− 15.7, − 4.3)	7.88×10^{-8}	77.2 (10)	− 0.80 (− 6.49, 4.88)	0.78
cg11449935	20	35,202,477	*TGIF2*	2.2 (0.4)	0.29 (0.16, 0.43)	4.20×10^{-8}	10.2 (2.2)	0.31(− 0.52, 1.13)	0.47
cg18036763	22	45,404,910	*PHF21B*	10.2 (3.6)	3.24 (1.75, 4.74)	7.62×10^{-8}	6.2 (1.7)	0.38 (− 0.34, 1.11)	0.30

Abbreviations: *SD* standard deviation, *CI* confidence interval, *NA* excluded from the replication cohort (Generation R Study) after standard quality control
[a]Adjusted for maternal age, parity, race/ethnicity, smoking (never, former and during pregnancy), pre-pregnancy BMI, mode of delivery, education and infant sex, gestational age, and estimated cord blood nucleated cells (CD8, CD4, Mono, NK, B cells, granulocytes and nRBCs)
[b]Adjusted for the same covariates as above and included sample plate as an additional covariate
[c]Refers to sample size for cg22159528, whereas other models from Generation R Study excluded samples that failed at specific CpG sites (smallest sample size $n = 970$ for cg18036763)

magnitude of the effect. Using a blood and brain DNA methylation reference database, we observed moderate correlations among three brain regions with blood cells at the discovered and validated CpG site in the *ZNF575* gene.

The Zinc Finger Protein 575 gene (*ZNF575*) is part of a large family of zinc finger proteins with multiple diverse functions that are abundant across multiple eukaryotic genomes [18]. This protein is involved in transcriptional regulation and has been previously associated with lung cancer [19]. Otherwise, there is very little known about the function of the *ZNF575* gene and its role in health or development. This top finding was persistent in early, at approximately 3 years of age, but not in mid-childhood in the discovery cohort. This is important, as the first 1000 days of life represent a period of rapid development and vulnerability able to influence the life course further stressing the need to fully characterize the function of the *ZNF575* gene.

There were an additional 15 CpG sites in cord blood associated with maternal antidepressant use that survived Bonferroni correction in Project Viva, but they were not replicated in The Generation R Study. Six prior studies of in utero antidepressant exposure and offspring DNA methylation were recently systematically reviewed by Viuff et al. [20]. The authors concluded that there was no consistent association among studies and highlighted the need for untargeted epigenetic assays with external validation [20]. None of the prior studies reported differentially methylated sites at/near *ZNF575*. Three of the studies used a candidate gene approach [21–23]; two used an earlier epigenome-wide array which analyzed only 27,000 CpG sites [24, 25]. Only one prior study by Non et al. examined associations between maternal SSRI use and offspring DNA methylation using the same DNA methylation platform as we did [26]. They used a case-control design of 22 exposed infants and 23 unexposed infants and found no significant association between SSRIs and offspring DNA methylation. In addition, Non and colleagues selected infants exposed to SSRIs that differ from the medications used in our population and did not adjust for cell-type composition. Lack of consistency found in the literature may be a result of differences in study design, population, technology for DNA methylation assessment, and smaller sample sizes as well as exposure timing and ascertainment.

A few of the cohort-specific associations of high levels of pregnancy-related anxiety with DNA methylation in Project Viva were consistent with prior literature. For

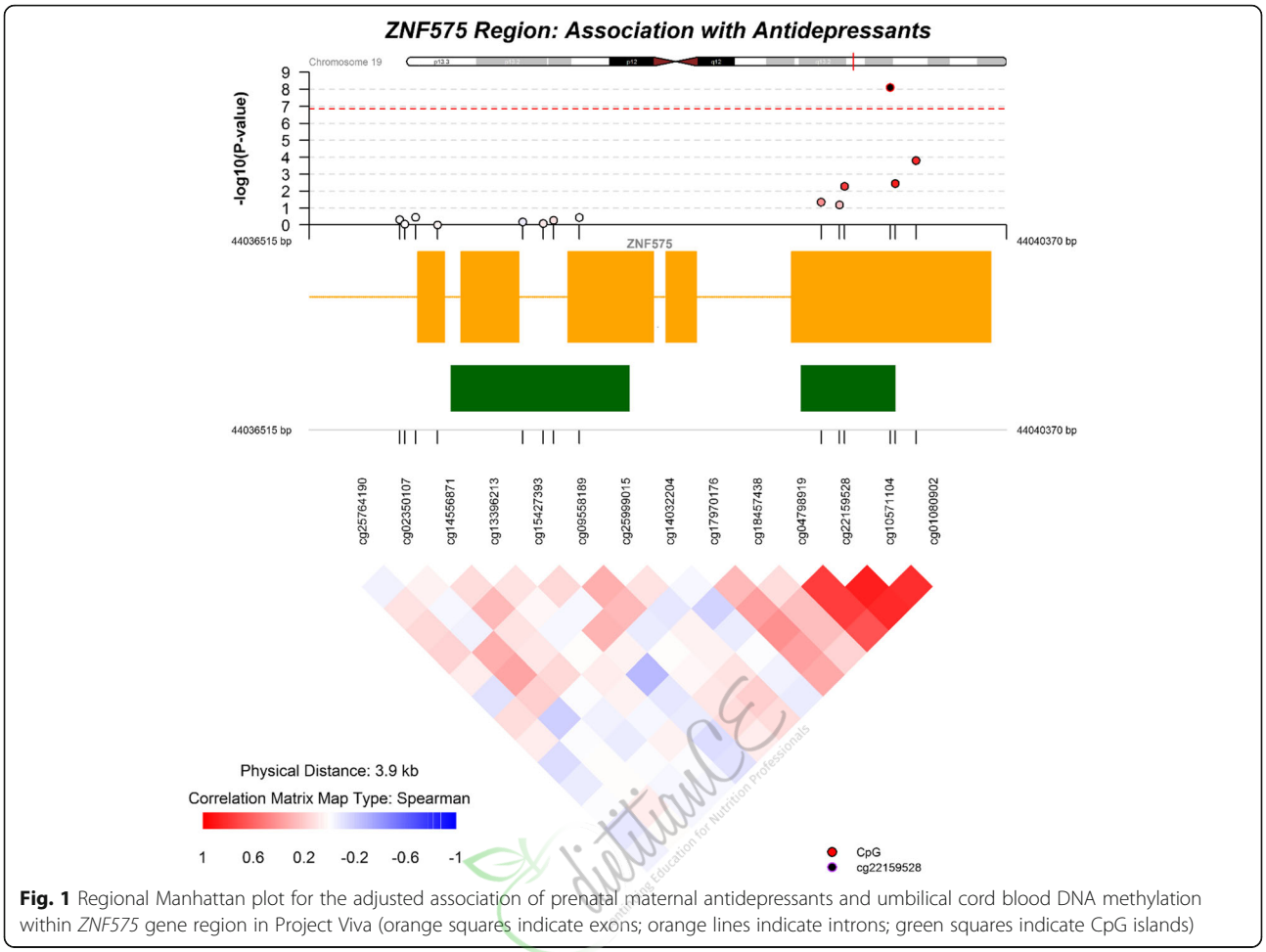

Fig. 1 Regional Manhattan plot for the adjusted association of prenatal maternal antidepressants and umbilical cord blood DNA methylation within *ZNF575* gene region in Project Viva (orange squares indicate exons; orange lines indicate introns; green squares indicate CpG islands)

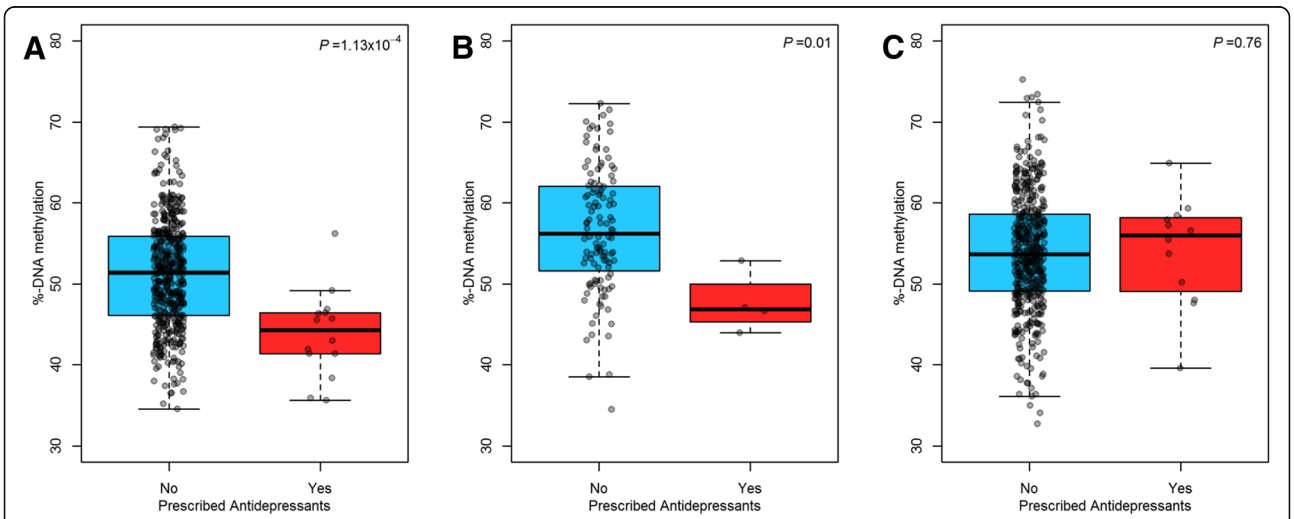

Fig. 2 Unadjusted %-DNA methylation distribution for antidepressants exposed and unexposed infants at the replicated CpG site (cg22159528) in the *ZNF575* gene and unadjusted Wilcoxon-rank sum test *P* value in the discovery cohort, Project Viva, measured at three time points: **a** umbilical cord blood ($n = 479$), **b** early childhood ($n = 120$), and **c** mid-childhood peripheral blood ($n = 460$). One hundred twelve participants in early childhood also had cord blood measurements, and 235 participants from mid-childhood also had cord blood measurements

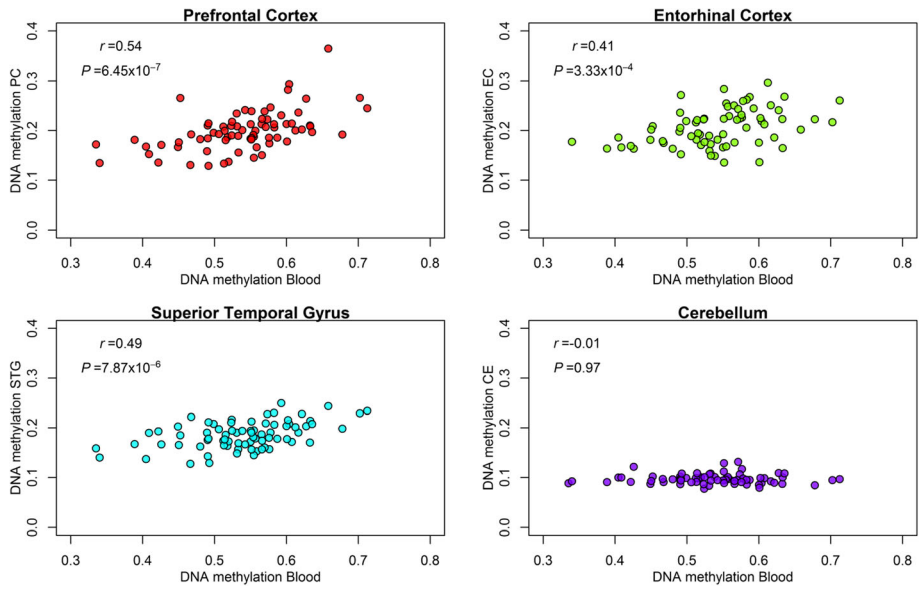

Fig. 3 Scatterplots and correlations for cg22159528 (*ZNF575* gene) methylation levels of blood DNA and four brain regions: prefrontal cortex (PC, $n = 74$), entorhinal cortex (EC, $n = 71$), superior temporal gyrus (STG, $n = 75$), and cerebellum (CE, $n = 71$). Samples from external paired dataset of blood and brain tissue of adults [51].

example, a CpG site in the glial cell-derived neurotrophic factor (*GDNF*) gene showed higher DNA methylation compared to low or moderate pregnancy-related anxiety. Using blood samples, a study on inflammatory markers of women with antenatal depression found DNA methylation at another CpG site near *GDNF* to be higher among depressed pregnant women [27]. Also, in a mouse model, DNA methylation of the *GDNF* gene in experimentally stressed mice has been shown to be differentially methylated relative to stress. These experiments also showed that chronic stress reduced levels of a histone modification, H3K4me3, in the promoter region of the *GDNF* gene and this effect was reversed by antidepressants [28].

Our study has several limitations. In Project Viva, over seven types of antidepressants were used during pregnancy with some women prescribed more than one single type, although 12/14 were SSRIs (Additional file 1: Table S2). In the Generation R Study, antidepressant prescriptions were limited to tricyclic antidepressants and SSRIs. In addition, in the Generation R Study, general anxiety during pregnancy was ascertained while pregnancy-related anxiety was evaluated in Project Viva. These are different scales and could capture different sources and levels of anxiety. Moreover, we measured DNA methylation only in blood and it is likely that blood may not accurately reflect DNA methylation variability in other relevant tissues. However, we used external brain and blood reference DNA methylation data to compare correlations at the externally replicated site. Our hypothesis was based on DNA methylation programming during fetal development. However, another possibility is that of cellular polycreodism—or the systematic variability of cell fate to yield a distinctive repertoire of cells [29]. Yet, without experimental data, it is impossible to determine the true causal effects of these exposures in the epigenome and therefore results should be interpreted as biomarkers. It would be nearly impossible to conduct a randomized trial for these prenatal maternal exposures and conditions. Additionally, timing, severity, and accuracy of self-reported depression and pregnancy-related anxiety along with medication adherence for antidepressants and repeated exposure could introduce substantial exposure misclassification making it challenging to capture underlying associations. Further, any study of the effects of medications can be affected by confounding by indication. Specifically, it may be the most depressed or anxious women who were treated with antidepressants and that those underlying causes were truly responsible for the observed associations. This issue is further complicated by the small overlap of women who exhibited symptoms of anxiety ($n = 2$) or depression ($n = 4$) among the treated women in Project Viva. The two cohorts also differed from one another, especially with respect to ethnicity (Table 1), which may have limited our ability to replicate findings across different populations. Lastly, our antidepressant-exposed sample was small, limiting statistical power.

Our study also has important strengths. First, we implemented an epigenome-wide approach to agnostically capture associations with a relatively large sample size. Our prospective design reduces the chance of bias

that might arise from case-control studies and allowed us to collect valuable confounder information early during pregnancy. Another major strength is the replication of findings in an independent birth cohort. Our use of an external reference dataset demonstrated moderate to strong correlations between DNA methylation of blood and three brain regions in the replicated site (*ZNF575*), suggesting that this finding may be relevant to long-term mental health or neurodevelopment. Yet, more work is needed to fully characterize the function of the *ZNF575* gene.

Conclusion

In conclusion, we found DNA methylation of the *ZNF575* gene in infant cord blood to be associated with maternal antidepressant use in pregnancy in two independent cohorts. We also demonstrated that this association persists into early childhood. These findings warrant further study to confirm the association and to determine its clinical significance.

Methods

Discovery cohort: Project Viva

We studied mother-child pairs participating in Project Viva, a prospective pre-birth cohort study recruited between 1999 and 2002 from Atrius Harvard Vanguard Medical Associates in MA, USA [30]. Mothers provided written, informed consent, and the institutional review board of Harvard Pilgrim Health Care approved the study. Of the total 2128 singleton births, there were 485 infants with cord blood DNA methylation data and information on prenatal maternal antidepressants, anxiety, and depression. We excluded 6 infants with gestational age < 34 weeks and analyzed 479 mother-infant pairs with cord blood DNA methylation. We evaluated persistence of epigenetic associations observed at birth in 120 children ($n = 112$ included in cord blood analyses) with peripheral blood DNA methylation measurements from early childhood (mean 3.4 years, range 2.9 to 5.3) and 460 children ($n = 235$ included in cord blood analyses) with peripheral blood DNA samples from mid-childhood (mean 7.9 years, range 6.7 to 10.5).

We defined women as exposed to antidepressants if the medical record included a prescription during pregnancy (Additional file 1: Table S2). To assess anxiety, at the mid-pregnancy visit, we administered the 7-item Pregnancy-Related Anxiety Scale (PRAS) [31]. Answers are on a 4-point Likert scale (very much, moderately, somewhat, and not at all). The scale captures worry about fetal growth, health, and delivery method. The PRAS specifies three categories of anxiety levels (low, moderate, and high) with good reliability (Cronbach alpha = 0.78) [32]. We classified mothers as having high pregnancy-related anxiety if they chose "very much" to three or more questions on the PRAS and all other women served as the reference group. To assess depression at the mid-pregnancy visit, we administered the Edinburgh Postnatal Depression Scale (EPDS) [33], a 10-item questionnaire screening for depressive symptoms. Answers are on a 4-point Likert scale from 0 to 3. The EPDS is a validated screener for probable depression but it is not intended to diagnose clinical depression. The scale has been validated in pregnant women and has a sensitivity of 86% and a specificity of 78% for the diagnosis of depression [33, 34]. A score ≥ 13 on the 0–30 scale indicates probable prenatal depression [35, 36].

To assess DNA methylation, we used umbilical cord blood collected at delivery and whole blood samples from early and mid-childhood visits. Technicians extracted DNA using the Qiagen Puregene Kit (Valencia, CA) and stored aliquots at − 80 °C until analysis. DNA underwent sodium bisulfite conversion using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA). Samples were shipped to Illumina Inc. and analyzed for DNA methylation at > 485,000 CpG sites simultaneously using the Infinium Human Methylation450 BeadChip (Illumina, San Diego, CA).

We used a two-stage algorithm in which we randomized 12 samples to each chip and then randomly assigned eight chips to each of the 15 plates used to ensure balance by sex across chips and plates. We excluded samples as potentially mislabeled if they were mismatched on sex, genotype or were deemed to be low in quality. Background correction and dye-bias equalization was performed via the normal-exponential out-of-band (*noob*) method [37], and a β-mixture quantile intra sample normalization procedure (*BMIQ*) was applied to the data to reduce the potential bias that can arise from probe design [38]. For each CpG site, methylation is reported as average β value $= M/(M + U + \varepsilon)$, where M and U represent the average fluorescence intensity from each probe corresponding to the methylated and unmethylated target CpG and $\varepsilon = 100$, a small quantity to protect against division by zero. Thus, the average β value is an interval-scaled quantity between zero and one interpreted as the fraction of DNA molecules whose target CpG is methylated across all nucleated cells. We excluded individual probes if they had non-significant detection P values ($P > 0.05$) for more than 1% of the samples. Additionally, non-CpG probes (probes for SNPs (rs) and methylated sites other than cytosine (ch)), probes in X and Y chromosomes, SNP-associated probes at either the single base extension or within the target region were removed for SNPs that have a minor-allele frequency of > 5%. Any probe with a SNP ≤ 10 base pairs was excluded using annotation from the Bioconductor package IlluminaHumanMethylation450kanno.ilmn12.hg19 that utilized information from dbSNP. Previously identified

non-specific and cross-reactive probes within the array along with polymorphic CpG sites were also excluded from the analysis [39]. We excluded individual probes with values greater than three times the interquartile range (IQR) from the 75th percentile or values less than three times the IQR from the 25th percentile to eliminate potential DNA methylation outliers. We used ComBat [40] to correct for technical variability from plate and scanner. We visually inspected the effectiveness of adjustment for batch using principal components before and after batch adjustment. We calculated the genomic inflation factor (λ) for all three EWAS to evaluate systemic biases.

After quality control, there were 372,563 loci for analysis. We logit-transformed methylation values on the β values (bounded between 0 and 100%) to M values prior to analyses as previously described to be more appropriate for the differential analysis of DNA methylation [41] but report results as %-change in DNA methylation for interpretability.

Replication cohort: the Generation R Study

We pursued external replication of the top differentially methylated sites in Project Viva in an independent birth cohort study, the Generation R Study, based in Rotterdam, the Netherlands. For the Generation R Study, all pregnant women living in Rotterdam with an expected delivery date between April 2002 and January 2006 were asked to participate. In total, 9778 mothers were enrolled [42]. Cord blood DNA methylation was measured using Illumina Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego, USA).

Preparation and normalization of the HumanMethylation450 BeadChip array data were performed according to the CPACOR workflow [43] using the software package *R* [44]. In detail, the idat files were read using the *minfi* package. Probes that had a detection *P* value above background (based on the sum of methylated and unmethylated intensity values) $\geq 1 \times 10^{-16}$ were set to missing per array. Next, the intensity values were stratified by autosomal and non-autosomal probes and quantile normalized for each of the six probe type categories separately: type II red/green, type I methylated red/green, and type I unmethylated red/green. Beta values were calculated as the proportion of methylated intensity value on the sum of methylated + unmethylated + 100 intensities. Arrays with observed technical problems such as failed bisulfite conversion, hybridization or extension, and arrays with a mismatch between sex of the proband and sex determined by the chr X and Y probe intensities were removed from subsequent analyses. Additionally, only arrays with a call rate > 95% per sample were processed further.

A subset of $N = 999$ mother-child pairs had complete information on maternal antidepressant use in pregnancy, and $N = 969$ had complete information on maternal depression and anxiety. Maternal prenatal depression and anxiety were assessed at 20 weeks of pregnancy with the Brief Symptom Inventory [45, 46]. This questionnaire comprises 53 items which provided nine scales of various psychiatric symptoms. The scale has a global index and includes two subscales for anxiety and depressive symptoms. The subscales for anxiety and depression contained six items each on a 5-point scale, from 0 to 4 where a higher score indicates a higher level of symptoms. Antidepressant use was reported during each trimester of pregnancy using a self-reported questionnaire. Use of SSRI was confirmed with prescription records from pharmacies with participant consent. These measurements have been previously described in detail [42, 47, 48].

Statistical analyses

For each covariate in both discovery and replication cohorts, we calculated means and standard deviations (SD), or sample sizes and percentages, to describe the discovery and replication cohorts. In the discovery cohort, we performed epigenome-wide DNA methylation analyses on a CpG-by-CpG basis to assess DNA methylation differences at each site in cord blood relative to prenatal maternal exposure to (1) antidepressant prescription, (2) anxiety, and (3) depression compared to non-exposed infants. We used separate robust linear regression models with heteroskedasticity-consistent estimators to model the methylation levels of each individual CpG on the M value scale as the dependent variable and antidepressants, high pregnancy-related anxiety, and depression as predictors. We adjusted all regression models for variables selected a priori: maternal age, parity, self-reported race, smoking during pregnancy, body mass index (BMI), mode of delivery, education and infant sex, gestational age at birth, and nucleated cell-type proportions in cord blood (CD8+ T cells, CD4+ T cells, monocytes, natural killer cells, B cells, granulocytes, and nucleated red blood cells for cord blood analyses) estimated from the DNAm data using *minfi* [49]. Statistical significance for the CpG-by-CpG analyses was adjusted by controlling the false discovery rate at 5% (FDR < 0.05) for each of the three-independent epigenome-wide analyses. As secondary analyses, we tested for differentially methylated regions in relationship to antidepressant prescription, anxiety, and depression using DMRcate [50] with an FDR < 0.05.

Similarly, in the replication cohort, we fit a robust linear regression with each of the top CpGs from discovery as the outcome for each prenatal maternal exposure and adjusted for similar covariates as we had in discovery. We tested CpG sites associated with prenatal maternal antidepressant use that passed

Bonferroni correction in the discovery cohort due to an early departure from the expected uniform distribution for this EWAS (Additional file 2: Figures S1-S2) and for depression and anxiety among significant differentially methylated sites that passed FDR < 0.05. In replication analyses, we deemed a $P < 0.05$ as statistically significant in addition to having the association be consistent in direction with the discovery cohort.

We also evaluated the persistence of associations in early and mid-childhood in Project Viva by carrying forward individual loci found to be associated with DNA methylation in cord blood analyses that also replicated in the Generation R Study. Persistence of DNA methylation differences was evaluated in peripheral blood samples collected during early and mid-childhood using multivariate robust linear regression models adjusting for the same covariates as cord blood models with the addition of child age at the time of the blood draw. We considered $P < 0.05$ as statistically significant for the persistence of epigenetic alterations in early or mid-childhood peripheral blood analyses. We also investigated unadjusted DNA methylation differences between exposed and unexposed children using boxplots and a Wilcoxon-rank sum test. We present the unadjusted distribution of DNA methylation levels in boxplots by antidepressant prescription given the relative small number of exposed infants. All analyses were carried out using the R statistical package, version 3.4.1 (www.r-project.org/).

Blood-brain DNA methylation samples

We evaluated co-variation between blood DNA methylation and methylation levels of brain regions using publicly available data from the Gene Expression Omnibus (GEO) repository (GSE59685). Briefly, to generate reference data, investigators collected whole blood samples prior to death and matched those samples to postmortem samples from the prefrontal cortex, entorhinal cortex, superior temporal gyrus and cerebellum of $N = 75$ men and women (40–105 years old) [51]. They measured DNA methylation using the Illumina HumanMethylation450 BeadChip Array. Scatter plots and person correlation coefficients for the relationship between blood and brain DNA methylation was examined among sites that replicated in the external cohort.

Additional files

Additional file 1: Table S1. Differentially methylated CpG sites in umbilical cord blood DNA associated with high anxiety and depression (FDR < 0.05 for the discovery cohort, Project Viva) and replication results from the Generation R Study. **Table S2.** Type of prenatal maternal antidepressants prescribed to the 14 unique participants in Project Viva. (DOCX 19 kb)

Additional file 2: Figure S1. Quantile-Quantile plots of observed vs expected *P* values and genomic inflation factor (λ) for Epigenome-Wide Associations of prenatal maternal A) prenatal antidepressants B) high pregnancy-related anxiety and C) depression. **Figure S2.** Manhattan plots for Epigenome-Wide Associations of prenatal maternal A) antidepressants B) high pregnancy-related anxiety and C) depression. (DOCX 852 kb)

Abbreviations

BMI: Body mass index; CI: Confidence interval; EPDS: Edinburgh Postnatal Depression Scale; EWAS: Epigenome-wide association study; PRAS: Pregnancy-Related Anxiety Scale; SD: Standard deviation; SSRIs: Serotonin reuptake inhibitors; *ZNF575*: Zinc Finger Protein 575 gene

Competing interests

The authors declare that they have no competing interests.

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Prenatal adverse environment is associated with epigenetic age deceleration at birth and hypomethylation at the hypoxia-responsive *EP300* gene

Abstract

Background: Obstetric complications have long been retrospectively associated with a wide range of short- and long-term health consequences, including neurodevelopmental alterations such as those observed in schizophrenia and other psychiatric disorders. However, prospective studies assessing fetal well-being during pregnancy tend to focus on perinatal complications as the final outcome of interest, while there is a scarcity of postnatal follow-up studies. In this study, the cerebroplacental ratio (CPR), a hemodynamic parameter reflecting fetal adaptation to hypoxic conditions, was analyzed in a sample of monozygotic monochorionic twins (60 subjects), part of them with prenatal complications, with regard to (i) epigenetic age acceleration, and (ii) DNA methylation at genes included in the polygenic risk score (PRS) for schizophrenia, and highly expressed in placental tissue.

Results: Decreased CPR measured during the third trimester was associated with epigenetic age deceleration ($\beta = 0.21$, $t = 3.362$, $p = 0.002$). Exploration of DNA methylation at placentally expressed genes of the PRS for schizophrenia revealed methylation at cg06793497 (*EP300* gene) to be associated with CPR ($\beta = 0.021$, $t = 4.385$; $p = 0.00008$, FDR-adjusted $p = 0.11$). This association was reinforced by means of an intrapair analysis in monozygotic twins discordant for prenatal suffering ($\beta = 0.027$, $t = 3.924$, $p = 0.001$).

Conclusions: Prenatal adverse environment during the third trimester of pregnancy is associated with both (i) developmental immaturity in terms of epigenetic age, and (ii) decreased CpG-specific methylation in a gene involved in hypoxia response and schizophrenia genetic liability.

Keywords: DNA methylation, Obstetric complications, Prenatal stress, Hypoxia, EP300 gene, Epigenetic clock, Monozygotic twins, Schizophrenia

Main text

Background

Prenatal environment constitutes the first modulating agent the developing fetus encounters as it progresses through gestation. The tremendous impact of any environmental threat occurring during this period for both short- and long-term consequences is now widely accepted and well-known as the Developmental Origins of Health and Disease (DOHaD) hypothesis [1]. Also known as the theory of fetal programming, the embedding of early life and its ability to exert long-term effects in late-life is thought to rely on epigenetic mechanisms [2, 3].

Recently, several DNA methylation-based epigenetic clocks have been developed in order to predict chronological age with high accuracy [4, 5]; afterward, Knight and colleagues developed a new predictor specifically aimed to predict gestational age (GA) in perinatal samples [6]. Although epigenetic and chronological age

robustly show high correlation across studies, the difference between both variables allows the estimation of the so-called age acceleration (i.e., when epigenetic age is higher than chronological age).

On the one hand, epigenetic age acceleration in adult subjects has been associated with cumulative lifetime stress, lifestyle, and all-cause mortality, among others, suggesting its utility as a better predictor for life expectancy than chronological age itself [7–9]. On the other hand, epigenetic GA deceleration (i.e., when chronological age is higher than epigenetic age), as measured in cord blood, has been described in newborns born to women with low socioeconomic status, Sjögren syndrome, insulin-treated gestational diabetes mellitus, and experiencing antenatal depressive symptoms [6, 10, 11]. Such findings suggest that newborns exposed to prenatal stressors are born in an immature state independently of their chronological GA. In this regard, boys—but not girls—who exhibited lower epigenetic GA at birth exhibited more internalizing problems, such as anxious-depressive symptoms or somatic complaints, at follow-up (mean age 3.7 years), suggesting they are born with a developmental disadvantage [11].

Nevertheless, there is a dearth of studies examining the putative relationship between ultrasound parameters acquired during pregnancy and epigenetic GA acceleration. In this regard, the cerebroplacental ratio (CPR) has been reported to be associated with adverse perinatal outcomes not only in growth-restricted fetuses, but also in low-risk population [12, 13]. Briefly, CPR is calculated by dividing the middle cerebral artery (MCA) pulsatility index (PI) by the umbilical artery (UA) PI [14]. The PI is a parameter reflecting vascular impedance or resistance, i.e., decreased blood flow. Specifically, fetal brain blood supply is known to increase in front of hypoxic stimuli thus decreasing PI in the MCA [15]; while placental insufficiency decreases umbilical blood flow hence increasing UA PI, and has been associated with both short- and long-term detrimental outcomes, including increased cardiovascular risk and deficits in cognition [16, 17]. Consequently, a decreased CPR reflects the combination of both alterations and is an indicator of fetal adaptation to adverse conditions [12].

Obstetric complications (OCs) constitute one of the risk factors more reliably associated with psychopathology, particularly with neurodevelopmental disorders; specifically, the putative association between OCs and schizophrenia has been debated since the 1970s [18–20]. In this regard, a recent umbrella review evaluating all published meta-analysis regarding risk factors and biomarkers for schizophrenia spectrum disorders revealed a history of OCs to significantly increase the risk for developing the disorder with an odds ratio of 2 [21]. Furthermore, exposure to severe OCs together with increased genetic vulnerability, as measured with the polygenic risk score (PRS) for schizophrenia, interact to increase the risk to suffer the disorder up to an odds ratio of 8.36 [22]. In the same study, authors further explored the putative relevance of placental expression of genes included in the PRS; following this approach, they reported (i) an enrichment of PRS genes expressed in placental tissue and (ii) differential expression of PRS genes in placentae from complicated pregnancies (specifically in pre-eclampsia and intrauterine growth restriction). Specifically, the described gene-environment interaction between exposure to OCs and the PRS for schizophrenia was driven by those genes highly expressed in placenta and/or dynamically regulated in complicated pregnancies [22]. Since CPR is a robust indicator of prenatal stress and a predictor of perinatal and long-term morbidity, DNA methylation analysis of genes included in the placental PRS for schizophrenia could shed light on the epigenetic mechanisms mediating the interaction between OCs and neurodevelopmental disorders.

Monozygotic twins have been instrumental for the elucidation of environmental and genetic risks in the etiology of complex traits and disorders. Actually, the differential role of the prenatal environment in shaping psychopathological proneness was first described thanks to monozygotic twin designs [23–25]; these pioneering studies focused on dermatoglyphic measures assessed at birth, which can be used as surrogate measures of altered neurodevelopment during the second trimester of pregnancy [26]. Furthermore, monozygotic twin pregnancies and, more specifically, monochorionic twin pregnancies—i.e., those in which both fetuses share the placenta—are at a higher risk of obstetric complications, the more prevalent being twin-to-twin transfusion syndrome (TTTS) and selective intrauterine growth restriction (sIUGR) [27–29]. Thus, the thorough and prospective ultrasound assessment of prenatal development through monochorionic twin pregnancies offers a quasi-experimental study design in which the genetic and environmental components of epigenetic variability can be dissected.

The objective of the current study was to investigate whether prenatal adverse environment (i) alters human development in terms of epigenetic age, and if (ii) it can get embedded through epigenetic mechanisms in genes previously identified as risk factors for schizophrenia acting during prenatal stages. We hypothesized that a higher exposure to prenatal adverse environment would be associated with (i) delayed development and (ii) DNA methylation at genes involved in the pathogenesis of schizophrenia. While CPR can have diverse effects on genome-wide DNA methylation, with potential relevance for a multitude of phenotypes, the present study a priori

examined how CPR epigenetically regulates risk genes for schizophrenia, previously described to interact with the presence of OCs [22].

Results

GA estimation using Knight's epigenetic clock

After exclusion of two twin pairs (see Methods section), the final sample size was 30 twin pairs. The mean GA at birth of our twin cohort (n = 30 twin pairs) was 35.3 weeks (range = 31.7–37.1) and the mean DNA methylation GA at birth was 35 weeks (range = 31.4–37.7). To validate the epigenetic clock predictor in our sample, DNA methylation-based GA was tested for correlation with chronological GA (r = 0.76, p = 1.68 × 10^{-12}; Fig. 1). The average absolute difference between epigenetic GA and chronological GA—hereinafter referred as ΔGA—was 0.9 weeks (range = 0.03–4.02), i.e., 6.3 days. In agreement with previous studies, there was a significant negative correlation between ΔGA and chronological GA (r = – 0.47; p < 0.001).

Association between ΔGA and CPR

ΔGA was tested for associations with CPR measured during the third trimester (mean = 33.8 weeks, range = 28.3–36.4), a few days before childbirth (median = 6.5 days). CPR was significantly associated with ΔGA (β = 0.21, t = 3.362, p = 0.002) when adjusting for sex, birthweight, diagnostic of either TTTS or sIUGR, surgery time interval (when laser fetoscopy had been applied), and gestational age at ultrasound as covariates. The positive association between CPR and ΔGA remained significant after correction for cell type proportion (β = 0.21, t = 2.616, p = 0.01). Figure 2 shows the positive association between third trimester CPR and ΔGA.

Epigenetic exploration of placental PRS for schizophrenia with regard to CPR

Following the approach developed by Ursini and collaborators (2018), association between CPR and DNA methylation was tested in all CpG sites included in the DNA methylation array located within genes of the PRS for schizophrenia expressed in placental tissue (placental PRS) [22]. There were 1400 CpG sites annotated to placental PRS genes out of 866,091 CpG sites included in the array. After FDR correction for multiple testing, methylation at one single CpG site, cg06793497, was significantly associated with CPR (β = 0.021, p = 0.00008, t = 4.385; $q_{\text{FDR adjusted}}$ = 0.11; Fig. 3a), such that increased

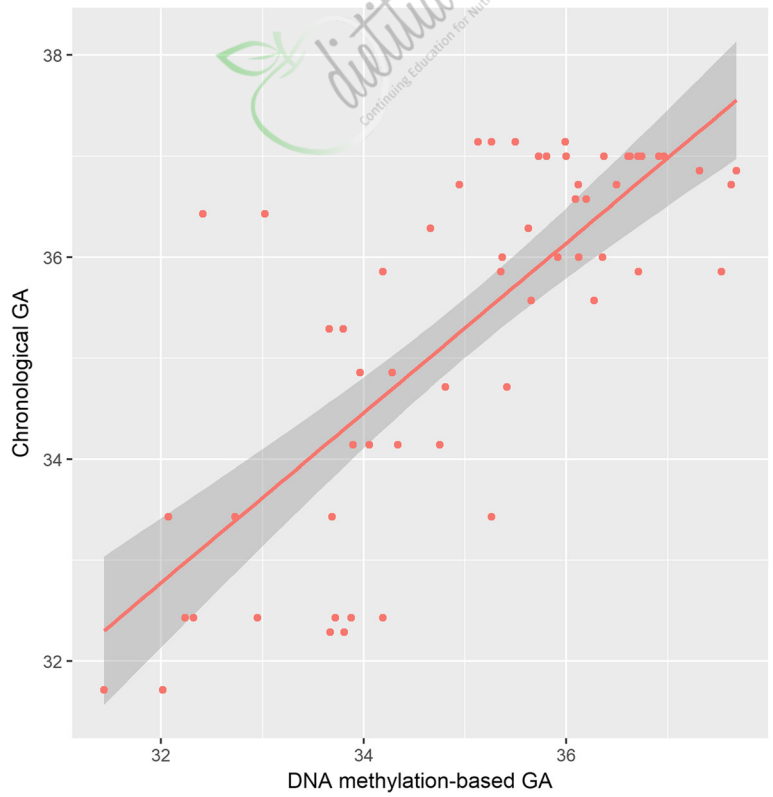

Fig. 1 Correlation between chronological GA and epigenetic GA. Chronological GA was calculated using first-trimester crown-rump length measurement of the larger twin, and epigenetic age was calculated based on DNA methylation-based Knight's clock. Both GA estimations were significantly correlated (r = 0.76; p = 1.68 × 10^{-12})

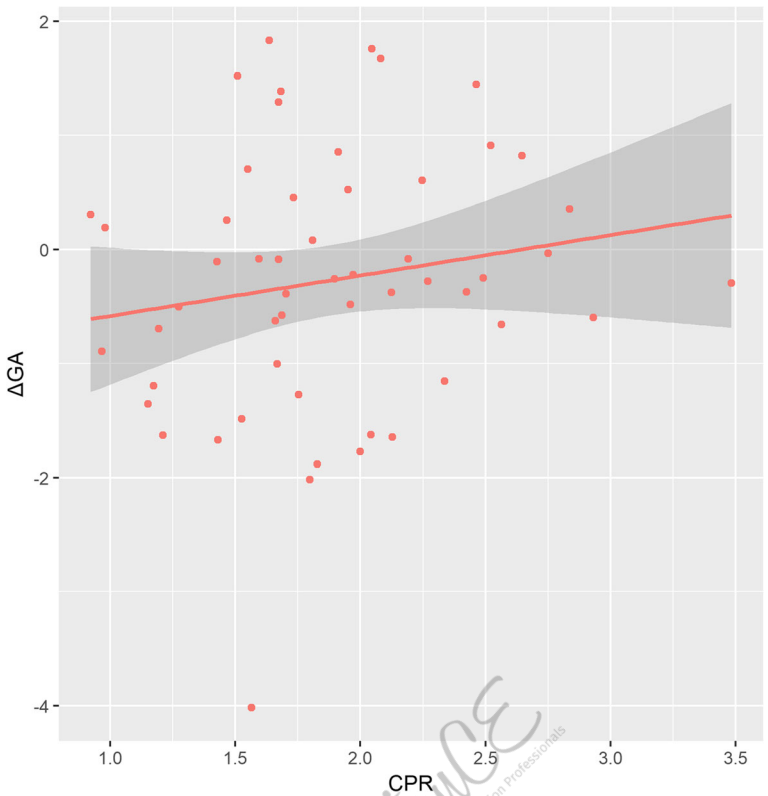

Fig. 2 Association between epigenetic age acceleration and cerebroplacental ratio measured during the third trimester. Epigenetic age delta (ΔGA) corresponds to estimated epigenetic age minus chronological age. Thus, ΔGA-positive values reflect epigenetic age acceleration while negative values point out the presence of epigenetic age deceleration. The cerebroplacental ratio (CPR) is calculated as the ratio between the MCAPI and UAPI. Both variables were significantly correlated when adjusting for sex, chronological gestational age, birth weight, and gestational age at ultrasound

cg06793497 methylation was associated with increased CPR. The top 10 CpG sites yielded by this approach are summarized in Table 1 (all q values > 0.75).

To further explore the association between cg06793497 methylation and CPR, it was analyzed in a monozygotic twin intrapair design. The intrapair twin design further allows controlling for chronological GA, sex, and timing of the Doppler ultrasound, since these variables are shared by co-twins of a pair. Four observations were removed from the analysis due to missingness for any of the variables in one of the co-twins of a pair. Thus, intrapair differences for these measures were calculated for all twin pairs of the sample were both measures were available for both twins of a pair (n = 27 twin pairs). Intrapair differences in cg06793497 methylation and CPR, measured during the third trimester, were significantly correlated (r = 0.64, p < 0.001; Fig. 3b). The association between both variables remained significant after adjusting for cell type count intrapair differences (β = 0.027, t = 3.924, p = 0.001). Intrapair exploration of the top 10 CpG sites (Table 1) revealed significant associations between CPR and DNA methylation at CpG probes cg00262246 (β = 0.012, p = 0.029), cg01024069 (β = – 0.01, p = 0.033), and cg12955069 (β = – 0.021, p = 0.026).

DNA methylation exploration of EP300 gene

To further explore the putative relevance of DNA methylation at other CpG sites located within the *EP300* gene and its surrounding regions, DNA methylation at 27 CpG sites included in the array and annotated to this region was also explored with regard to CPR (see Table 2). All analyses were adjusted for cell sex, birthweight, gestational age at ultrasound, and cell type count. In addition to cg06793497, two additional CpG sites—cg12968540 and cg19011939—were significantly associated with CPR (p < 0.05); moreover, methylation at four additional CpG sites—cg04452260, cg24349919, cg11931284 and cg25888227—showed trend associations with CPR (p < 0.01). The intrapair approach was then applied for these newly identified six CpG sites revealing cg11931284 (β = 0.028, t = 2.985, p = 0.008) and cg19011939 (β = – 0.021, t = – 2.343, p = 0.03) to be significantly associated with CPR, when adjusting for cell types intrapair differences.

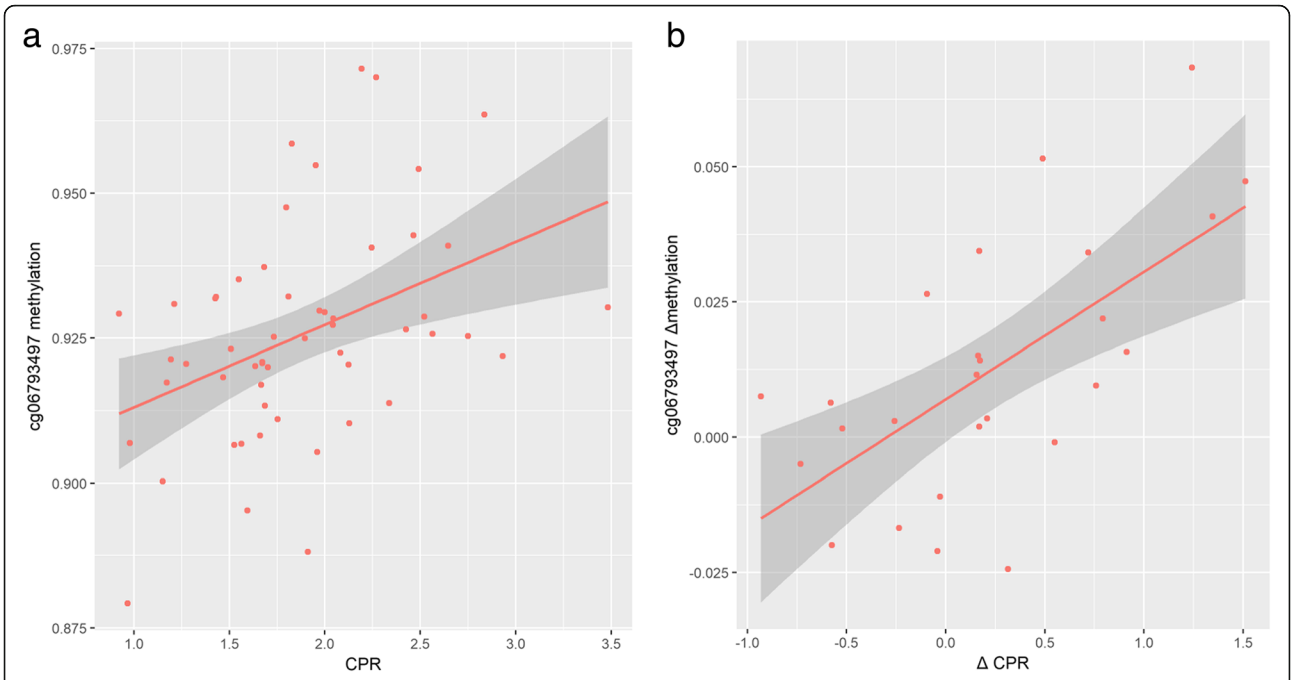

Fig. 3 Schizophrenia PRS methylation exploration with regard to cerebroplacental ratio measured during the third trimester. **a** Methylation at cg06793497 (*EP300* gene) was significantly associated with CPR in the whole sample (n = 54 twin subjects). **b** Intrapair methylation difference at cg06793497 was significantly associated with intrapair CPR difference (n = 26 twin pairs)

Discussion

To the best of our knowledge, this is the first study analyzing the epigenetic age in association with adverse prenatal environment as measured by a hemodynamic ultrasound parameter. Firstly, we describe the significant association between CPR measured during the third trimester of pregnancy with epigenetic age acceleration. Specifically, subjects exhibiting decreased CPR—exposed to prenatal adverse conditions—were born with decelerated epigenetic age, i.e., prenatally stressed subjects were born immature adjusting for their gestational age at birth. Additionally, methylomic exploration of schizophrenia PRS genes known to be expressed in placenta revealed the association between CPR and *EP300* gene CpG-specific methylation, at the cg06793497 probe, in our monochorionic twin sample.

Table 1 Top 10 CpG sites of the PRS methylomic exploration in association with CPR (1400 CpG sites tested)

#	CpG probe[a]	Genomic coordinates[b]	Gene	PRS exploration[c]			Intrapair[d]	
				beta	*p* value	q value	beta	*p* value
1	cg06793497	22: 41,542,898	*EP300*	0.021	8.2E-05	0.115	0.027	0.001
2	cg15620905	1: 44,024,150	*PTPRF*	0.040	0.002	0.804	0.008	0.563
3	cg12252443	2: 198,364,630	*HSPD1*	-0.006	0.002	0.804	− 0.007	0.055
4	cg24936500	2: 233,499,637	*EFHD1*	0.022	0.004	0.804	0.001	0.849
5	cg00262246	3: 136,007,461	*PCCB*	0.012	0.004	0.804	0.012	0.029
6	cg22495590	5: 138,161,059	*CTNNA1*	-0.020	0.004	0.804	− 0.010	0.203
7	cg14902598	5: 138,210,650	*CTNNA1*	0.017	0.005	0.804	0.006	0.371
8	cg01024069	14: 104,158,878	*KLC1*	-0.010	0.005	0.804	− 0.010	0.033
9	cg16362480	16: 30,077,084	*ALDOA*	0.031	0.005	0.804	0.024	0.126
10	cg12955069	16: 58,593,852	*CNOT1; SNORA50*	-0.016	0.006	0.804	− 0.021	0.026

[a] CpG site code according to the Illumina annotation
[b]Genomic coordinates correspond to hg19 built
[c]Refers to the statistics of each analysis in the first model encompassing 1400 CpG sites located in genes of the PRS for schizophrenia highly expressed in the placenta as described by Ursini et al.
[d]Refers to intrapair comparison of DNA methylation values and CPR. Thus, the *n* for these analyses was of 26 twin pairs

Table 2 List of CpG sites included in the array located in the *EP300* gene and its surrounding CpG island and antisense ncRNA (*EP300-AS1*)

CpG probe	Genomic coordinates	beta	*p* value
cg00500400	41487283	0.001	0.67
cg04452260	41487569	0.003	0.09
cg09331127	41487734	− 0.0006	0.79
cg02046995	41487740	0.004	0.49
cg24349919	41487761	0.005	0.09
cg02107564	41488750	− 0.005	0.48
cg03427564	41489051	0.004	0.21
cg03656483	41490340	− 0.001	0.65
cg00187244	41492007	0.011	0.23
cg11931284	*41492370*	*0.015*	*0.07*
cg13028324	41501680	− 0.0005	0.89
cg20730595	41513219	− 0.001	0.85
cg17439569	41513539	− 0.005	0.16
cg05997318	41542772	− 0.014	0.12
cg06793497	*41542898*	*0.021*	*0.00008*
cg06329185	41544246	− 0.0008	0.75
cg07345240	41556691	0.002	0.73
cg25299898	41563501	0.003	0.74
cg12968540	41572924	0.009	0.008
cg26901641	41573032	− 0.006	0.12
cg03950371	41573046	− 0.003	0.48
cg14455139	41573155	− 0.0001	0.98
cg05601844	41573176	− 0.0005	0.89
cg19011939	*41591607*	*− 0.01*	*0.01*
cg12917725	41592634	0.002	0.76
cg25888227	41593581	0.004	0.07
cg22037654	41593650	0.0001	0.94

DNA methylation at CpG sites highlighted in italics was significantly associated with CPR measured during the third trimester in an intrapair approach

Developmental deficits and developmental delays have been previously described in children who would later develop schizophrenia [30]; although such prodromal symptoms were in accordance with the neurodevelopmental hypothesis for schizophrenia, biological mechanisms mediating these effects remain largely unknown. Epigenetic immaturity in response to prenatal stress could be contributing to this developmental delay. Interestingly, epigenetic age deceleration has been previously described in association with maternal pathologies during pregnancy, such as maternal depression or Sjögren's syndrome, suggesting it can be a robust biomarker of prenatal suffering [10, 11]. It is worth noting that CPR was measured a few days prior to childbirth; thus, it can be used as a surrogate marker of prenatal adaptation to adverse conditions experienced at the end of the pregnancy, i.e., as a marker of perinatal risk.

Integration of the schizophrenia PRS [31] with obstetric and placental information [22], allowed the identification of *E1A binding protein p300* (*EP300*) gene CpG-specific methylation as a putative marker of exposure to prenatal stress. Interestingly, the *EP300* gene encodes a histone acetyltransferase (HAT) involved in several cell pathways such as cell proliferation and differentiation. Mutations at *EP300* gene have been described to cause Rubinstein-Taybi syndrome, a rare autosomal dominant neurodevelopmental disorder characterized by intellectual disability, psychomotor and language delay, and facial dysmorphisms [32]. Likewise, these symptoms, including developmental delay, learning problems, and cleft palate, characterize the 22q11.2 deletion syndrome, a well-defined congenital condition caused by the deletion of the 22q11.2 segment [33]. Notably, this syndrome is associated with a higher risk to develop schizophrenia, among other psychiatric conditions [34]; interestingly, *EP300* gene is located on chromosome 22 at position 22q13.2.

Further exploration of differential DNA methylation in and around the EP300 gene revealed cg19011939 to be differentially methylated in association with prenatal adversity. While higher exposure to a prenatal adverse environment, as reflected by lower CPR during the third trimester, is associated with decreased methylation at cg06793497 in the hypoxia-responsive *EP300* gene, there appears to be increased methylation at cg19011039 at *EP300-AS1* gene. Thus, we speculate that higher exposure to prenatal stress might be associated with reciprocal patterns of *EP300* and *EP300-AS1* epigenetic regulation that could act synergistically, a hypothesis that may be explored in future studies [35].

Remarkably, *EP300* has been identified as a co-activator of the hypoxia-inducible factor 1 alpha (*HIF1A*). In this regard, hypoxic conditions stimulate *EP300* expression, which has a neuroprotective role [36]. Accordingly, genetic variability at *EP300* gene has been associated with human adaptations to high altitude regions, e.g., the Tibet [37]. Likewise, pre- and peri-natal hypoxia have been associated with schizophrenia spectrum disorders, particularly by decreasing hippocampal volume [38, 39]; complementarily, a decreased or impaired response to hypoxia via neurotrophic factors has also been implicated in the etiology of schizophrenia [40]. Furthermore, DNA methylation at the *IGF2BP1* gene, also involved in prenatal development [41], has been associated with both adult working memory and birthweight [42]; further highlighting the advantage of twin study designs to identify environmentally-driven epigenetic consequences of prenatal stress. Overall, these findings point to the existence of a GxE interaction between genetic vulnerability and exposure to prenatal hypoxia, as already highlighted by Ursini and

collaborators [22]. In this framework, *EP300* methylation could be one of the mediators of such interaction.

A number of limitations of the present study should be noted. First, the moderate sample size (n = 60 subjects, 30 twin pairs) limits the statistical power of the analysis; however, smaller sample sizes (n = 22 MZ twin pairs) have been described to be sufficient to identify methylation differences of 6% with > 80% power [43]. Moreover, a lenient significance threshold after correction multiple testing was used; however, previous epigenetic studies have described FDR values between 5 and 20% as markers of medium-confidence sites [44]. Another limitation regards the moderately small reported effect sizes (around 2%) questioning the biological relevance of our findings [45]; however, these findings are in agreement with a larger body of evidence regarding cord blood methylation after exposure to a number of prenatal stressors. Such small DNA methylation changes may act in conjunction with a myriad of other epigenetic signatures and biological processes in order to maintain homeostasis in the face of threats. Additionally, although epigenomic information was available from a methylomic array including more than 800,000 CpG sites distributed throughout the whole human genome, only 1400 CpG sites were analyzed; alternative approaches including the total of CpG sites included in the array would have yield different findings, probably pointing to genes involved in other neurodevelopmental disorders besides schizophrenia. Furthermore, while the set of genes analyzed in the current approach were described to be highly expressed in placental tissue [22], placentae were not available for this sample and cord blood was thus analyzed as the proxy tissue of choice with regard to exposure to prenatal adversity. Finally, MZ twin pregnancies are characterized by lower gestational ages at birth than singleton pregnancies; besides, obstetric scales commonly used in psychiatric studies include twin pregnancies as an obstetric complication. Thus, findings derived from the present design might not be generalizable to the general population.

Conclusions

Further studies are needed to test the time stability of the hereby identified methylation signature. It will be equally relevant to explore neurobehavioral correlates of EP300 methylation during early childhood along with its putative association with neurodevelopmental outcomes, including psychosis liability. Additionally, a longitudinal follow-up is required to test the role of postnatal environment in these phenotypes since both epigenetic age deceleration and CpG-specific differential methylation in association with CPR could return to basal levels after birth. Finally, genetic exploration of these subjects regarding schizophrenia PRS will be instrumental for the study of GxE interactions and genetic liability for an impaired hypoxia response during human development.

Methods

Study population

This was a prospective study including fetal pairs from monochorionic diamniotic twin pregnancies attended at Hospital Clínic de Barcelona (Spain) during a 2-year recruitment period. Monochorionic monoamniotic twin pregnancies were excluded from the present study to avoid putative confounding with regard to differential exposure to stress in both types of twin pregnancies. The study protocol was approved by the hospital ethics committee (HCB/2016/0046), and all patients provided written informed consent.

We included 32 monochorionic pregnancies (n = 64 samples). The sample was enriched for two monochorionic-specific severe obstetric complications: twin-to-twin transfusion syndrome (TTTS, n = 8) and selective intrauterine growth restriction (sIUGR, n = 9). All TTTS cases were treated upon detection by means of laser fetoscopy [46].

Maternal age and pre-pregnancy BMI were retrieved from hospital records. Gestational age was dated using first-trimester crown-rump length measurement of the larger twin [47].

Fetal ultrasound assessment

Ultrasound assessment was performed on a Voluson Expert 8 (General Electrical Medical Systems, Milwaukee, WI, USA) or a Siemens Sonoline Antares (Siemens Medical Systems, Erlangen, Germany) with 8- to 4-MHz or 6- to 4- MHz curved array probes, respectively. All fetuses underwent detailed ultrasound evaluation including fetal anatomy and Doppler measurements such as UAPI, MCAPI and ductus venosus PI. All Doppler evaluations were acquired at a normal fetal heart rate (FHR) in the absence of fetal body or respiratory movements and at an angle of insonation as close to 0° as possible (but always < 15°), and the mechanical and thermal indices were maintained below 1. CPR was calculated as the ratio between MCAPI and UAPI, according to previous studies [12].

DNA methylation

Umbilical vein cord blood samples were obtained from the clamped umbilical cord immediately after delivery of the fetus. All blood samples were collected in EDTA-treated tubes and processed within 1 h. Plasma was separated by centrifugation at 3000 rpm for 10 min at 4 °C, and stored at − 80 °C until further use. Genomic DNA was extracted from fetal cord blood using QIAamp DNA Mini Kit (Qiagen). DNA quality and quantity were assessed by NanoDrop One (Thermo Scientific).

Genomic DNA was bisulfite converted using the Zymo EZ-96 DNA Methylation Kit (Zymo Research). Genome-wide DNA methylation levels were assessed over 850,000 CpG sites by means of the Infinium MethylationEPIC BeadChip Kit (Illumina Inc., CA, USA) according to the manufacturer's protocol. Pre-processing and normalization were performed using the Bioconductor minfi package [48]. CpG probes containing common SNPs were discarded. All probes mapping to the X and Y chromosomes were also removed. Finally, cross-hybridizing probes as previously identified were excluded from further analysis [49]. All samples (n = 64) were run on the same plate.

Absence of maternal contamination was confirmed after retrieving DNA methylation values at 10 CpG sites previously described to identify sample contamination by maternal blood during sample collection [50]. None of the samples assayed exhibited DNA methylation values above the threshold at 5 or more of those CpG sites (see Additional file 1 for specific methylation values). Two samples (from the same twin-pair) were excluded from further analyses due to lack of monozygosity as assessed by 59 SNPs included in the array. One of the samples was removed from analysis due to insufficient DNA concentration, the co-twin sample was also excluded from further analysis.

Statistical analyses

All statistical analyses were conducted in R version 3.5.0 [51]. DNA methylation-based GA prediction was performed using the R code and statistical pipeline developed by Knight, based on the methylation profile of 148 CpG sites [6]; this predictor was developed using 15 Illumina DNA methylation datasets (n = 1434 neonates). Following Simpkin et al. recommendations, the Knight clock was preferred for our analysis as it was developed and tested in preterm infants datasets such as our monozygotic twin population, characterized by a mean gestational age at birth of 35.3 weeks [52]. The EPIC array lacks 6 of the CpG sites originally included in the Knight clock, these values were imputed manually as non-available. Interestingly, DNA methylation-based age estimation relying on EPIC array data has already been described to accurately predict age despite the lack of several CpG sites originally included in Horvath's and Hannum's clocks [53].

Gestational age acceleration (ΔGA) was calculated as the absolute difference between epigenetic GA and chronological GA. Since ΔGA was associated with chronological GA (r = − 0.47; p < 0.001), the latter was included as a covariate in all statistical models; this association has been already reported in prior studies exploring epigenetic-based GA estimations at birth [6, 10, 11].

Cell counts of $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells, granulocytes, monocytes, and nucleated red blood cells (nRBCs) were estimated using the R code and statistical pipeline developed by Houseman [54].

A multiple linear regression model was built to analyze the correlation between ΔGA and CPR. Fetal sex, birthweight, diagnostic of either TTTS or sIUGR (binary variable), post-surgery interval (in TTTS cases where laser fetoscopy had been applied), and gestational age at ultrasound were included as independent variables in the model as they are known to influence either DNA methylation (from which ΔGA is calculated) or CPR. This analysis was conducted in the total MZ twin sample (n = 60).

DNA methylation at CpG sites annotated to the 43 genes of the Placental PRS1 as described by Ursini et al. [22] was retrieved to test their association with CPR. A second multiple linear regression model was then designed to explore putative effects of CPR upon methylation of PRS genes, testing 1,400 associations. The aforementioned confounding variables along with cell types proportions ($CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells, granulocytes, monocytes, and nRBCs) were included as covariates, as they are known to affect methylation values. False discovery rate (FDR) correction for multiple testing was applied, considering q values under 20% to be indicative of medium-confidence probes following prior studies [44].

A twin-based approach previously developed in our group [55] was also applied to refine the association between cg06793497 methylation and CPR. Briefly, intrapair differences for both variables of interest were computed for each twin pair; afterward, a regression model was fitted with an estimated intrapair cg06793497 methylation (Δmethylation) and intrapair CPR (ΔCPR). This last model was not adjusted for either sex or chronological gestational age since both variables are identical for both twins of a pair.

Additional file

Additional file 1: DNA methylation values for CpG probes used to discard the presence of maternal contamination. (DOCX 29.6 kb)

Competing interests
The authors declare that they have no competing interests.

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DNA methylation changes related to nutritional deprivation: a genome-wide analysis of population and in vitro data

Abstract

Background: DNA methylation has recently been identified as a mediator between in utero famine exposure and a range of metabolic and psychiatric traits. However, genome-wide analyses are scarce and cross-sectional analyses are hampered by many potential confounding factors. Moreover, causal relations are hard to identify due to the lack of controlled experimental designs. In the current study, we therefore combined a comprehensive assessment of genome-wide DNA methylation differences in people exposed to the great Chinese famine in utero with an in vitro study in which we deprived fibroblasts of nutrition.

Methods: We compared whole blood DNA methylation differences between 25 individuals in utero exposed to famine and 54 healthy control individuals using the HumanMethylation450 platform. In vitro, we analyzed DNA methylation changes in 10 fibroblast cultures that were nutritionally deprived for 72 h by withholding fetal bovine serum.

Results: We identified three differentially methylated regions (DMRs) in four genes (*ENO2*, *ZNF226*, *CCDC51*, and *TMA7*) that were related to famine exposure in both analyses. Pathway analysis with data from both Chinese famine samples and fibroblasts highlighted the nervous system and neurogenesis pathways as the most affected by nutritional deprivation.

Conclusions: The combination of cross-sectional and experimental data provides indications that biological adaptation to famine leads to DNA methylation changes in genes involved in the central nervous system.

Keywords: Chinese famine, Nutrition deprivation, Genome-wide DNA methylation, Pathway analysis

Background

DNA methylation is one of the epigenetic mechanisms that plays an important role in the cellular responses to detrimental environmental influences that are involved in the etiology of many diseases [1]. Studies show that early life exposure to nutritional deprivation is associated with stable DNA methylation differences [2, 3]. Nutritional deprivation, especially in utero and early in life, has detrimental effects on human development and significantly increases the risk of multiple chronic diseases later in life [3–6].

A seminal example of the impact of in utero exposure to nutritional deprivation is the cohort study on offspring from mothers that were pregnant during the Dutch hunger winter during the Second World War, which was intense and well-documented but with brief duration [7]. This study identified persistent differential methylation of the insulin-like growth factor II (IGF2), as a key human growth and development factor involved in the response to famine in utero [3]. Subsequent studies of this cohort identified DNA methylation changes as mediators of the association between maternal famine and metabolic disease in adulthood [6, 8]. Other epigenetic differences associated with famine exposure in utero have been related to schizophrenia [9] and type 2 diabetes [10].

While the Dutch famine is the most extensively studied famine in the literature, the Chinese great famine (1959–1961) was one of the largest famines recorded around the world and had more severe consequences resulting in an estimated 30 million deaths [11]. The offsprings of those mothers who suffered famine were shorter in length [5], had worse midlife health [12], and had a higher rate of chronic diseases [13, 14]. Studies also showed a twofold increased risk to develop schizophrenia among offspring conceived at the height of the famine [15, 16]. However, only one genome-wide DNA methylation study is reported in the Chinese famine population [17]. To further understand the impact of maternal famine on DNA methylation changes in offspring, we compared genome-wide DNA methylation from whole blood of Chinese participants exposed to famine in the first trimester to unexposed controls from the same populations.

Since a cross-sectional population-based study is subject to residual confounding and does not allow the examination of the direct effect of nutritional deprivation, we subsequently performed an in vitro study of human fibroblasts before and after exposure to nutritional deprivation. By combining the result of a genome-wide methylation approach of both studies, we aim to provide an unbiased investigation of DNA methylation changes induced by nutritional deprivation.

Methods

Chinese famine sample

The sample of Chinese famine is part of our previous study and has been described in more detail elsewhere [9]. In short, volunteers were recruited in the northern province of Jilin, China. Considering the almost complete penetration of famine during January 1960 and September 1961, it is assumed that those born during that period will have been exposed. A total of 79 healthy participants were included of which 25 were exposed to famine during the first 3 months in utero. All participants provided written informed consent. Table 1 gives the full details of the participants.

Fibroblast in vitro study

The in vitro fibroblast experiment was described in more detail previously [9]. In short, fibroblasts were obtained by skin biopsies from five healthy participants of Dutch descent, of which one was male and four were female (mean age = 38.4 years, sd = 7.0) (see Table 1). All participants provided written informed consent. Fibroblasts were plated in two T25 flasks in Minimum Essential Medium (MEM) (Gibco®) with 15% fetal bovine serum (FBS)(Gibco®) and 1% penicillin-streptomycin PenStrep (Gibco®) and in an atmosphere of 95% atmospheric air and 5% CO_2 at 37 °C (normal conditions). After reaching 70–80% confluence, the supernatant was removed and the cells were washed three times with phosphate buffered saline (PBS) (BioWhittaker® Reagents, Lonza). Next, one of the T25 flasks from each donor was cultured in the non-famine condition with Minimum Essential Medium (MEM) (Gibco®) supported with 15% FBS, while the other T25 flasks were cultured in only Minimum Essential Medium (MEM) as famine condition. After 72 h, cells were harvested from each flask and stored as cell pellet for DNA isolation.

Table 1 Summary of characteristics of the Chinese famine samples

	Unexposed	Exposed to maternal famine
N	54	25
Age (sd)	46.8 (1.0)	50.3 (0.5)
Male *N* (%)	21 (39%)	10 (40%)

DNA processing

DNA from the Chinese famine samples was extracted from whole blood using the Gentra Puregene Kit (Qiagen, Valencia, CA, USA). Fibroblast cell pellets were used for DNA isolation according to the manufacturer's instructions (Qiagen, Hilden, Germany). The DNA concentration and quality were examined using NanoDrop (Thermo Fisher Scientific, Massachusetts, USA). Bisulfite conversion of each DNA sample was conducted according to the manufacturer's instructions of the Zymo EZ DNA Methylation™ Kit (Zymo, Irvine, CA, USA). Quality and quantity of the bisulphite treated single stranded DNA was examined using NanoDrop.

Genome-wide analysis of DNA methylation

One hundred and fifty nanograms of bisulfite-converted DNA from the Chinese famine study was used to quantify genome-wide patterns of DNA methylation using the Illumina Infinium HumanMethylation450 BeadChip. Genome-wide DNA methylation levels of fibroblasts were obtained using Illumina HumanMethylation EPIC BeadChip arrays. For the Chinese famine samples, intensity readouts, beta and *M* value calculation, and cell-type proportion estimates were obtained using the minfi package (version 1.10.2) in Bioconductor [18]. Probes were excluded based on a bead count less than three ($n = 279$ probes) or a detection p value larger than 0.001 in at least 5% of the samples ($n = 2125$ probes). Non-autosomal or cross-hybridizing probes were discarded as were loci with SNPs of minor allele frequency larger than 1% within 1 bp of the primer [19]. None of the blood samples had over 1% of failed probes. All 79 DNA samples survived quality control [20], and 397,985 loci were left in the dataset for further analysis. The normalization was performed using the functional normalization procedure which is implemented in the minfi package. Additional adjustments were made using the genetic principal components estimated according to Barfield et al. [19]. Moreover, the blood-based

analysis included an adjustment for cell-type (B cells, CD8 T cells, CD4 T cells, natural killer cells, monocytes, and granulocytes) [21].

The quality control for fibroblasts was performed in a similar workflow as the Chinese famine samples but adjusted to the newer EPIC methylation beadchip. The dataset was pre-processed in R version 3.3.1 with the meffil package [22] using functional normalization [23] to reduce the non-biological differences between probes. To account for technical batch variables, pre-processing was performed in a larger dataset (n = 80), including DNA samples of other studies that included brain and blood DNA. However, normalization was conducted for the fibroblast samples only. No mismatches between methylation-predicted sex and actual gender were present nor were there samples with outliers on mean of methylated and unmethylated channels. Probes were removed if they failed quality control (a detection p value > 0.01 for > 10% of samples (n = 4610) or a bead count < 3 for > 10% of samples (n = 68)), were non-specific [20], or were one of the SNP probes included on the array for quality control purposes. All 10 fibroblast DNA samples survived quality control, and 862,160 probes were left in the dataset for further analysis.

For both the Chinese sample and fibroblast samples, the level (percentage) of methylation is expressed as a β value, ranging from 0 (unmethylated cytosine) to 1 (completely methylated cytosine), but analyses were performed using M values (log2 of β values), for better statistical validity [24]. To examine the overlap between the results of the two datasets, DMR and pathway analyses were performed for the 397,985 CpGs that were present on the EPIC as well as the 450 k arrays.

Pathway analysis

We performed Gene Set Enrichment Analysis (GSEA) for the nominal significant CpGs that overlapped from the Chinese famine and fibroblasts samples. SetRank tool was chosen in the current study for GSEA analysis since it could eliminate many false positive hits [25], especially those biased toward neuronal pathways as these genes are much more abundant and larger in size. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), WikiPathways, and Reactome pathway database are included in the SetRank tool.

Permutation analysis

The significance level of the identified DMRs was confirmed by permutation analysis whereby p values were calculated from all potential DMRs with the same number of CpGs throughout the genome. From the fit of the actual identified DMR in this distribution, an empirical p value was derived. The probability of finding the number of overlapping DMRs that we presented from all potential matches was established. All these analyses were based on 10,000 permutations.

Statistical analyses

Statistical analyses were carried out using R [26]. Analysis of the association of DNA methylation with famine in the Chinese famine samples was performed using linear regression with DNA methylation as dependent and famine, age, gender, and cell-type proportion estimates based on the Houseman algorithm [21] as well as the first two DNA methylation-based ancestry principal components as indicators [19]. In addition, similar as previously, we adjusted for the effects of smoking by deriving a proxy for smoking based on methylation levels of CpGs that were previously associated with smoking [27]. For the fibroblast experiment, methylation changes under the famine condition were assessed using Wilcoxon's paired rank test. The QQ plots were inspected to assess type I error inflation and power (Additional file 1). DMRcate (version 1.4.2) was used to identify differentially methylated regions (DMRs). Nominal significance for the DMR analysis was set at 0.01 [28]. Only DMRs with the same direction of effect (hyper- or hypomethylation) in both samples were considered overlapping.

Results

Identification of differentially methylated regions

Analysis of single CpG methylation did not identify significant differences after adjustment for multiple testing due to insufficient power. The QQ plot indicated the analysis was underpowered to detect genome-wide differentially methylated probes (Additional file 1 shows the QQ plots). Additional file 2 provides the information and test statistics of the nominally associated loci (18,871 for the Chinese famine and 56,375 for the fibroblast experiment). Two thousand seven hundred six CpGs overlapped between nominally associated loci of both experiments. The probability to end up significant in both analyses was higher for CpGs from the famine study (chi-squared = 843.97, df = 1, p value < 0.001) as a logical result from the larger number of loci on the methylation array of the fibroblast experiment. However, the odds of identification as nominal significant was also significantly larger in the famine study (chi-squared = 1398.4, df = 1, p < 0.001) most likely as a result of a larger power. Analysis of DMRs in the Chinese famine cohort identified 613 different methylated regions (DMRs) and 1080 DMRs in fibroblast samples. Among these significant DMRs, three DMRs were similarly associated (significant and same direction of effect) in both samples. The three replicated DMRs are all hypomethylated in relation to famine exposure and highlight four gene promoters: DMR1, enolase 2 (*ENO2*) (cg08003732, cg13334990, cg18912645, cg19720347), and DMR2, zinc finger protein 226 (*ZNF226*) (cg19331658, cg03559973,

cg19836894, cg19599862, cg03573702). DMR3 is related to 2 gene promoters: coiled-coil domain containing 51 (*CCDC51*) and translation machinery associated 7 homolog (TMA7) (cg00329014, cg06625258, cg07744328, cg01538982, cg24981564, cg12370248, cg07095599, cg11196693, cg03629318, cg15853329, cg21856689, cg26094714, cg25858682). The study from Hannon et al. [29] was used as a lookup for the relation between methylation in blood and brain for the identified loci. cg08003732 and cg13334990 loci in *ENO2* gene were all significantly correlated between blood and four brain regions: the prefrontal cortex (PFC), entorhinal cortex (EC), superior temporal gyrus (STG), and cerebellum (CER). Other loci with significant correlation between blood and brain were cg19331658 in *ZNF226* and cg26094714 in *CCD51*/*TM7* that were correlated with PFC, cg18912645 in *ENO2* and cg12370248 and cg15853329 in *CCD51*/*TM7* that were correlated with EC, and blood cg19720347 methylation in *ENO2* that was correlated with CER. Table 2 shows the characteristics of the DMRs consistently associated to famine in both experiments. Permutation analysis confirmed the significance of most of the presented associations with the exemption of the association of ENO2 to famine in the Chinese sample that showed an empirical p value of $p = 0.099$, although combined p values of both analyses remain significant ($p = 0.0016$). Additional file 3 presents the results of the permutation analysis.

Pathway analysis of identified CpG loci

Figure 1 shows the significant pathways that are associated with all the 2706 overlapping CpGs from the Chinese famine sample and fibroblast experiments. The pathway analysis is based on GO, KEGG, WikiPathways, and Reactome pathway databases. GO pathway analysis highlighted three significant molecular function pathways, among which cell adhesion molecule binding is mostly prevalent. Adherens junction is most relevant regarding the cellular components. In addition, we found that the famine condition influenced a wide range of biological processes, among which neuronal systems are most strongly implied. For example, pathways in nervous system development, both positive and negative neurogenesis, and neuron projection morphogenesis are highly involved. The pathway analysis from significant Reactome and WikiPathways analysis showed that DNA damage response and signaling by nerve growth factor (NGF) are mostly involved by nutritional deprivation.

Discussion

This is the first study that combines genome-wide DNA methylation analysis of famine exposure with an in vitro study of nutritional deprivation to explore the effect of famine on DNA methylation. The results highlight several gene promoters that are differentially methylated due to nutritional deprivation. Further pathway analysis showed that the nervous system development and signaling by nerve growth factor (NGF) are sensitive to nutritional deprivation.

Analysis of the overlapping DMRs from Chinese famine samples and in vitro fibroblast samples identified three DMRs in four gene promoters (*ENO2*, *ZNF226*, *CCDC51*, and *TMA7*) that are consistently hypomethylated in relation to nutrition deprivation in both Chinese famine and fibroblast in vitro samples (Table 2). The fact that famine is consistently linked to hypomethylation and no occurrences of hypermethylation were identified suggests reduced methylation efficacy, for instance, due to the limited production of the methyl donor *S*-adenosyl methionine (SAMe) which is dependent on nutrients such as folate, vitamin B1, B6, and B12. Genes identified in the current study have a wide range of functions, but the involvement of the gene *ENO2* is one of the most interesting findings. *ENO2* is abundantly expressed neurons and peripheral neuroendocrine tissue [30] and often used as neuron-specific reference genes [31–33]. Functional studies showed that *ENO2* promotes cell proliferation, glycolysis, and glucocorticoid resistance [34], and silencing of this gene was found to inhibit the growth of glioblastoma cells [35]. Consistently, *ENO2* serves as a biochemical marker for tumors derived from neuronal and peripheral neuroendocrine tissues [34]. Furthermore, *ENO2* is found to be expressed higher in the brain of schizophrenia (SCZ) patient as compared to controls and may affect glucose metabolism in SCZ patients [36]. Moreover, a recent study

Table 2 Three DMRs consistently associated with famine in both experiments (Chinese famine samples and fibroblasts samples)

DMRs	Gene promoters	CHR	Region (hg19)	CpG numbers	β value (Chinese)	p value (Chinese)	β value (Fibroblasts)	p value (Fibroblasts)
DMR1	*ENO2*	chr12	7023752–7024121	4	− 0.0243	1.19E−04	− 0.1523	7.42E−04
DMR2	*ZNF226*	chr19	44669146–44669354	5	− 0.0636	9.21E−03	− 0.3155	1.07E−03
DMR3	*CCDC51* *TMA7*	chr3	48481268–48481793	13	− 0.0290	7.87E−04	− 0.2318	1.25E−06

DMR differentially methylated regions, *CHR* chromosome, *hg19* human genome version 19. The first column of the table shows the DMR identifier and followed by the gene name which belongs to the DMR. The chromosome of the gene is provided and followed by the more precise region in hg19 (human genome version 19). The number of significant CpGs response to nutrition deprivation in both studies is presented, and β value and p value of DMRs in both studies are also presented. β value in each study refers to the mean β values of identified CpG in each DMR

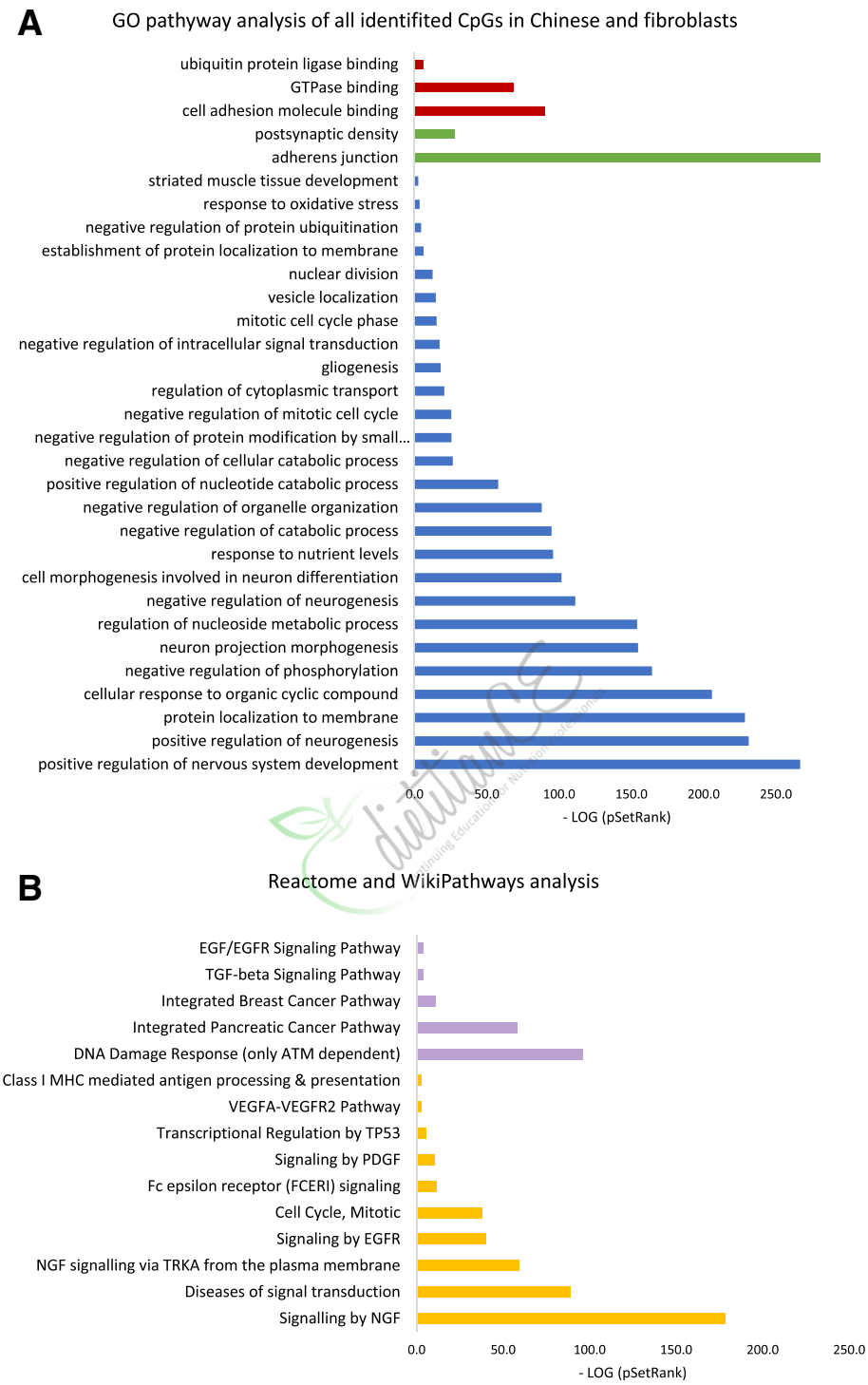

Fig. 1 Significant pathway analysis based on CpGs (2706) associated with famine in both the Chinese famine and fibroblast study. **a** Significant pathways from GO analysis. Pathways in red represent molecular functions, in green represent cellular components, and in blue represent biological processes. *X*-axis displays the minus log *p* value of the association with the SetRank value of the gene set. **b** Significant pathway analysis from Reactome and WikiPathways. Reactome pathway is in purple, and WikiPathways is in orange. *X*-axis displays the minus log *p* value of association with the SetRank value of the gene set

found *ENO2* hypermethylation in autism alongside with decreased transcription and translation of this gene [37]. A look-up in BECon [38], an online database to compare the methylation pattern between brain and blood, suggests that part of the DMR in *ENO2* (cg08003732) has a similar DNA methylation pattern in blood and brain tissue.

Interpretation of the involvement of zinc finger protein gene *ZNF226* is less straightforward as not much is currently known about this specific gene. Zinc finger proteins have a broad range of molecular functions, and they are widely targeted for aberrant DNA hypermethylation during toxicant-induced malignant transformation [39] and as a driver of detrimental environment factor-associated carcinogenesis, leading to suggestions of their suitability for cancer prevention [40]. The third DMR identified, *CCDC51*, is a protein-coding gene, which is present in endosomes [41]. This gene is involved in several signaling pathways, such as B cell receptor activation [42], micronucleus formation regulation [43], cellular senescence [44], liver-specific microRNA binding [45], and tumor suppressor activity [46], as well as kidney disease [47]. Mouse *Ccdc51* gene is the target gene of miR-672-5p, which is highly expressed after steroid-induced osteonecrosis [48]. Considering that nutritional deprivation could potentially disturb steroid levels, the current finding of *CCDC51* hypomethylation raises the possibility of a relation between famine and steroid imbalance. The final DMR gene *TMA7* codes for the TMA7 protein, and deletion of this gene is consistent with loss of proteins involved in ribosome biogenesis [49]. Though the current finding is based on blood and fibroblasts, the database from Hannon et al. shows that methylation in blood of the four identified loci from the current study is correlated with the prefrontal cortex, five are correlated with the entorhinal cortex; two are correlated with the superior temporal gyrus, and three are correlated with the cerebellum. This suggests that blood methylation levels of these DMRs in part may serve as a proxy for methylation in these brain areas.

In the previous genome-wide methylation study of the Dutch hunger winter, 181 genes were identified through reduced representation bisulfite sequencing (RRBS) and a further 6 genes were verified in mass spectrometry-based EpiTYPER assay [8]. Later, in a Bangladesh famine cohort, seven epialleles were identified [4]. Although the DMRs from these previous studies do not overlap with our DMRs, the DMRs are near genes from the same pathway. For example, *ZNF251* and *CCDC57* were identified in the Dutch hunger cohort, whereas in our study, *ZNF226* and *CCDC51* are found differentially methylated. The different genetic background of the three famine cohort studies could be one of the explanations of these differences since the vulnerability to environmental factors could be inherent genetically [50]. Another explanation for the diverging results could be that although all three populations suffered from famine, the remaining food consumption pattern probably was quite different between countries. Differences in dietary nutrient intake could eventually lead to different patterns of malnutrition and to different outcomes.

The pathways most commonly related to malnutrition exposure are in the nervous system and neurogenesis, specifically, positive regulation of nervous system development in the GO pathway analysis (blue in Fig. 1) and nerve growth factor (NGF) signaling in the WikiPathways analysis (orange in Fig. 1). This points to the high relevance of epigenetic adaptations to famine for the brain [51] (even though current study did not analyze brain). Impact of famine on the brain has been shown in rodent studies that showed large epigenetic changes in the hippocampus in offsprings of nutritional deprived rats [52].

Performing DNA methylation analysis on fibroblasts in addition to whole blood increases the diversity of the tissue types and strongly reduces the risk that residual confounding factors are driving the results. Fibroblasts provide a different tissue type, and using longitudinal analysis within the same participants poses the opportunity to directly relate DNA methylation changes to famine. The replicating DMRs from fibroblasts and blood therefore provide compelling evidence that these are relevant genes that are involved in the response to malnutrition.

Some limitations should be considered when interpreting the current study. Replication of our findings in the Dutch famine [8] study was not possible due to the fact that these loci were eliminated in their analysis based on a low variance in whole-genome bisulphite sequencing data. Also, lookup of the presented DMRs in the studies of James et al. [53] and Finer et al. [4] did not identify an overlap. However, considering these studies essentially used candidate gene approaches in very different populations, this does not refute our findings. The merit of the current approach is the triangulation identifying epidemiological associations combined with an experimental biological response [54]. Inherent to the case-control setup of this study, other residual confounding factors such as for instance diet, cannot be ruled out. The identified DMRs from the current study are based on two different tissues and different experimental setups. Considering these differences, we expected a small number of overlapping DMRs from these two experiments. We expect that only truly strong biological effects will be detected in both experiments consistent with the concept of triangulation of research findings [54]. Nevertheless, the small overlaps between DMRs from both experiments underscore the limited similarity between studies and therefore have limited value as a replication. The sample sizes are relatively small, and therefore, power and significance level are limited. Also, although two tissue types were used, both the blood and fibroblast methylation may still not represent the situation in the developing brain. Finally, the genetic background from this study limits our conclusion on malnutrition response to Chinese and Dutch ancestry and may not represent other ethnic groups.

Conclusions

Using an unbiased genome-wide approach, the current study examined the association between DNA methylation and severe nutritional deprivation in two unique samples separately (Chinese famine and in vitro fibroblasts) and leads to the identification of DMRs that were consistently hypomethylated in both samples. The three DMRs in the four gene promoters *ENO2*, *ZNF226*, *CCDC51*, and *TMA7* and the involvement of the nervous system development and signaling by nerve growth factor (NGF) that are suggested by pathway analyses can provide new leads to understand the pathways from nutrition deprivation to disease.

Additional files

Additional file 1: A. QQ plot of the *p* value distribution for the regression of DNA methylation and Chinese famine samples. B. QQ plot of the *p* value distribution for the paired *t* test of DNA methylation and fibroblast in vitro samples. (PDF 58 kb)

Additional file 2: Nominally associated loci of Chinese and fibroblast samples. The file contains the loci ID and *p* value of each locus associated with nutritional deprivation in Chinese and fibroblast samples. (XLSX 4646 kb)

Additional file 3: Permutation analysis of DMRs. The file contains the results of the permutation analysis. (DOCX 14 kb)

Abbreviations

CHR: Chromosome; CpG: Cytosine-phosphate-guanine; DMRs: Differentially methylated regions; DNA: Deoxyribonucleic acid; GO: Gene Ontology; GSEA: Gene Set Enrichment Analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; NGF: Nerve growth factor; RRBS: Reduced representation bisulfite sequencing

Competing interests

The authors declare that they have no competing interests.

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