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Adult global DNA methylation in relation to pre-natal nutrition

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Background Exposure to a pre-natal famine environment has been associated with a persistent decrease in DNA methylation of the *IGF2* gene, although study findings on other loci have been highly variable. There have been no studies to date of the relation between pre-natal famine and overall global DNA methylation in adulthood.

Methods Our study population includes 350 births with pre-natal exposure to the Dutch famine of 1944–45 selected from three birth clinics, 290 births from these clinics born before or after the famine as unexposed time controls and 307 same-sex siblings of either birth group as unexposed family controls. All study subjects were interviewed and underwent a medical examination at a mean age of 58 years when blood samples were also collected. As measures of genomic DNA methylation, we analysed two repetitive elements, LINE-1 (long interspersed nucleotide element 1) and Sat2 (Satellite 2 DNA sequence) by pyrosequencing and MethyLight, respectively, and overall genomic DNA methylation using the Luminometric methylation assay (LUMA).

Results Mean DNA methylation by LUMA was 75.2% [standard deviation (SD) 4.7], by LINE-1 was 77.1% (SD 2.5) and by Sat2 was 122.2 (SD 56.2). Pre-natal famine exposure was associated with negligible changes in all three assays {LUMA: −0.16% [95% confidence interval (95% CI) −0.49 to 0.81], $P=0.63$; LINE-1: −0.05 % (95% CI −0.33 to 0.22), $P=0.70$; and Sat2: −0.51% (95% CI −7.38 to 6.36), $P=0.88$} relative to unexposed controls, adjusting for age at examination and within family clustering.

Conclusion Our results show no relation between overall global DNA methylation in adults and pre-natal famine exposure. Further work should focus on selected regions in the genome that may be differentially methylated in response to changes in early life exposures and predict adult health outcomes.

Keywords Pre-natal exposure delayed effects, Dutch famine, The Netherlands, World War II, DNA methylation, LINE-1, Sat2, LUMA

Introduction

Global DNA methylation has been associated with chromosomal instability and with a number of health outcomes. Although more data exist at the tissue level, a number of recent studies of global white blood cell (WBC) DNA methylation have shown a relation between lower methylation and cancers of the colon,[1,2] bladder,[3–5] stomach,[6] breast[7] and liver.[8] Other studies have reported on associations with schizophrenia and myelodysplastic syndrome.[9,10] Lower methylation levels have also been associated with benzene exposure, persistent organic pollutants, lead and air pollution.[5,11–14] Although most data do not support an association between active cigarette smoking and global DNA methylation in WBC, we have found associations between pre-natal smoke exposure and WBC methylation measured in adulthood.[15,16] There is ample suggestion, therefore, that global DNA methylation may be sensitive to environmental exposures at several periods during the life course.

Among men and women with pre-natal exposure to the Dutch famine of 1944–45 who underwent medical examinations at age ~58 years, we have previously documented persistent methylation changes on the imprinted *IGF2* gene and other loci but global DNA methylation studies in this population have not yet been undertaken.[17,18] We therefore use here various methods to examine potential changes in global DNA methylation in relation to pre-natal famine.

We employ the Luminometric methylation assay (LUMA)[19] that targets all CCGG sequences in the genome using methylation-sensitive restriction enzymes to discriminate methylated and unmethylated DNA and also assays of genomic methylation of retrotransposable elements and repeat sequences as surrogate markers, using pyrosequencing of the long interspersed nucleotide element-1 (LINE-1) and quantitative real-time PCR (qRT-PCR) with Taqman probes (Methylight) of satellite repeat-2 (Sat2). We selected LUMA and LINE-1 because of their extensive use in epidemiological studies and Sat2 because of our previous results suggesting pre-natal exposure to smoking was associated with decreased Sat2 methylation in WBC.[16]

Methods

Study population

As described elsewhere,[20] we identified a birth cohort of 3307 live-born singleton births at three institutions in famine-exposed cities in The Western Netherlands. We selected all 2417 births between 1 February 1945 and 31 March 1946 (infants whose mothers were exposed to the famine during or immediately preceding that pregnancy). We sampled 890 births from 1943 and 1947 (infants whose mothers were not exposed to famine during their pregnancy) to serve as comparison groups.

We provided the names and addresses at birth of all 3307 infants to the Population Register in the municipality of birth. Of these, 308 persons (9%) were reported to have died and 275 (8%) to have emigrated. For 294 persons (8.9%), a current address could not be located and the Population Registry in Rotterdam declined to trace 130 individuals born out of wedlock. A current address was obtained for 2300 individuals (70% of the birth cohort).

All 2300 traced persons were invited by mail to participate. Initially, our study design called for the recruitment of same-sex sibling pairs and the lack of an available sibling was a reason for ineligibility. We received a reply from 1767 persons, of whom 347 expressed willingness to participate together with a sibling. Of those who declined (1420), 67% (951) reported not having a same-sex sibling available for study and 381 of these later agreed to participate. This resulted in a total of 1075 individuals who were interviewed by telephone, and 971 of this group also underwent a medical examination.

All study protocols were approved by the Human Subjects (medical ethics) committees of the participating institutions. Study participants provided verbal consent at the start of the telephone interview and written informed consent at the start of the clinical examination.

Data collection

Telephone interviews

A 1-h telephone interview included questions on socio-demographic characteristics, including age, gender, education and smoking and drinking habits. Following the telephone interview, we scheduled a clinic visit to the Leiden University Medical Center.

Clinical examinations and measures

Following registration and the obtaining of informed consent, we measured height to the nearest 1 mm with a portable stadiometer (SECA, Hamburg, Germany) and weight to the nearest 100 g with the participant standing in underclothing without shoes on a portable digital scale (SECA). Further details on the obtained anthropometric and other measurements are provided elsewhere.[21]

DNA extraction

Drawn blood samples were spun, processed and stored into labelled vials at −70°C according to standard protocols. Subsequent DNA extraction was done from WBCs using Qiagen Blood Maxi kits (QI-51194) eluting 2 times with 1 ml. The DNA solution was collected in 1.5 ml micro tubes with screw lids labelled with study numbers assigned to study participants that had no relation to exposure status. DNA concentrations were measured using the Picogreen Quant-it kit preceded by a standard A260 spectrophotometer measurement for the initial concentration estimation that is required for the picogreen assay. The first

elution containing 90% of the DNA was split in two batches and kept in microtubes. One batch was previously used for the epigenetic analysis of *IGF2* methylation with the Leiden University Medical Center. For this study, we are using DNA from the second batch. Working sets were prepared of a 50 ng/µl in 10 mM Tris/0.01 mM EDTA buffer, pH8 (10 µg) in ABgene 96-wells 0.8 ml storage plates (ab-0765 96) and covered with ABgene adhesive sealing foil (ab-0626).

DNA methylation assays

The LUMA assay as developed by Karimi *et al*.[19] is based on the ability of two isoschizomers to differentially digest sequences depending on the methylation status of the CpG site within the sequence. Isoschizomers *Msp*I and *Hpa*II target the same sequence 5′-CCGG-3′; however, *Msp*I will digest all CCGG sites and *Hpa*II digestions will be blocked by the presence of a 5 m-C in the second position. An additional enzyme, *EcoR*I, is used to digest the DNA and create an overhang that has a sequence that does not contain C and G nucleotides and serve as a reference for DNA amount present in the reaction mixture. Enzymatic digests are set up as double digest by either *EcoR*I and *Hpa*II or *EcoR*I and *Msp*I. Pyrosequencing with a PyroMark Q24 system is used to sequence the overhangs left by each double digest. We ran a modified version of the assay.[22] The percentage of DNA methylation was expressed as [1-(*Hpa*II/*EcoR*I ΣG/ΣT)/ (*Msp*I/ *EcoR*I ΣG/ΣT)]*100. The coefficient of variation for this assay was 4.9% for samples run on the same day, and 6.2% for samples run on separate days.

For the analysis of LINE-1 and Sat2 methylation, DNA was bisulphite treated using EZ DNA Methylation Kit (Zymo Research Corporation, Irvine, CA, USA) following the manufacturer's recommendations. Pyrosequencing for LINE-1 was carried out as described previously by others.[12] The biotinylated PCR products were purified and pyrosequencing was run on a PyroMark Q24. We used non-CpG cytosine residues as internal controls to verify efficient sodium bisulphite DNA conversion, and ran universal unmethylated and methylated DNAs as controls. Methylation quantification was performed using the PyroMark Q24 1.010 software. The degree of methylation was expressed for each DNA locus as percentage of the methylated cytosine over the sum of methylated and unmethylated cytosine. The average of three CpG sites was used in the analysis. The coefficient of variation for this assay was 3.6% for samples run on the same day and 1.2% for samples run on different days.

As it is not possible to design a pyrosequencing assay for the short Sat2 repeat, the MethyLight assay was carried out using the sequences of probes and forward and reverse primers for the Sat2M1 consensus sequence described in Weisenburger *et al*.[23] Standard curves for the AluC4 repeat control reaction were generated from 1:25 serial dilutions of bisulphite-converted, CpGenome universal methylated and unmethylated DNAs (Zymo Research Corporation, Irvine, CA, USA). Assays were run on an ABI Prism 7900 Sequence Detection System (Perkin-Elmer, Foster City, CA, USA). Universal methylated DNA served as a methylated reference, and AluC4 was used to measure the levels of input DNA to normalize the signal for each methylation reaction. MethyLight data were expressed as a percent of methylated reference (PMR) values. PMR = 100%*2exp − [ΔC_t (Sat2 in sample – AluC4 in sample) − ΔC_t (100% methylated Sat2 in reference sample − AluC4 in reference sample)]. Each MethyLight reaction was performed in duplicate, and the PMR values represent the mean. The coefficient of variation for the Sat2 measurement was 25.2% for samples run on the same day and 28.5% for samples run on different days.

Exposure to famine

We used the date of last menstrual period (LMP) as noted in the hospital records to define the start of gestation unless it was missing or implausible (12%). In those cases, we inferred the LMP date from relevant annotations on the birth record and estimated gestational age from birthweight and date of birth, using cut-points from tables of gender-, parity- and birthweight-specific gestational ages from the combined birth records of the Amsterdam midwives school (1948–57) and the University of Amsterdam Obstetrics Department (1931–65).[20,24]

We characterized exposure to famine during gestation by determining the gestational ages (in weeks after the LMP) during which the mother was exposed to an official ration of <900 kcal/day. We considered the mother exposed in gestational Weeks 1–10, 11–20, 21–30 or 31 to delivery if these gestational time windows were entirely exposed. Thus, pregnancies with LMP between November 26, 1944 and March 4, 1945 were considered exposed in Weeks 1–10; between September 18, 1944 and December 24, 1944 in Weeks 11–20; between July 10, 1944 and October 15, 1944, in Weeks 21–30; and between May 2, 1944 and August 24, 1944, in Weeks 31 through delivery. By these definitions, any participant could have been exposed to famine during at most two adjacent 10-week periods. Individuals exposed in at least one of the 10-week periods were considered to have had some pre-natal famine exposure. We also compared the exposure windows as previously used in the study of IGF2-DMR methylation in this population.[17]

Statistical methods

We computed means and SDs, and categorical frequencies for selected exposure groups as appropriate and assessed differences among exposure groups with ANOVA or chi-square tests. We used linear regression

analyses to estimate the association between methylation percentage of the three selected DNA measures in relation to famine exposure in one or more of the four 10-week gestation periods. These were either combined into one group ('any' exposure) or analysed separately as four distinct exposure groups each defined by an indicator variable. Testing for the significance of the four 10-week exposure periods combined was carried out with a four degrees of freedom Wald's test.

We carried out gender-specific analyses and evaluated possible significant interactions between exposure and gender. In the absence of such interactions (all with *P*-values between 0.30 and 0.90), we only present pooled models with adjustment for gender. We included age at examination (with linear and quadratic terms) in all regressions. Models without adjustment for age and gender gave essentially the same results as adjusted models and are not further reported.

We first analysed the entire study population of 947 individuals (famine exposed, hospital controls and family controls), and then repeated all analyses on the subset of 296 sibling pairs, ignoring the 355 individuals without a same-sex sibling control. The subset analyses did not materially change any of the findings and are not reported separately. All analyses of SAT2 were repeated on the log-transformed variable to normalize distributions. As the findings did not materially change, these analyses are not reported separately. Statistical analyses were conducted with SAS v.9.2 software (SAS Institute, Cary, NC, USA) using hierarchical linear regression models with a random intercept (PROC MIXED) to account for within family clustering where indicated. The findings were confirmed with STATA v.11 software (StataCorp, College Station, TX, USA), using the xtreg command.

Results

LUMA measures were missing for seven individuals, Sat2 for six and all three methylation measures for four individuals. As the number of missing samples was small, we limited our analysis to the 947 participants with available DNA measures from all (LUMA, LINE-1 and Sat2) assays. This study group includes 350 hospital births with pre-natal famine exposure, 290 hospital births without famine exposure (time controls born before or after the famine) and 307 same-sex sibling controls without famine exposure.

The siblings in our study were somewhat younger than the hospital birth series and had a mean age of 57.3 years (range: 36–76 years). The age range of the famine exposed participants was 57–60 years and of hospital controls was 55–62 years. The other selected demographic and behavioural characteristics of the exposure groups did not differ (Table 1). The cumulative distributions for LUMA, LINE-1 and Sat2 for each exposure group are shown in Figure 1. Although the range for the Sat2 distribution is much wider than for the other two measures, a visual inspection shows no differences in distributions of any of the measures in any of the exposure categories. This is confirmed by comparing the mean values of all DNA methylation measures in men and women by exposure category (Table 2).

When analysing the change in DNA methylation by the three measures in famine-exposed participants relative to controls, we observed no interaction of exposure and sex (data not shown). We did not see any significant changes in DNA methylation by any of the measures in relation to pre-natal famine exposure either defined by exposure in any of the four 10-week periods combined or considered separately (Table 3). The same lack of association was seen when the analysis was limited to the 296 sibling pairs in the study and when we analysed potential differences in DNA methylation using the exposure windows from our study of methylation of the IGF2-gene in this population[17] (data not shown).

With regard to the ranked inter-correlation of the methylation measures, there was no correlation between the LUMA measure and LINE-1 (Spearman's

Table 1 Demographic and lifestyle characteristics of 947 adults examined between 2003 and 2005 at age ~58 years, by pre-natal famine exposure category

	Hospital controls[a] ($n=290$)	Sibling controls ($n=307$)	Famine exposed ($n=350$)	*P*-value[b]
Male	47	43	46	0.54
Age (years)	58.8 (1.6)	57.3 (6.3)	58.9 (0.5)	<0.001
Education exceeding primary or lower vocational schooling	73	69	66	0.15
Current smoker	24	22	26	0.61
Alcohol: one drink a week or more	76	74	79	0.27

Values are represented as mean (SD) or % unless otherwise specified.
[a]Born in the same institutions as exposed, but before the famine or after the famine and not exposed to famine during gestation.
[b]Comparing the three exposure categories by analysis of variance or chi-square test, as appropriate.

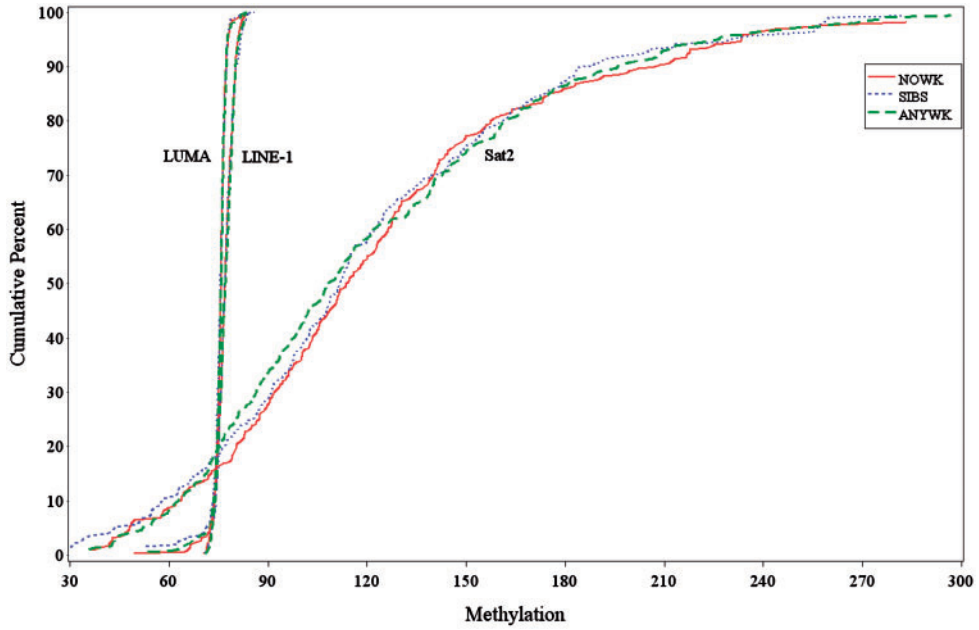

Figure 1 Cumulative frequency distributions of selected DNA genomic methylation percent measures for 947 adults examined between 2003 and 2005 at age ~58 years, by pre-natal famine exposure category.
Nowk: Hospital controls, not exposed in pregnancy. Sibs: Sibling controls, not exposed in pregnancy. Anywk: Famine exposed, any week in pregnancy

Table 2 Selected DNA genomic methylation measures for 947 adults examined between 2003 and 2005 at age ~58 years, by pre-natal famine exposure category

	Hospital controls ($n=290$)	Sibling controls($n=307$)	Famine exposed($n=350$)	P-value[a]
LUMA				
Men	75.5 (2.4)	74.8 (6.3)	75.1 (4.1)	0.41
Women	75.5 (2.7)	74.6 (7.4)	75.4 (3.1)	0.18
All	75.5 (2.6)	74.7 (7.0)	75.3 (3.6)	0.10
LINE-1				
Men	77.4 (2.4)	77.4 (2.4)	77.5 (2.4)	0.93
Women	76.8 (2.4)	77.1 (2.6)	76.8 (2.5)	0.51
All	77.1 (2.4)	77.2 (2.5)	77.1 (2.5)	0.74
Sat2				
Men	128.2 (57.6)	119.9 (50.2)	117.4 (50.4)	0.24
Women	121.7 (58.8)	120.9 (56.5)	125.2 (61.9)	0.75
All	124.7 (58.1)	120.5 (53.8)	121.6 (56.9)	0.58

Values are expressed as mean (SD) or % unless otherwise specified.
[a]P-value for exposure contrasts in two-way ANOVA with interaction term.

$\rho=0.04$; $P=0.22$) or Sat2 ($\rho=-0.03$; $P=0.42$) but the correlation between LINE-1 and Sat2 was −0.29 (95% CI −0.35 to −0.23; $P<0.0001$).

Discussion

In this study of 947 men and women examined at age ~58 years, we found no relation between any of three measures of genomic DNA methylation (LUMA, LINE-1 and Sat2) and pre-natal famine exposure. The absence of an association was confirmed when we limited the analysis to the 296 sibling pairs in the study. We therefore rule out potential confounding at the family level having any significant effect on the nature of this association. As some sibling controls were much younger than either participants with pre-natal famine exposure or hospital controls, we adjusted for age at examination in all analyses although this did not affect any regression estimates.

Table 3 Change in selected DNA genomic methylation measures (percent units) for selected pre-natal famine exposure categories relative to unexposed controls; study population includes 947 adults examined between 2003 and 2005 at age ~58 years

	Exposure during any week of gestation ($n = 350$)	Exposure during weeks 1–10 of gestation ($n = 73$)	Exposure during weeks 11–20 of gestation ($n = 125$)	Exposure during weeks 21–30 of gestation ($n = 143$)	Exposure during weeks 31–40 of gestation ($n = 129$)	Wald test, *P*-value[a]
LUMA						
β[b]	0.16	0.02	0.48	−0.64	0.28	
95%CI	−0.49 to 0.81	−1.16 to 1.19	−0.51 to 1.46	−1.58 to 0.29	−0.66 to 1.21	
P-value[a]	0.63					0.69
LINE-1						
β[b]	−0.05	−0.36	0.32	−0.12	0.02	
95% CI	−0.33 to 0.22	−0.89 to 0.17	−0.11 to 0.75	−0.53 to 0.29	−0.40 to 0.44	
P-value[a]	0.70					0.50
Sat2						
β[b]	−0.51	−5.56	0.47	1.48	−3.48	
95%CI	−7.38 to 6.36	−18.6 to 7.49	−10.2 to 11.2	−8.74 to 11.7	−13.8 to 6.86	
P-value[a]	0.88					0.88

[a]*P*-values either for exposure during any week of gestation, or for exposures during Weeks 1–10, 11–20, 21–30 or 31–40 of gestation considered as a set by four degrees of freedom Wald's test.
[b]Adjusted for age, age^2 and gender, as well as clustering within siblings.

It is a recognized challenge in large epidemiological studies of DNA methylation to select an assay that is both cost efficient and valid. This is particularly important for measures of genomic DNA methylation that may exhibit some bias unless they are whole genome based. To minimize these potential limitations, we used three different assays as measures of genomic DNA methylation. The selected methods each have their strengths and limitations, but none suggests that there is any association between pre-natal famine exposure and DNA methylation at age ~58 years in this cohort.

LUMA provides a measure of 5 mC at CpG island and non-CpG island regions, which is more informative of overall genomic DNA methylation levels. However, the CCGG sequence accounts for only 8% of CpG sites of the genome.[25] Contrasting uniform LUMA methylation measures of men and women with and without pre-natal famine exposure is still valid and highly relevant, however, for study questions pertaining to potential long-term changes in selected areas of the genome.

In this study, we also determined repetitive element methylation targeting two different sequences, by two different methods. LINE-1 are the only human retrotransposable elements and account for 17–20% of the genome. However, only 80–100 of these elements are still active and have transcriptional capacity due to loss of the 5′-UTR, mutations and rearrangements. Most of the transcriptionally active remaining elements are polymorphic, a fact that limits our ability to accurately detect DNA methylation percentages at these genomic regions.[26] LINE-1 was measured by pyrosequencing, a methodology that provides an accurate quantitative measure of DNA methylation.[27] The main limitation of this assay is the bisulphite conversion step, which can be incomplete. However, internal controls provide a measure of the quality of the conversion.

Sat2 elements belong to the satellite family of non-coding tandem repeats. These are located in heterochromatic regions and can interfere with transcription by inducing gene relocalization. Sat2 DNA methylation levels were measured by MethyLight, a high-throughput methodology that can be easily applied to large population studies. This methodology also required bisulphite conversion and does not provide a truly quantitative measure of DNA methylation. Unlike pyrosequencing that provides information on the average methylation level at each CpG site, MethyLight requires that all the CpG sites in the primers and Taqman probe are fully methylated for amplification to occur. An internal standard (Alu) accounts for the amount of template in the reaction. Since the number of copies of the elements may vary among individuals, accurate quantification is difficult. This may also account for the values >100%. The large variability of the assay is also a limitation. The PMR is calculated using four threshold cycle values each with their own variability. The lack of association of the two repetitive element measures with pre-natal famine exposure is again informative, however, as

any differences could guide further targeted research along specific lines into biological mechanisms.

The inverse correlation between LINE-1 and Sat2 methylation observed was unexpected. We previously observed a positive correlation between LINE-1 and Sat2 methylation when methylation of both repetitive elements was measured by MethyLight.[28] Quantification of fully methylated sequences by MethyLight clearly does not perfectly correlate with the average methylation levels obtained by pyrosequencing. This, in conjunction with the many samples with >100% methylation, suggests that the MethyLight may not be an appropriate assay for analysis of genomic DNA methylation.

In summary, our results using three different assays as a measure of genome-wide methylation do not support an effect of pre-natal famine on adult global DNA methylation levels in WBC. This is in contrast to our previous results on *IGF2* methylation. Further work should focus on selected regions in the genome that may be differentially methylated in response to changes in early life exposures and predict adult health outcomes.

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Conflict of Interest: None declared.

KEY MESSAGES

- This study shows no relation between pre-natal famine exposure and three measures of global DNA methylation (LUMA, LINE-1, Sat2) at age ~58 years.
- Earlier study in this population showed a persistent decrease in DNA methylation of the *IGF2* gene after pre-natal famine early in pregnancy.
- Further work should focus on possible methylation changes of other selected loci in the genome.

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Commentary: Marking the epigenome—in search of the fingerprints of intrauterine nutritional deficiencies

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Epigenetics is increasingly attracting the attention of epidemiologists for many reasons: unlike the genetic code, epigenetic marks are modifiable; environmental exposures influence the epigenome; epigenetics may provide the mechanistic underpinning for certain exposure–disease associations; and, the epigenome provides a wealth of opportunities to identify new disease risk and early detection biomarkers.[1] Moreover, the relevance of epigenetic aberrations for health and disease has been suggested in numerous studies, linking epimutations to a variety of illnesses ranging from childhood disorders to cancer. The prenatal establishment of the epigenome has made it a favourite also among researchers of the Developmental Origins of Health and Disease (DOHaD), as transient perturbations during intrauterine life may affect the reprogramming of the epigenetic code.[2]