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Persistent epigenetic differences associated with prenatal exposure to famine in humans

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Abstract

Extensive epidemiological studies suggested that adult disease risk is associated with adverse environmental conditions early in development. While the mechanisms behind these relationships are unclear, an involvement of epigenetic dysregulation has been hypothesized. Here we show that individuals who were prenatally exposed to famine during the Dutch Hunger Winter in 1944/45 had, six decades later, less DNA methylation of the imprinted *IGF2* gene than their unexposed same-sex siblings. The association was specific for periconceptional exposure reinforcing that very early mammalian development is a crucial period for establishing and maintaining epigenetic marks. Exposure late in gestation was not related to *IGF2* methylation. These data are the first to contribute empirical support for the hypothesis that early-life environmental conditions can cause epigenetic changes in humans that persist throughout life.

Introduction

Superimposed on the DNA sequence is a layer of epigenetic information that is heritable, particularly during mitosis, and controls the potential of a genomic region to become transcribed¹. The two main molecular marks that make up this information and regulate chromatin structure and DNA accessibility are methyl groups coupled to a cytosine in CpG dinucleotide and histone modifications that package the DNA².

Although generally stable, animal studies indicated that certain transient environmental influences can produce persistent changes in epigenetic marks that have life-long phenotypic consequences^{3,4}. Early embryonic development is of special interest in this respect since it is a crucial period in establishing and maintaining epigenetic marks⁵. Indeed, culturing of preimplantation mice embryos showed that epigenetic marks are susceptible to nutritional conditions in the very early stages of mammalian development^{6,7}. One of the rare opportunities for studying the relevance of such findings to humans, is presented by individuals who were prenatally exposed to famine during the Dutch Hunger Winter⁸. This period of famine was the consequence of a German imposed food-embargo in the western part of The Netherlands towards the end of World War II in the winter of 1944/45. During this period registries and health care remained intact so that individuals who were prenatally exposed to this famine can be traced. Moreover, the period of famine was clearly defined and official food rations documented. These unique features allow us to assess whether prenatal exposure to famine is associated with persistent epigenetic differences in humans.

One of the best characterized epigenetically regulated loci is insulin-like growth factor II (*IGF2*). *IGF2* is a key factor in human growth and development and is maternally imprinted⁹. Imprinting is maintained through the *IGF2* differentially methylated region (DMR) whose hypo-methylation leads to bi-allelic expression of *IGF2*¹⁰. We recently studied *IGF2* DMR methylation in 372 twins¹¹. *IGF2* DMR methylation was a normally distributed quantitative trait and was largely determined by genetic factors in both adolescence and middle age indicating that the methylation mark is stable up to middle age.

If affected by environmental conditions early in human development, altered *IGF2* DMR methylation may therefore be detected many years later.

Here we use our ongoing Hunger Winter Families Study⁸ to investigate whether prenatal exposure to famine is associated with persistent differences in methylation of the *IGF2* DMR. Our primary focus was exposure during periconception, thus ensuring that the exposure was present during the very early stages of development that are critical in epigenetic programming. To further investigate the role of timing, we also studied individuals who were exposed late in gestation.

Results

Our primary goal was to test whether periconceptional exposure to famine was associated with differences in *IGF2* DMR methylation in adulthood. Using a quantitative mass spectrometry-based method^{11,12}, the methylation of 5 CpG dinucleotides within the *IGF2* DMR was measured. Three CpG sites were measured individually and 2 simultaneously, because they could not be resolved due to their close proximity.

Periconceptional exposure

We selected the 60 individuals from the Hunger Winter Families Study who were conceived during the famine six decades ago. The exposure period thus included the very early stages of development. The exposed individuals were compared to their same-sex sibling to achieve partial genetic matching. All but one CpG site were significantly less methylated among periconceptionally exposed individuals as compared to their siblings ($1.5 \times 10^{-4} \leq P \leq 8.1 \times 10^{-3}$; see **Table 1**). The average methylation fraction of the *IGF2* DMR based on all 5 CpG sites was 0.488 among exposed and 0.515 among unexposed siblings. Periconceptional exposure therefore was associated with -5.2% lower methylation ($P=5.9 \times 10^{-5}$) corresponding to 0.48 standard deviations of the controls. The association was independent of sex ($P_{\text{interaction}}=0.20$).

Table 1. IGF2 DMR methylation among individuals periconceptionally exposed to famine and their unexposed, same-sex siblings.

IGF2 DMR methylation	Mean methylation fraction (SD)				Relative change exposed	Difference in SDs	P ¹
	Exposed (n=60)		Controls (n=60)				
Average	0.488	(0.047)	0.515	(0.055)	-5.2%	-0.48	5.9x10 ⁻⁵
CpG 1	0.436	(0.037)	0.470	(0.041)	-6.9%	-0.78	1.5x10 ⁻⁴
CpG 2&3	0.451	(0.033)	0.473	(0.055)	-4.7%	-0.41	8.1x10 ⁻³
CpG 4	0.577	(0.114)	0.591	(0.112)	-2.3%	-0.12	0.41
CpG 5	0.491	(0.061)	0.529	(0.068)	-7.2%	-0.56	1.4x10 ⁻³

1. P-values were adjusted for age.

Figure 1 displays the difference in *IGF2* DMR methylation within sib ships according to the estimated conception date of the famine-exposed individual. *IGF2* DMR methylation was lowest in the famine-exposed individual among 72% (43/60) of sib ships and this lower methylation was observed in conceptions across the famine period. Official daily rations were set weekly and the same for every individual. The average daily rations during the famine were 667 kcal (SD, 151) (**Figure 1**).

As a technical validation of this finding, *IGF2* DMR methylation was re-measured among 46 out of 60 periconceptionally exposed individuals and their same-sex sibling, repeating the whole procedure from bisulfite treatment to quantification. A similarly lower 5.6% *IGF2* DMR methylation was observed (P=2.1x10⁻³), confirming our initial findings.

Late gestational exposure

To further investigate the influence of timing, we selected the 62 individuals who were exposed to famine late in gestation for at least 10 weeks so that they were born in or shortly after the famine. This time no difference in *IGF2* DMR methylation was observed between exposed individuals and their unexposed siblings (**Table 2** and **Figure 1B**).

To formally test whether the association with lower *IGF2* DMR methylation depended on timing of the exposure, we analyzed the periconceptional

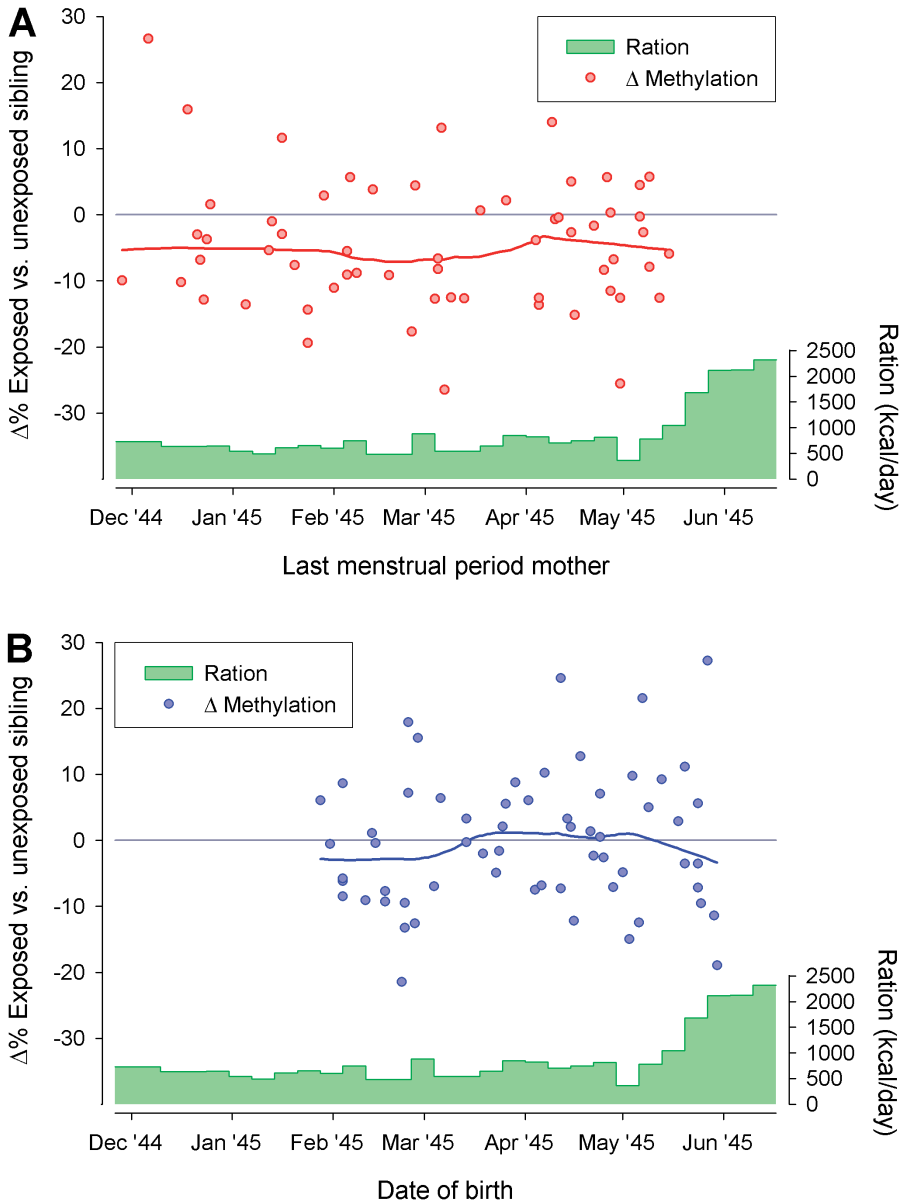


Figure 1. Within sibling pair difference in IGF2 DMR methylation set against the estimate of conception or data of birth of the famine-exposed individual.

A lowess curve is drawn to describe the difference in methylation. In green, the average distributed rations (kcal/day) between December 1944 and June 1945 are depicted. **A.** To describe the difference in methylation according to the data of the last menstrual period of the mother (a common estimate of conception), a lowess curve (red) was drawn. **B.** The lowess curve (blue) describes the difference in methylation according to the birth dates.

and late exposure groups together with all 122 controls in a single model (**Table 3**). Periconceptional exposure was associated with lower methylation ($P=1.5 \times 10^{-5}$) whereas late exposure was not ($P=0.69$). Furthermore, there was statistically significant evidence for an interaction between timing and exposure ($P_{\text{interaction}}=4.7 \times 10^{-3}$) indicating that the association was timing-specific.

Table 2. IGF2 DMR methylation among individuals exposed to famine late in gestation and their unexposed, same-sex siblings.

IGF2 DMR methylation	Mean methylation fraction (SD)				Relative change exposed	Difference in SDs	P ¹
	Exposed (n=62)		Controls (n=62)				
Average	0.514	0.045	0.519	0.036	-0.9%	-0.12	0.64
CpG 1	0.460	0.044	0.464	0.048	-0.9%	-0.09	0.68
CpG 2&3	0.462	0.039	0.471	0.039	-1.7%	-0.21	0.46
CpG 4	0.602	0.085	0.612	0.073	-1.5%	-0.12	0.30
CpG 5	0.529	0.060	0.531	0.060	-0.3%	-0.02	0.77

1. P-values were adjusted for age.

Birth weight

The mean birth weight of the 62 individuals exposed late in gestation was 3126 g (SD, 408), which is 296 g lower (95% CI, -420 to -170 g) than the mean (3422 g; SD, 464) of 324 reference births in 1943 at the same institutions ($P=4 \times 10^{-6}$)¹³. The lower birth weight underscores the impact of the famine during the Hunger Winter notwithstanding the absence of an association with IGF2 DMR methylation. The mean birth weight of the 60 individuals periconceptionally exposed was 3612 g (SD, 648) and was not lower compared to the reference births (95% CI, +15 to +365 g; $P=0.03$). IGF2 DMR methylation was not associated with birth weight ($P=0.39$).

Age association

To place the 5.2% lower *IGF2* DMR methylation association with periconceptual famine exposure into perspective, we assessed the relationship between age and *IGF2* DMR methylation in the 122 control individuals. Within the age range studied of 43 to 70 years, a 10 year older age was associated with a 3.2% lower methylation ($P=0.037$).

Table 3. Timing of famine exposure during gestation, IGF2 DMR methylation and birth weight.

	Periconceptual exposure	Late gestational exposure	all controls
N	60	62	122
Males, %	46.7	45.2	45.9
Mean age, years	58.1 (SD, 0.35)	58.8 (SD, 0.4)	57.1 (SD, 5.5)
Birth weight, g	3612 (SD, 648)	3126 (SD, 408)	-
<i>IGF2</i> DMR Methylation			
Average	0.488 (SD, 0.047)	0.514 (SD, 0.045)	0.517 (SD,0.047)
$P_{vs\ all\ controls}^1$	1.5×10^{-5}	0.69	
$P_{interaction}$			4.7×10^{-3}

1. P values were adjusted for age.

Discussion

Here we report that periconceptual exposure to famine during the Dutch Hunger Winter is associated with lower methylation of the *IGF2* DMR six decades later. The hypo-methylation we observed is remarkably comparable to that found in offspring of female rats fed a protein deficient diet starting before pregnancy (-5.2% in our human study versus -8.2% and -10.2% of rat *Nr3c1* and *Ppara* genes, respectively)³. The similarity substantiates that famine is the main culprit in *IGF2* hypo-methylation. An additional contribution of other stressors like cold and emotional stress⁸, however, cannot be ruled out. Our study provides the first evidence that transient environmental

conditions early in human gestation can be recorded as persistent changes in epigenetic information.

In contrast to periconceptual exposure to famine, exposure late in gestation was not associated with *IGF2* DMR methylation. Epigenetic marks may be particularly vulnerable during the very early stages of mammalian development which are crucial in establishing and maintaining epigenetic marks⁵. Experiments in which mouse zygotes were cultured to blastocyst stage favour this hypothesis^{6,7}. The timing-dependence of the association we observed may, however, also relate to timing of tissue development¹⁴. We studied blood and adult blood cells stem from the hematopoietic system that is established in the first stages of development (e.g. day 10.5 of the mouse embryo¹⁵). Detailed future studies are required to establish whether the susceptibility of epigenetic marks is an intrinsic property of early mammalian development or a general feature of newly developing tissues throughout gestation.

The developmental origins hypothesis states that adverse conditions during development contribute to adult disease risk¹⁶. While the mechanisms behind these relationships are unclear, it has been proposed that epigenetic dysregulation is involved¹⁶⁻¹⁸. Our results are a key element in elaborating this hypothesis. Human studies on the developmental origins of health and disease, however, often use low birth weight as a proxy for a compromised prenatal development¹⁶. Our data indicate that such studies are not necessarily sufficient to test the involvement of epigenetics and thereby extend our previous finding that birth weight is a poor surrogate for nutritional status during gestation¹³. Epigenetic differences were found among individuals who were exposed to famine early in gestation and had a normal birth weight. Exposure late in gestation was associated with low birth weight as expected but not with epigenetic changes. To monitor the crucial stages of early development, assessing maternal life style especially regarding nutrition¹⁹ and embryo growth using 3D ultrasound²⁰ will be more appropriate than birth weight.

The current study presents a first example of an association between a periconceptual exposure and DNA methylation in humans. It will be

of prime interest to investigate whether other exposures during early development that are more common in modern societies like overnutrition³ and assisted reproductive technologies²¹ give rise to similar associations. Also, the extent to which epigenetic marks at other genomic regions are vulnerable to such exposures remains to be established. A key question of future studies will be to assess the phenotypic consequences of changes in epigenetic marks. Diseases that have been associated with early gestational exposure to famine like schizophrenia²² and coronary heart disease²³ are of particular interest in this respect. Analogous to current studies in genetic epidemiology²⁴, such epigenetic epidemiological studies may need to be large and include replication. Understanding how epigenetic control depends on early exposures may shed light on the link between development and health over life time and ultimately suggest new ways to prevent human disease.

Materials and Methods

Study population

Design of and recruitment for the Hunger Winter Families Study was described previously⁸. Individuals prenatally exposed to famine were recruited by identifying and follow-up of live singleton births in 1945 and early 1946 at three institutions in famine-exposed cities (the midwifery training schools in Amsterdam and Rotterdam and the Leiden University hospital). As controls, same-sex siblings and unrelated individuals from the same institutions were recruited who were born before or conceived after the famine period. Clinical examination including obtaining a blood sample was completed for 311 exposed individuals, 311 same-sex siblings and 349 unrelated controls. Birth weight was abstracted from birth records from the three institutions. No birth weight data are available for the same-sex siblings, who were not born at these institutions.

For the current epigenetic study, we focused on exposed individuals and their sibling as control to achieve partial genetic matching in view of the high heritability of *IGF2* DMR methylation¹¹. From these, we selected the sib ships

with an individual periconceptionally exposed to famine and those with an individual exposed to famine late in gestation. Periconceptional exposure was defined as a last menstrual period of the mother before conceiving the exposed individual between November 28, 1944 and May 15, 1945. This yielded 60 sib ships. Exposure late in gestation was defined as a birth between January 28 and May 30 1945 so that the duration of the famine exposure was at least 10 weeks. This yielded 62 sib ships.

DNA methylation

Methylation of the *IGF2* DMR was measured using genomic DNA from whole blood extracted using the salting out method. One microgram of genomic DNA was bisulfite-treated using the EZ 96-DNA methylation kit (Zymo Research). Sib ships were bisulfite treated on the same plate. The region harbouring the *IGF2* DMR was amplified using primers described elsewhere¹¹. DNA methylation was quantified using a mass spectrometry-based method (Epityper, Sequenom)¹². All measurements were done in triplicate and CpG dinucleotides whose measurement was confounded by single nucleotide polymorphisms were discarded as part of quality controls¹¹. This resulted in 93% complete data. DNA methylation of 5 CpG dinucleotides could be measured, 3 of which individually and 2 of which as pair because they were directly adjacent and could not be resolved individually.

Statistical analysis

Mean methylation fractions of individual CpGs and their SDs presented in tables are based on raw data. To obtain the average methylation of the whole *IGF2* DMR presented in tables and figures, missing methylation data was first imputed using estimates from the mixed model thus exploiting the correlation between CpG sites¹¹. To test for differences between exposed individuals and their unexposed siblings, linear mixed models were used. This analysis accounts for family relations, correlated methylation of CpG dinucleotides and methylation data missing at random (thus p-values were

calculated without imputation of missing data). Exposure status, CpG dinucleotide and age were entered as fixed effects and sib ship as random effect. The model including both the periconceptual and the late exposure groups were extended with a variable indicating timing of the exposure and an interaction term exposure status times exposure time. To test for the association between *IGF2* DMR methylation and birth weight, birth weight was added as a fixed effect. P-values are two-sided and statistical analyses were performed using SPSS 14.0.

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