



Early Life Environment

Early gestation as the critical time-window for changes in the prenatal environment to affect the adult human blood methylome

Elmar W Tobi,¹ Roderick C Slieker,¹ Aryeh D Stein,² H Eka D Suchiman,¹ P Eline Slagboom,¹ Erik W van Zwet,³ Bastiaan T Heijmans^{1†} and LH Lumey^{1,4,*†}

¹Molecular Epidemiology, Leiden University Medical Center, Leiden, The Netherlands, ²Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, Georgia USA, ³Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, The Netherlands and ⁴Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, New York USA

*Corresponding author. Department of Epidemiology, Mailman School of Public Health, Columbia University, 722 West 168 Street, New York, NY 10032, USA. E-mail: lumey@columbia.edu

†These authors contributed equally to the work

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Abstract

Background: The manipulation of pregnancy diets in animals can lead to changes in DNA methylation with phenotypic consequences in the offspring. Human studies have concentrated on the effects of nutrition during early gestation. Lacking in humans is an epigenome-wide association study of DNA methylation in relation to perturbations in nutrition across all gestation periods.

Methods: We used the quasi-experimental setting of the Dutch famine of 1944–45 to evaluate the impact of famine exposure during specific 10-week gestation periods, or during any time in gestation, on genome-wide DNA methylation levels at age ~ 59 years. In addition, we evaluated the impact of exposure during a shorter pre- and post-conception period. DNA methylation was assessed using the Illumina 450k array in whole blood among 422 individuals with prenatal famine exposure and 463 time- or sibling-controls without prenatal famine exposure.

Results: Famine exposure during gestation weeks 1–10, but not weeks 11–20, 21–30 or 31-delivery, was associated with an increase in DNA methylation of CpG dinucleotides cg20823026 (*FAM150B*), cg10354880 (*SLC38A2*) and cg27370573 (*PPAP2C*) and a decrease of cg11496778 (*OSBPL5/MRGPRG*) ($P < 5.9 \times 10^{-7}$, $P_{FDR} < 0.031$). There was an increase in methylation of *TACC1* and *ZNF385A* after exposure during any time in gestation ($P < 2.0 \times 10^{-7}$, $P_{FDR} = 0.034$) and a decrease of cg23989336 (*TMEM105*) after exposure around conception. These changes represent a shift of 0.3–0.6 standard deviations and are linked to genes involved in growth, development and metabolism.

Conclusion: Early gestation, and not mid or late gestation, is identified as a critical time-period for adult DNA methylation changes in whole blood after prenatal exposure to famine.

Key words: DNA methylation, prenatal, Dutch Famine, Hunger Winter, exposure, nutrition

Key Messages

- Studies on prenatal nutrition and DNA methylation in humans have thus far been focused on early gestation. A systematic investigation of the influence of prenatal nutrition on DNA methylation patterns genome-wide during specific periods outside early gestation is missing.
- We have used the quasi-experimental setting of the Dutch Famine to study the impact of famine exposure during specific 10-week periods of gestation on genome-wide DNA methylation patterns in whole blood evaluated at age ~58 years.
- We found specific associations with famine exposure during weeks 1–10 of gestation but not weeks 11–20, 21–30 and 31–delivery. We also found associations with famine exposure across all gestation periods. An impact was also seen among individuals conceived between March and early May 1945, who do not meet the definition of early famine exposure throughout weeks 1–10 of gestation.
- Early gestation seems a uniquely sensitive period during which the blood methylome may be sensitive to the prenatal environment.

Introduction

Measures of prenatal adversity have been associated with adult disease,¹ but identifying the causal factors has been challenging.² Quasi-experimental studies using the setting of the Dutch Hunger Winter of 1944–45,³ a 6-month famine, show that exposure to famine during pregnancy may increase the risk of obesity, type 2 diabetes, dyslipidaemia and schizophrenia in the offspring, and have highlighted early gestation as a sensitive period.⁴

In animal experiments, perturbation of prenatal nutrition has been shown to alter epigenetic marks such as DNA methylation which control stable changes in gene expression potential⁵ and are associated with adult phenotype.⁶ Experiments perturbing early maternal nutrition have shown that blastocyst development and the period around implantation is a critical developmental window during which DNA methylation differences may arise.^{7,8} This may be related to the dynamic nature of DNA methylation during this period, as the genome is demethylated after fertilization and remethylated in the period after implantation.⁹ Most animal studies, however, evaluate nutritional perturbations across gestation in relation to DNA methylation differences.^{10,11}

In humans, we have shown that prenatal famine exposure is associated with persistent differences in DNA methylation in adulthood and that the relation can depend on the gestational timing of the exposure.^{12,13} We previously only examined famine exposure during the first or last 10

weeks of gestation in a candidate gene approach^{12,13} and the first 10 weeks in a genome-scale study.¹⁴ Studies by us and others on prenatal famine,^{12–15} seasonal food shortage¹⁶ and micro-nutrient supplementation^{17,18} have focused on early gestation as being one period that is critically sensitive in this respect. DNA methylation differences may also arise later in gestation, for example from smoking during pregnancy.¹⁹

It is unknown during which other specific periods of gestation the human methylome may be sensitive to prenatal perturbations in nutrition, as a systematic genome-wide investigation is still missing. Here we report on an epigenome-wide association study (EWAS) for famine exposure during specific gestation periods and for exposure to famine in any period during gestation.

Methods

Study setting

The Dutch Hunger Winter was a 6-month famine at the end of World War II that resulted from punitive measures imposed by occupying German forces after a national railway strike, winter conditions and fuel shortages. Food rations were distributed centrally and, during the famine, rations were below 900 kcal/day between 26 November 1944 and 15 May 1945. The percentage of calories from proteins, fat and carbohydrates in the diet was constant as the food rations diminished.^{20,21}

Study subjects

The Dutch Hunger Winter Families study is described in detail elsewhere.²² We identified live-born infants in three institutions in famine-exposed cities in the western Netherlands and selected the 2417 singleton births between 1 February 1945 and 31 March 1946 of infants whose mothers were exposed to the famine during or immediately preceding that pregnancy. The study population is characterized by low socioeconomic status (SES) pregnancies and does not differ on SES of origin. We sampled 890 births from 1943 and 1947, to include infants whose mothers were not exposed to famine during this pregnancy as time-controls. A current address was obtained for 2300 individuals (70% of the birth cohort). They were invited by mail to participate in a telephone interview and in a clinical examination, together with a same-sex sibling not exposed to the famine to serve as a family-control. This means that any of the famine-exposed individuals or time-controls from the birth series could have a matched unexposed sibling control. We conducted 1075 interviews and 971 clinical examinations (of 345 clinic births without a matched sibling and 313 with a matched sibling) between 2003 and 2005.

The study was approved by the Institutional Review Board (IRB) of Columbia University Medical Center and by the Medical Ethics Committee (MEC) of Leiden University Medical Center. Study participants provided verbal consent at the start of the telephone interview and written informed consent at the start of the clinical examination.

Famine exposure

We used the date of the last menstrual period (LMP) from the hospital records to define the start of gestation unless it was missing or implausible (12%). Otherwise, we estimated LMP from annotations on the birth record and from birthweight and date of birth.²² We characterized exposure to famine during gestation by determining the gestational ages (in weeks after LMP) during which the mother was exposed to an official ration of less than 900 kcal/day. As in prior studies of the population starting in 2007,²³ 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 contained within this period. Pregnancies with LMP between 26 November 1944 and 4 March 1945 were thus considered exposed in weeks 1–10; between 18 September 1944 and 24 December 1944 in weeks 11–20; between 10 July 1944 and 15 October 1944 in weeks 21–30; and between 2 May 1944 and 24 August 1944 in week 31 to delivery. Some participants therefore could be considered exposed to famine during two

adjacent 10-week periods. Individuals exposed in at least one of the 10-week gestation periods were considered to have had ‘any’ prenatal famine exposure. No individuals were exposed throughout gestation. The exposure definition outlined above represents an average exposure to famine post conception of <900 kcal/day during an entire gestation period of 10 weeks. This definition does not cover individuals with LMPs between 5 March 1945 and 15 May 1945, who were exposed to extreme famine but for shorter periods and do not meet this definition. To evaluate the impact of shorter periods of famine on these births, we evaluated changes in DNA methylation outcomes in this group. We also explored the potential influence of the duration of famine exposure during gestation and DNA methylation at age ~58 years.

Characteristics in 2003–2005

At interview and examination, we collected data on several adult characteristics that have been associated with differential DNA methylation in whole blood, including smoking,²⁴ dietary intake of macronutrients²⁵ or micronutrients²⁶ and SES.²⁷ These were evaluated as potential confounders in our analyses. Smoking was classified as never, current or past. Dietary intake in the past 12 months was ascertained from a 140-item food frequency questionnaire developed to assess dietary habits in an elderly Dutch population. This questionnaire provides estimates of macronutrient (total energy and fat, protein and carbohydrate) and micronutrient intake (folic acid, vitamin B₁₂, and vitamin B₆).²⁸ We classified study participants on a five-level education scale, identifying individuals with primary (ages 6–12 years), lower or middle level vocational, secondary, higher vocational and university education.

DNA methylation data generation and processing

Genome-wide DNA methylation data were generated using the Illumina Infinium Human Methylation 450k BeadChip (450k array). Briefly, 500 ng of genomic DNA from whole blood isolated using the salting-out technique was bisulfite-treated using the EZ-96 DNA methylation kit (Zymo Research, Orange County, USA). DNA of study participants was treated together with that of their control sibling on 96-well plates with even distributions of exposure periods, sex ratios and mean ages per 96-well plate and 450k array. Sibling pairs were placed on the same row of the 450k array with random allocation to the left or right column. Participants without a control sibling were randomly placed on a separate set of 96-well plates with even distributions in exposure periods, sex ratios and mean ages.

The 450k arrays were measured at the Erasmus Medical Center Human Genotyping Facility in Rotterdam, The Netherlands. The quality of the generated 450k array data was assessed using both sample dependent and sample independent quality metrics using the *Bioconductor* package *MethylAid* (29) with default settings. Bisulfite conversion efficiency was assessed using the dedicated probes on the 450k array and by Sanger Sequencing four random samples per 96-well plate. To prevent loss of power due to sample swaps, a subset ($N=21$) of the genotypes of single nucleotide polymorphisms (SNPs) yielded by the 450k array were compared by those measured with MASSARRAY and sample sexes were checked using X-chromosomal CpG dinucleotides (Supplementary Methods, available as Supplementary data at *IJE* online).

Normalization of the dataset was performed by Functional Normalization³⁰ implemented in the *minfi* package³¹ using six principal components. All measurements with a detection P -value >0.01 or zero intensity value were set as missing. The measurement success rate per sample was $>99\%$. Probes that did not map to unique genomic locations³² or with a $<95\%$ measurement success rate were then removed.

Because of background signal and measurement characteristics, few of the CpG dinucleotides have a methylation estimate (approximated by the array β value) equal to zero or one. It is known from bisulfite sequencing data that a significant proportion of CpG dinucleotides are fully methylated or unmethylated and show no interindividual variation.¹⁴ After consultation of 17 whole genome bisulfite sequencing datasets covering the major blood cell types, namely lymphocytes, neutrophils and monocytes,^{33,34} such uninformative CpG dinucleotides were identified and removed (Supplementary Methods, available as Supplementary data at *IJE* online).

After further removal of the X and Y chromosomal probes, outlier detection was performed using principal component analysis (PCA) on all remaining probes. High-quality DNA methylation data were obtained for 944 of the 971 individuals who completed the clinical examination for 342 596 autosomal CpG dinucleotides. The first three principal components of the variation in the data account for 16.5% of the total variation (Supplementary Figures 3 and 4, available as Supplementary data at *IJE* online) and reflect the cellular heterogeneity of whole-blood specimens, as indicated by a high correlation between these components and imputed cell mixture proportions.³⁵ The next 12 principal components each explained less than 1.6% of the variation and mainly reflect technical variation. Of these 944 individuals, 348 individuals were exposed during any 10-week period and 74 individuals were conceived during the famine between 5 March 1945 and 15 May 1945. As controls we

used for each EWAS 160 individuals born in the same institutions in 1943 or 1947 (time-controls) and 303 same-sex siblings recruited from the individuals in the 1943, 1945–46 and 1947 births series.

Statistical analysis

We used generalized estimation equations (GEE) with a Gaussian link function to evaluate the association between DNA methylation percentage (as reflected by the 450k array β -value) and famine exposure. Six separate EWAS analyses were run, one each for exposures to a specific 10-week period of gestation, one for ‘any’ exposure during gestation and one for conceptions from March to early May 1945. Thus for each EWAS, the individuals meeting an exposure definition were compared with all 463 unexposed time- and family-controls. GEE offer a flexible statistical framework to handle within-family correlations, unaffected from confounding by unmeasured family-level factors.³⁶ Using GEE, we controlled for correlation within sibships and we additionally adjusted for age, sex, row on the 450k array and batch type (bisulfite conversion plate and scan batch), and adjusted for cell heterogeneity by incorporating the first three principal variance components as their proxy measure. We additionally adjusted for potential confounders including smoking status, current macronutrient and micronutrient intake and socioeconomic status where noted. In view of sex-specific differences previously reported by us,¹³ we evaluated possible interactions between famine exposure and sex for the outcomes of interest by incorporating both interaction and main effects in the model. In the absence of statistical evidence for sex interactions, we present sex-adjusted results combining men and women. All P -values reported for the associations between DNA methylation and famine exposure are two-sided. P -values were corrected for genome-wide inflation by multiplying the standardized robust error with the square root of the inflation factor λ (Supplementary Methods, available as Supplementary data at *IJE* online)³⁷ and were adjusted for multiple testing using false discovery rate correction (FDR).³⁸ Methylation percentages in the figures and tables reflect β -value estimates. We used a 10kb-window around CpG dinucleotides associated with prenatal famine to investigate the existence of additional (nominal) significant associations. In a Dutch Reference panel³⁹ (GoNL version 5) we found no overlap between single nucleotide polymorphisms (SNPs) and the probes measuring the CpG dinucleotides associated with prenatal famine exposure. Annotation of the genomic regions to regulatory features such as enhancers was done using ENCODE annotations.⁴⁰

Results

A total of 348 individuals met our definition of being exposed during any of the four 10-week gestation periods. Of these, 73 individuals were classified as exposed during weeks 1–10 of gestation, 123 during weeks 11–20, 143 during weeks 21–30 and 128 during weeks 31 through to delivery. Some individuals meet the definition for exposure in two adjacent gestation periods. Of individuals born in 1945 or 1947 in the same institutions as the famine-exposed individuals, 160 are time-controls. The famine-exposed individuals and the time-controls had 303 unexposed same-sex siblings serving as family-controls (Table 1).

Comparing the individuals with prenatal famine exposure, time-controls and family-controls, we found a difference in the mean age ($P < 0.001$), with sibling-controls being slightly younger than the prenatal famine-exposed individuals (Table 1). Omnibus tests showed no other differences between exposure groups for any of the remaining characteristics. There were no differences in the proportion of complete sibships for exposure and time-control groups (Supplementary Table 1, available as Supplementary data at *IJE* online).

DNA methylation after famine exposure in specific 10-week gestation periods

We performed a separate EWAS of famine exposure during each of the four 10-week gestation periods. Quantile-Quantile plots (QQ-plots) representing the observed vs the expected test statistic for each evaluated CpG nucleotide given the number of performed tests are shown in Figure 1. For several CpGs, nominal P -values were lower than the expected distribution of P -values for famine exposure in weeks 1–10 of gestation. In contrast, no evidence for an association between DNA methylation and famine exposure in weeks 11–20, 21–30 or 31 to delivery was observed.

Four CpG dinucleotides (cg20823026, cg11496778, cg10354880 and cg27370573) were associated with famine exposure in weeks 1–10 of gestation after multiple testing correction ($P_{FDR} < 0.031$, Figure 2). No association was found for these CpG dinucleotides in any of the other exposure periods (Table 2). The effect sizes did not change after further adjustment for variables that could potentially mediate the effects of prenatal nutrition on DNA methylation, including current lifestyle factors such as smoking and dietary characteristics and SES.

CpG cg20823026 shows a 2.3% increase in methylation [95% confidence interval (CI): 1.5 to 3.1, $P = 3.1 \times 10^{-8}$] among individuals exposed in weeks 1–10 compared with time- and family-controls, representing a shift of 0.50 standard deviations (SD), and is located in an

intergenic region between *FAM150B* (genomic distance 77 kb) and *TMEM18* (302 kb). CpG cg11496778 shows a 2.3% decrease in methylation (95% CI: -3.1 to -1.5 , $P = 2.1 \times 10^{-7}$), a shift of 0.50 SD, and is located in a poised promoter distal from the *OSBPL5* (distance 37 kb) and *MRGPRG* (distance 14 kb) genes. Two adjacent CpGs show a nominally significant difference in methylation between the individuals prenatally exposed to famine and the time- and sibling controls (Supplementary Table 2, available as Supplementary data at *IJE* online). CpG cg10354880 shows an increase of 0.7% (95% CI: 0.5 to 0.9, $P = 5.9 \times 10^{-7}$), representing a shift of 0.56 SD in methylation. This CpG is located in an enhancer mapping to the downstream *SLC38A2* gene (15 kb distance). CpG cg27370573 shows an increase of 2.7% (95% CI: 1.7 to 3.7, $P = 3.6 \times 10^{-7}$), representing a 0.68-SD higher methylation. This CpG is located in the proximal promoter of *PPAP2C* (<1 kb) together with adjacent CpG dinucleotides that show a nominally significant difference in methylation (Supplementary Table 3, available as Supplementary data at *IJE* online).

There was no interaction of famine exposure and sex, and the cell composition of blood did not affect the observed patterns of famine exposure and methylation (Supplementary Table 4, available as Supplementary data at *IJE* online), except for cg11496778 (*OSBPL5/ MRGPRG*) for which the methylation change after famine exposure became more pronounced after cellular adjustment (from -1.5% to -2.3%).

DNA methylation and any exposure to famine

Next we performed an EWAS of famine exposure in any of the gestation periods. A QQ-plot representing the observed vs the expected test statistic given the number of performed tests for each evaluated CpG nucleotide is shown in Figure 3. Methylation differences were seen for the CpG dinucleotides cg15659713 and cg26199857 ($P_{FDR} = 0.034$, Figure 4). The estimates did not change after additional adjustment for smoking, current dietary characteristics or SES. There was no interaction between famine exposure and sex. The estimates were not affected by cell composition (Supplementary Table 4, available as Supplementary data at *IJE* online). Neither of these two CpG dinucleotides was associated with famine exposure limited to weeks 1–10, 11–20, 21–30 or weeks 31 to delivery, and the direction and the magnitude in each of these four exposure periods were similar to the estimate for the aggregate (Table 3). We further considered whether DNA methylation of these two CpG dinucleotides was dependent on the number of weeks of famine exposure. No relation between the number of weeks of famine exposure and DNA methylation of cg15659713 and

Table 1. Individual characteristics by famine exposure in selected gestation periods

Characteristics	Any exposure	Weeks 1–10 of gestation	Weeks 11–20 of gestation ^d	Weeks 21–30 of gestation ^e	Weeks 31-delivery ^f	Time-controls: 1943 & 1947	Family-controls: siblings
N	348	73	123	143	128	160	303
Age (y) [SD]*	58.9 [0.5]	58.6 [0.4]	58.8 [0.5]	59.0 [0.5]	59.1 [0.5]	59.2 (2.0)	57.3 [6.4]
% male	46	46.6	46.3	49.7	48.4	45.0	41.9
Paternal SES ^a [SD]	1.4 [0.8]	1.4 [0.7]	1.4 [0.8]	1.4 [0.8]	1.5 [0.9]	1.5 [0.8]	1.4 [0.8]
SES ^a [SD]	2.2 [1.3]	1.9 [1.2]	2.2 [1.3]	2.2 [1.2]	2.5 [1.3]	2.2[1.1]	2.2 [1.2]
% smokers ^b	25.3	24.7	27.6	23.8	24.2	20.2	22.4
Current dietary intake ^c							
Kcal [SD]	2249 [628]	2334 [612]	2293 [719]	2265 [684]	2233 [574]	2167[637]	2168 [631]
Protein (g) [SD]	96 [28]	100 [29]	100 [33]	98 [29]	94 [24]	95[26]	91 [26]
Fat (g) [SD]	91 [33]	94 [33]	94 [38]	92 [36]	89 [29]	86[33]	86 [32]
Carbohydrates (g) [SD]	234 [74]	243 [73]	234 [81]	235 [77]	235 [71]	226[74]	232 [74]
Folic acid (µg) [SD]	228 [131]	236 [73]	230 [80]	233 [188]	215 [65]	216[71]	209 [63]
Vitamin B ₁₂ (µg) [SD]	7.1 [6.2]	7.3 [4.5]	7.4 [5.0]	7.4 [8.0]	6.4 [4.1]	6.7[4.0]	5.9 [3.1]
Vitamin B ₆ (µg) [SD]	1.9 [0.5]	1.9 [0.5]	1.9 [0.6]	1.9 [0.5]	1.8 [0.5]	1.9[0.5]	1.8 [0.5]

^aSocioeconomic status as assessed by attained educational level (1–5 scale with 1 as lowest category).

^bThe percentage of current smokers.

^cEstimated from food frequency questionnaire. Entries include: total energy intake (kcal); macronutrient intake of protein, fat and carbohydrates (in grams); and micronutrients intake of folic acid, vitamin B₁₂ and vitamin B₆ (in micrograms).

^d24 individuals were classified as exposed in both weeks 1–10 and weeks 11–20.

^e52 individuals were classified as exposed in both weeks 11–20 and 21–30.

^f43 individuals were classified as exposed in both weeks 21–30 and 31-delivery.

* $P < 0.01$, comparing age at examination of family-controls with time-controls and famine exposed individuals. Other characteristics show no differences between exposure categories with omnibus tests across exposure categories.

cg26199857 was found in the individuals with any exposure to famine.

CpG cg15659713 shows a 1.2% increase (95% CI: 0.8 to 1.7, $P = 2.0 \times 10^{-7}$), representing a 0.32-SD shift in methylation which extends to two neighbouring CpG dinucleotides (Supplementary Table 5, available as Supplementary data at *IJE* online). These three CpG dinucleotides are located in the first exon of the *TAC1* gene. CpG cg26199857 shows a 2.0% methylation increase (95% CI: 1.3 to 2.7, $P = 1.5 \times 10^{-7}$), representing an effect size of 0.35 SD. Six neighbouring CpG dinucleotides show a nominal significant difference in methylation (Supplementary Table 6, available as Supplementary data at *IJE* online). These six CpG dinucleotides are located in the last exons of *ZNF385A* and in the promoter region of *GPR84* (6kb, which is located telomeric from *ZNF385A*).

Conceptions in March, April and May 1945

Our focus on famine exposures defined by 10-week gestation periods with rations averaging less than 900 kcal/

day excludes individuals conceived during the famine in March, April and early May 1945. These individuals ($N = 74$) were conceived during extreme famine, but exposed for a shorter period in gestation and also before conception. These individuals had both pre- and post-conception famine exposure and we performed an EWAS on this group. The QQ-plot showed several CpGs with a nominal P -value lower than the expected distribution of P -values (Supplementary Figure 1, available as Supplementary data at *IJE* online). One CpG dinucleotide, cg23989336, was associated with pre- and post-conception famine exposure ($P_{FDR} < 0.037$). No association was found however for cg23989336 in any of the 10-week gestation periods (Supplementary Table 7 available as Supplementary data at *IJE* online). The effect size did not change after adjustment for potentially confounding variables, including current lifestyle factors such as smoking and dietary characteristics or for SES.

CpG cg23989336 was associated with a –3.5% decrease in methylation (95% CI: –4.6 to –2.3, $P = 1.0 \times 10^{-7}$), representing a shift of 0.54 standard deviations (SD), and is located in an enhancer region between

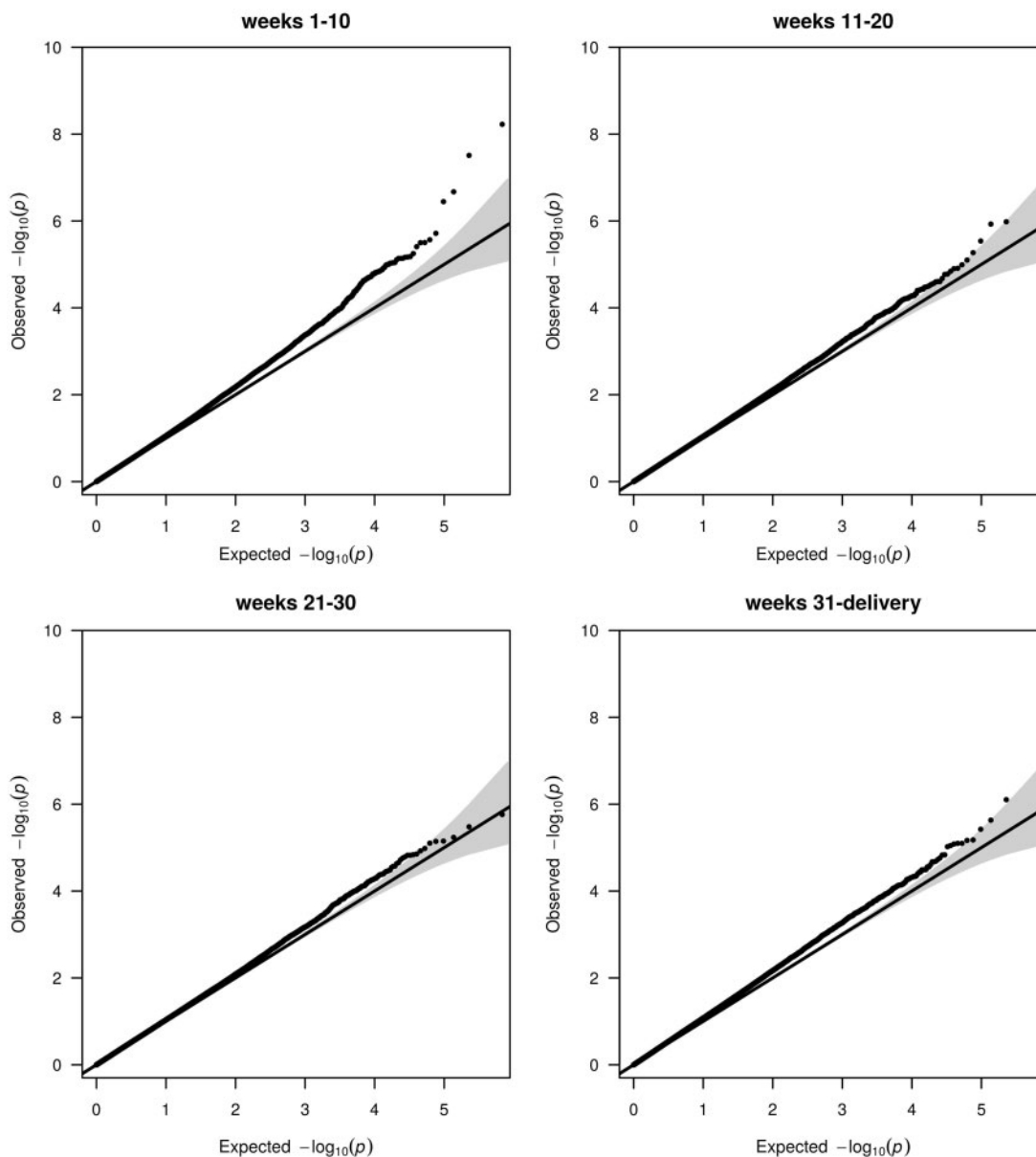


Figure 1. QQ-plots of prenatal famine exposure during specific 10-week periods. Plots depicting the Observed statistic (y-axis) with the statistic as expected by chance given the number of tests (x-axis). The 95% confidence interval of this relationship is given by the grey area around the expected line (black) for the instance that the observed statistic exactly follows the expected statistic. Each dot is the test statistic for one CpG dinucleotide. Enrichments for associations that go beyond that expected by chance can be seen as deviations upward from the expected line and 95% CI area. The *P*-values were corrected for the inflation factor [weeks 1–10 ($\lambda = 1.12$), weeks 11–20 ($\lambda = 1.12$), weeks 21–30 ($\lambda = 1.12$), weeks 31–delivery ($\lambda = 1.20$)] and uncorrected QQ-plots are shown in [Supplementary Figure 8](#), available as Supplementary data at *IJE* online.

TMEM105 (1 kb) and *SLC38A10* (15 kb). The association extended to four adjacent CpG dinucleotides ([Supplementary Table 8](#), [Supplementary Figure 2](#), available as Supplementary data at *IJE* online).

Discussion

We found that DNA methylation in four CpG dinucleotides measured at age ~ 58 years in whole blood was associated with prenatal famine exposure during the first 10

weeks of gestation. No associations were found however with famine exposure in other 10-week periods (weeks 11–20, 21–30 or 31 to delivery). We found an association between any famine exposure and DNA methylation of two CpG dinucleotides. Individuals conceived in March, April and early May 1945 with pre- and post-conception famine exposure showed an association with DNA methylation of one CpG dinucleotide. The CpG dinucleotides identified are linked to genes with roles in growth, differentiation and metabolism.

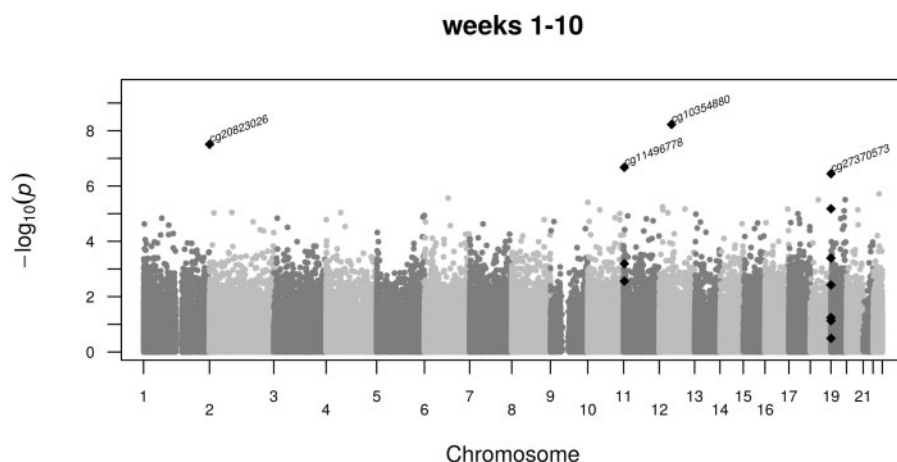


Figure 2. Manhattan plot of specific CpG associations with prenatal famine exposure during weeks 1–10 of gestation. Shown are the $-\log_{10} P$ -values (y-axis) of the association between DNA methylation at single CpG dinucleotides and famine exposure along the autosomal chromosomes (x-axis). Marked by the CpG dinucleotide identifier are the CpG dinucleotides significant after multiple testing. These and adjacent nominally significant CpG dinucleotides are depicted as black diamonds.

Of the four CpG dinucleotides, CpG cg20823026 is located between *FAM150B*, involved in cell growth and differentiation,⁴¹ and *TMEM18*, a known obesity gene.⁴² CpG cg10354880 lies within a strong enhancer⁴³ mapping to the *SLC38A2* gene, whose function includes matching nutrient supply to fetal demand, together with *IGF2*,⁴⁴ for which we previously reported differential methylation in a subset of this cohort.^{12,15} CpG dinucleotide cg11496778 is located distal from *OSBPL5* which is implicated with intracellular cholesterol transport;⁴⁵ and CpG dinucleotide cg27370573 lies in a CpG island in the proximal promoter of *PPAP2C* involved in cell cycle regulation.⁴⁶

With regard to the two CpG dinucleotides associated with any famine exposure, the region around CpG dinucleotide cg26199857 overlaps several exons of *ZNF385A*. Moreover, ENCODE data⁴⁷ indicate that the region overlaps an enhancer that interacts with the promoter of *ZNF385A* under the influence of *CTCF* protein binding.⁴⁸ *ZNF385A* is implicated in cell division and survival upon genotoxic stress⁴⁹ and early adipogenesis.⁵⁰ CpG cg15659713 is located in an exon of *TACCC1*, which is dynamically expressed during development⁵¹ and has a role in cell division⁵² and thyroid hormone and retinoic acid signalling cascades.⁵³

We also found evidence for an association in the group with pre- and post-conception famine exposure. CpG dinucleotide cg23989336 overlaps an enhancer and a CpG island downstream of *TMEM105*, a gene of unknown function, and *SLC38A10*, shown to influence body size in mouse knock-out studies.⁵⁴ This association was not found after prenatal famine exposure throughout any of the 10-week gestation periods, including weeks 1–10. More

detailed studies on famine exposure before and around the time of conception should therefore be undertaken.

The CpG dinucleotides identified can be linked to genes in pathways involved in growth, development and lipid metabolism and are mostly located in exons and regulatory regions such as enhancers, matching findings from a previous genome-scale study of DNA methylation and early gestation famine exposure in a small subset of this population.¹⁴ In that study we measured more CpG dinucleotides (1.2 M) and more of these important regulatory regions^{55,56} which are sparsely covered by the 450k array. In that study we found 181 of these regions associated with famine exposure during early gestation that were not covered in our current study; overall there was an overlap of just 9000 CpG dinucleotides between studies. The preferential coverage of CpG islands and promoters by the 450k array, assessing $\sim 1.5\%$ of all CpG dinucleotides in the genome, may explain why we only see a limited number of CpG dinucleotides associated with prenatal famine. In both studies we covered only a fraction of CpG dinucleotides in regulatory regions³³ and of the 28 M CpG dinucleotides in the genome; this highlights the need for new cost-effective high-throughput platforms for assessing DNA methylation genome-wide.⁵⁷ We previously studied DNA methylation of several candidate regions in a subset of the cohort with EpiTYPER.¹³ The *LEP*, *INSIGF* and *ABCA1* promoter regions were represented by one or more CpG dinucleotides in the 450k array, and although these assays do not cover the same CpG dinucleotides, methylation of the *LEP* and *INSIGF* promoters was also associated with famine exposure during weeks 1–10 in the 450k data (data not shown).

Table 2. CpG dinucleotides associated with famine exposure during weeks 1–10 of gestation

CpG	CpG dinucleotide Location (hg19)	Any exposure		Weeks 1–10		Weeks 11–20		Weeks 21–30		Weeks 31–delivery		
		Mean Beta-value, Estimate (95% CI) ^a	P_{FDR}	Estimate, (95% CI)	P_{FDR}	Estimate (95% CI)	P_{FDR}	Estimate (95% CI)	P_{FDR}	Estimate (95% CI)	P_{FDR}	
		% (SD) controls										
cg20823026	chr2:366,113	84.8 (4.8)	0.5 (-0.1–1.1)	0.89	2.3 (1.5–3.1)	5.27x10 ⁻³	0.6 (-0.3–1.4)	0.94	0.8 (0.0–1.5)	0.90	0.4 (-0.5–1.3)	0.95
cg11496778	chr11:3,225,076	26.4 (4.7)	-0.5 (-1.0–0.0)	0.86	-2.3 (-3.1–-1.5)	0.024	-1.1 (-1.8–-0.5) ^b	0.69	-0.3 (-1.0–0.4)	0.96	0.1 (-0.6–0.8)	0.98
cg10354880	chr12:46,737,123	87.6 (1.3)	0.1 (-0.1–0.2)	0.95	0.7 (0.5–0.9)	2.03x10 ⁻³	0.1 (-0.1–0.3)	0.96	0.1 (-0.1–0.3)	0.98	0.1 (-0.1–0.3)	0.93
cg27370573	chr19:292,167	84.2 (4.1)	0.7 (0.1–1.2)	0.81	2.7 (1.7–3.7)	0.031	0.9 (0.1–1.7)	0.86	0.3 (-0.5–1.1)	0.97	0.8 (0.0–1.6)	0.84

Estimates, confidence intervals and P -values are given for an analysis for famine exposure using a GEE within the dependent beta value and, as covariates, technical batch, row on the 450k array, age, gender and principal components 1, 2 and 3.

^a95% confidence interval (CI) is related to the nominal P -values.

^b P -value corrected for multiple testing.

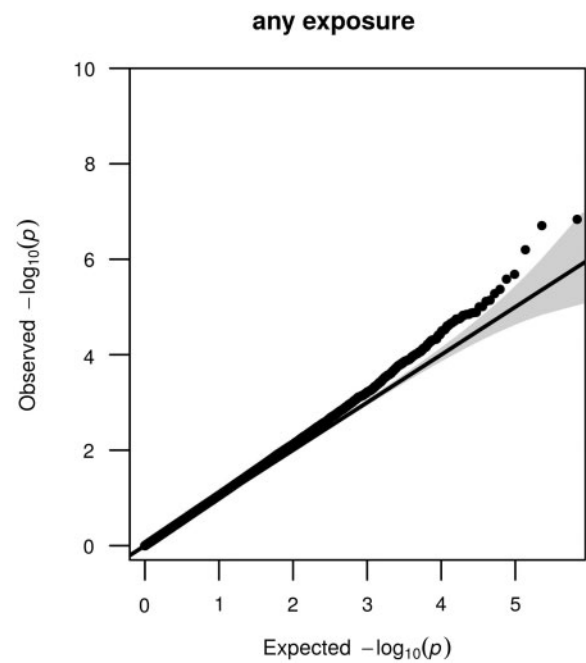


Figure 3. QQ-plots of any prenatal famine exposure. Plots depicting the Observed statistic (y-axis) with the statistic as expected by chance given the number of tests (x-axis). The 95% confidence interval of this relationship is given by the grey area around the expected line (black) for the instance that the observed statistic exactly follows the expected statistic. Each dot is the test statistic for one CpG dinucleotide. Enrichments for associations that go beyond that expected by chance can be seen as deviations upward from the expected line and 95% CI area. The P -values were corrected for the inflation factor ($\lambda = 1.14$).

In our current and previous studies,^{12–15} we found differences in DNA methylation percentages that are small in absolute terms, but that are of medium effect size (~0.5 standard deviations) from an epidemiological perspective.⁵⁸ Such small methylation differences are often found in epigenetic epidemiology⁵⁷ and are thought to exert an effect through the modulation of gene networks^{59,60} or to mark other larger molecular differences in a regulatory region, such as in histone modifications.⁵⁷ Animal experiments suggest that small absolute differences in methylation of medium effect size may still exert an effect.¹⁰

The mechanisms underlying DNA methylation differences from prenatal exposures await elucidation, but the observed timing-specific associations in blood may hint at an intrinsic sensitivity of newly developing tissues.⁶¹ We measured DNA methylation in whole blood, and the haematopoietic system is formed during prenatal weeks 1–10. Animal^{8,62} and human studies¹⁶ suggest that similar methylation changes are likely to be found in other tissues. Consultation of an external dataset suggests a broad agreement in methylation levels across six other tissues for the seven CpG dinucleotides in humans.⁶³ We do not have tissue specimens available other than whole blood in this population and are unable to address tissue-specificity of

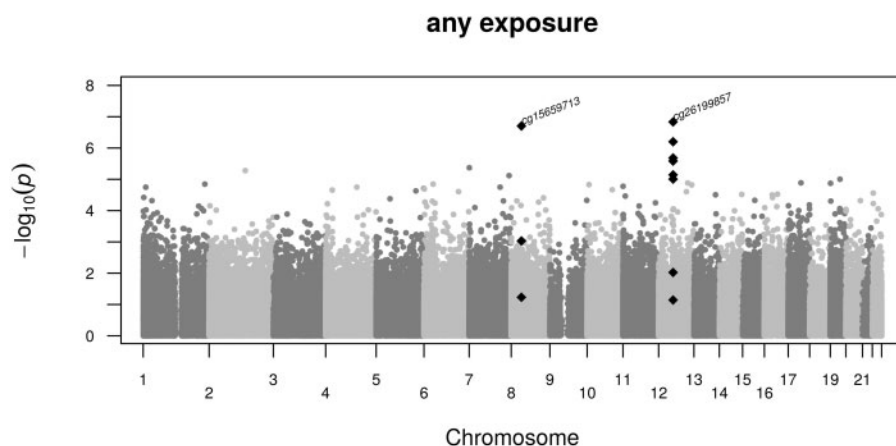


Figure 4. Manhattan plot of specific CpG associations with any prenatal famine exposure. Shown are the $-\log_{10} P$ -values (y-axis) of the association between DNA methylation at single CpG dinucleotides and famine exposure along the autosomal chromosomes (x-axis). Marked by the CpG dinucleotide identifier are the CpG dinucleotides significant after multiple testing. These and adjacent nominally significant CpG dinucleotides are depicted as black diamonds.

Table 3. CpG dinucleotides associated with any prenatal famine exposure

CpG	CpG dinucleotide Location (hg19)	Any exposure			Weeks 1–10		Weeks 11–20		Weeks 21–30		Weeks 31-delivery	
		Mean Beta-value, % (SD) controls	Estimate, (95% CI) ^a	P_{FDR} ^b	Estimate (95% CI)	P_{FDR}	Estimate (95% CI)	P_{FDR}	Estimate (95% CI)	P_{FDR}	Estimate (95% CI)	P_{FDR}
cg15659713	chr8:38,586,183	24.2 (3.9)	1.2 (0.8–1.7)	0.034	1.1 (0.2–1.9)	0.74	1.5 (0.8–2.1)	0.47	1.2 (0.7–1.8)	0.47	1.7 (1.1–2.3)	0.09
cg26199857	chr12:54,764,265	68.1 (5.8)	2.0 (1.3–2.7)	0.034	1.9 (0.6–3.2)	0.68	1.7 (0.7–2.7)	0.68	1.9 (1.0–2.9)	0.53	2.3 (1.3–3.4)	0.41

Estimates, confidence intervals and P -values are given for an analysis for famine exposure using a GEE within the dependent beta-value and, as covariates, technical batch, row on the 450k array, age, gender and principal components 1, 2 and 3.

^a95% confidence interval (CI) is related to the nominal P -values.

^b P -value corrected for multiple testing.

the differential methylation we observed here. Associations with any prenatal famine exposure may have arisen by other mechanisms than those associated with early gestation, as also suggested by animal experiments,⁷ and may be independent of the dynamics of DNA methylation during early development. A recent study on prenatal smoking also described select methylation changes after any smoking during pregnancy.¹⁹ In contrast to our study, some of these associations showed a relation between the duration of the prenatal exposure and DNA methylation. Further studies that are able to adequately account for potential confounding by postnatal and life-course factors will be required to unravel the mechanisms involved.⁶¹

In view of previous sex-specific methylation outcomes,¹³ we tested for exposure by sex interactions for the loci of interest, but none were detected. We therefore combined men and women in all analyses and added sex as a covariate. Because of the recognized low power of formal interaction tests in this setting, sex-specific effects cannot

be excluded but will need further evaluation in larger study samples.

Our study has other limitations. DNA methylation was measured six decades after the exposure; the differences could have been larger at birth, could have changed during life or could represent sub-clinical health differences.⁶⁴ The associations persisted after adjustment for variables that could potentially mediate the effect of prenatal nutrition on DNA methylation, including current lifestyle factors such as smoking and dietary characteristics and SES. It will be interesting to study the relation between famine exposure, health outcomes and the methylation of single CpG dinucleotides or along entire pathways, as both approaches appear to be promising in relation to complex phenotypes.⁶⁵ Confounding by postnatal and life-course factors remains possible, but this requires that selected confounding factors for individuals born after exposure in different gestational windows and DMPs only apply to births in the early gestation window. After prenatal smoking, DNA

methylation differences present in blood at birth may persist for up to two decades in the same individuals.¹⁹ Likewise, the current DNA differences associated with prenatal famine exposure could reflect the situation at birth. More longitudinal studies, both in animal experiments and in human cohorts, will be required to gain additional insights into this important issue in epigenetic epidemiology.⁵⁷ Finally, the small number of individual CpG dinucleotides associated with prenatal famine exposure may not only be related to the sparse coverage of the 450k array, but also to the limited number of famine-exposed individuals.

Although our study findings await replication, the results of candidate locus studies previously reported for this population^{12,13} were also sensitive to gestational diabetes,⁶⁶ prenatal smoking⁶⁷ and folic acid supplementation.¹⁸ Except for prenatal smoke exposure and gestational diabetes, no genome-wide studies on other prenatal exposures using the 450k array have been reported. With the exception of prenatal smoking,¹⁹ these studies have been small in terms of sample size. There was no overlap in the CpG dinucleotides differentially methylated after prenatal famine exposure and 450k array studies on prenatal smoking and gestational diabetes.^{19,68–71}

Our study builds on a strong design. It exploits the quasi-experimental conditions of the Dutch famine that imposed severe nutrition restrictions upon previously well-fed populations, avoiding confounding by social characteristics related to nutrition in pregnancy. The famine was well defined in place and time, so that exposed and unexposed populations can be distinguished from their place and date of birth. Moreover, we enrolled same-sex siblings to serve as family-controls. This avoids confounding due to unmeasured family factors common to siblings.

In summary, we show in a genome-wide DNA methylation study that prenatal famine exposure in early, but not in mid or late gestation, affects DNA methylation at specific CpG dinucleotides. These findings provide additional evidence that the early gestation period is a critical time-window during which the prenatal environment may affect the human blood methylome. The functional implications of these findings need further exploration.

Supplementary Data

Supplementary data are available at *IJE* online.

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