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Brief Report

Dietary and supplemental maternal methyl-group donor intake and cord blood DNA methylation

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Abstract

Maternal nutrition is critically involved in the development and health of the fetus. We evaluated maternal methyl-group donor intake through diet (methionine, betaine, choline, folate) and supplementation (folic acid) before and during pregnancy in relation to global DNA methylation and hydroxymethylation and gene specific (*IGF2 DMR, DNMT1, LEP, RXRA*) cord blood methylation. A total of 115 mother-infant pairs were enrolled in the MAternal Nutrition and Offspring's Epigenome (MANOE) study. The intake of methyl-group donors was assessed using a food-frequency questionnaire. LC-MS/MS and pyrosequencing were used to measure global and gene specific methylation, respectively. Dietary intake of methyl-groups before and during pregnancy was associated with changes in *LEP*, *DNMT1*, and *RXRA* cord blood methylation. Statistically significant higher cord blood *LEP* methylation was observed when mothers started folic acid supplementation more than 6 months before conception compared to 3-6 months before conception (34.6 ± 6.3% vs. 30.1 ± 3.6%, $P = 0.011$, *LEP* CpG1) or no folic acid used before conception (16.2 ± 4.4% vs. 13.9 ± 3%, $P = 0.036$ for *LEP* CpG3 and 24.5 ± 3.5% vs. 22.2 ± 3.5%, $P = 0.045$ for *LEP* mean CpG). Taking folic acid supplements during the entire pregnancy resulted in statistically significantly higher cord blood *RXRA* methylation as compared to stopping supplementation in the second trimester (12.3 ± 1.9% vs. 11.1 ± 2%, $P = 0.008$ for *RXRA* mean CpG). To conclude, long-term folic acid use before and during pregnancy was associated with higher *LEP* and *RXRA* cord blood methylation, respectively. To date, pregnant women are advised to take a folic acid supplement of 400 µg/day from 4 weeks before until 12 weeks of pregnancy. Our results suggest significant epigenetic modifications when taking a folic acid supplement beyond the current advice.

Key words

methyl donors, folic acid supplementation, preconception, pregnancy, DNA methylation, *LEP, RXRA, DNMT1*, *IGF2*

Introduction

Maternal nutrition is critically involved in the growth, development, and health of the fetus. This has been most clearly shown in studies from the Dutch Hunger Winter (1944 - 1945). Severe cold and wartime resulted in a 5-month period of extreme food shortage in the Netherlands. Long-term follow-up studies from this cohort found that adults who had been exposed to the famine early in gestation showed low birth weight and increased risk of obesity as adults.[1, 2] This adaptive process in response to famine (nutritional insult) during a vulnerable period early in life is known as fetal metabolic programming.[3] Among the underlying mechanisms responsible for fetal programming are epigenetic modifications, such as DNA methylation.[4] DNA methylation takes place when a methyl-group (CH_3) is added to the carbon-5 position of cytosine in CpG dinucleotides.[5] Methylation of gene promoters and regulatory regions hinder the binding of transcription factors, leading to altered gene expression.[6] The one-carbon (I-C) metabolism plays a central role in DNA methylation, since it determines the flux of methyl-groups towards methylation of DNA. Folate, betaine, choline, and methionine are the main sources of dietary methyl-group donors in the I-C metabolism. Folic acid is the synthetic form of folate used in supplements and for food fortification. Women who wish to become pregnant are advised to take a folic acid supplement of 400 µg per day starting 4 weeks before conception until 12 weeks of pregnancy for the prevention of neural tube defects.[7] All methyl-group donors enter the I-C metabolism at different sites and are, in the end, all converted to the universal methyl-group donor S-adenosylmethionine (SAM).[8] The transfer of methyl-groups from SAM to the DNA is catalyzed by three DNA methyltransferases (DNMTs), with DNMT1 as a maintenance DNMT that is required to maintain DNA methylation patterns.[5] DNMT3A and 3B are responsible for the establishment of *de novo* DNA methylation patterns, from fertilization until implantation. During this short period, DNA methylation marks on the maternal and paternal genome are globally lost and gained. Later in pregnancy, during organogenesis and tissue differentiation, there is a progressive increase in DNA methylation. Thus, there are several critical windows during fetal development where dietary factors can influence the fetal epigenome.[9] Therefore, to assess the effect of maternal nutrition during pregnancy on offspring DNA methylation levels, maternal dietary information and supplement use should be obtained at several time points during pregnancy (early, mid, and late gestation), because offspring DNA methylation levels and health risk are found to be different depending on the time of exposure during gestation. For example, individuals who were exposed to famine early in gestation (Dutch Hunger Winter) showed 5.2% lower methylation levels at the insulin-like growth factor 2 (*IGF2*) differentially methylated region (DMR) as compared to non-exposed siblings. Exposure in late gestation, on the other hand, showed decreased glucose tolerance and no difference in *IGF2 DMR* methylation between exposed and non-exposed siblings.[2, 4] *IGF2* is a maternally imprinted gene that is regulated by two differentially methylated regions (DMRs) and is important for fetal growth and development. Since the *IGF2 DMRs* are only methylated on the maternal allele, this region might be particularly susceptible to nutritional insults and supplementation in the pre- and peri-conceptional period.[10] Another study from the Dutch Hunger Winter found a significant increase in leptin (*LEP*) methylation of adults (men only) exposed to famine in early and late gestation.[11] *LEP* produces the hormone leptin, which is involved in food intake (inhibition) and energy expenditure, and, thus, a regulator of body weight. *LEP* promoter DNA methylation has been linked to adverse pregnancy outcomes and is plausibly involved in fetal metabolic programming.[12] Another metabolic gene that can be affected by maternal nutrition is the retinoid X receptor alpha (*RXRA)* gene, which is known to be involved in insulin sensitivity, adipogenesis, and fat metabolism. Lower maternal carbohydrate intake in early pregnancy was associated with higher *RXRA* cord blood methylation and with greater offspring's adiposity (fat mass and percentage fat mass) in 9-year old children.[13]

Besides changes in maternal diet, several studies have shown that maternal supplement use can induce alterations in offspring epigenetic marks. For example, periconceptional folic acid use of 400 µg/day was associated with a 4.5% increase in *IGF2 DMR* methylation in infants (17 months old), compared to children who were not exposed to folic acid. [14] However, according to Hoyo et al.,[15] no differences in cord blood *IGF2* methylation of infants born to women taking no, moderate (400 -- 1000 µ/day), or high doses (> 1000 µg/day) of folic acid before and during pregnancy were found. Maternal choline supplementation during pregnancy has also been shown to modify the neonate epigenome. One study examined the effect of choline intake (480 mg vs. 930 mg/day) in the third trimester of pregnancy on offspring DNA methylation. They found a decrease in placental DNA methylation of cortisol regulating genes (*CRH* and *NR3C1*) with higher maternal choline intake. Global DNA methylation and site-specific DNA methylation (*LEP*, *IL10*, *IGF2*, and *GNASAS1*) was, however, not altered by maternal supplemental choline intake.[16] The long-term effects of these methylation changes, due to maternal supplementation, on offspring health remain unknown.

In this study, we aimed to determine the effect of maternal dietary methyl-group donor intake (methionine, folate, choline, and betaine) and supplemental intake (folic acid) before and during each trimester of pregnancy on global DNA methylation and hydroxymethylation and gene specific methylation in cord blood in patients from the MAternal Nutrition and Offspring's Epigenome (MANOE) cohort. Promoter regions of *RXRA*, *LEP*, and *DNMT1*, and *IGF2 DMR* were selected for gene specific DNA methylation analysis.

Results

Maternal and neonatal characteristics

For the 115 mothers in our cohort, mean maternal age was 31 years (range: 25 - 41), mean BMI was 23.1 ± 3.4 kg/m^2, and mean gestational weight gain was 14.8 kg (range: 1.9 - 28.9) (table 1). Only five women smoked before and during the first trimester of pregnancy. Three of them continued smoking during the second and third trimester. The newborns, of which 55 were girls (47.8%), had a mean birth weight of 3518 ± 405.4 g and mean gestational age of 39.6 ± 0.9 weeks. Birth weight-for-gestational age z-scores were calculated and a mean z-score of 0.57 ± 0.93 was obtained (range: -1.38 - 2.91).

The mean maternal intake of dietary methyl-group donors before and during each trimester of pregnancy is presented in table 2. The intake of dietary methyl-group donors was stable during the course of pregnancy. Supplemental intake of folic acid before and during each trimester of pregnancy is presented in table 3. Folic acid intake was significantly higher in the first trimester of pregnancy (504.6 µg, $P = 0.000$), compared to the intake before (371.5 µg) or during the other trimesters of pregnancy (386.9 and 3564 µg). Women are advised to take a folic acid supplement four weeks prior to conception. The majority of women in our study followed this guideline; however, 25.2% (n = 29) did not take a folic acid supplement before conception. On the other hand, 26.1% (n = 30) took a folic acid supplement more than 6 months prior to conception. Most women (43.8%) stopped the folic acid supplementation in the second trimester, but 38.3% of the women took the supplement during their entire pregnancy.

Cord blood DNA methylation levels

The 115 newborns had a mean global DNA methylation level of 6.51 ± 1.65% and a mean global DNA hydroxymethylation level of 0.23 ± 0.14%. The mean methylation percentage of *IGF2* DMR, *DNMT1*, *LEP*, and *RXRA* was 51.39 ± 4%, 1.53 ± 0.3%, 22.91 ± 3.36%, and 11.73 ± 1.97%, respectively.

Impact of dietary methyl-group donor intake before and during each trimester of pregnancy on cord blood DNA methylation

We next determined the effect of maternal dietary methyl-group donor intake before and during each trimester of pregnancy on offspring global DNA methylation and hydroxymethylation and gene specific methylation. Associations between maternal dietary methyl-group donor intake and cord blood methylation are presented in table 4. Before pregnancy, higher intakes of betaine and methionine were associated with higher cord blood methylation levels of *DNMT1* CpG4 (0.68% per 100 mg increase, 95% CI: 0.04 - 01.31, P = 0.039) and *LEP* CpG4 (0.43% per 100 mg increase, 95% CI: 0.01 - 0.85, P = 0.048), respectively. In the second trimester of pregnancy, high methyl-group donor intakes (except for methionine) were negatively associated with gene specific cord blood methylation (betaine with *LEP* CpG2; choline with *DNMT1* CpG4; folate with *LEP* CpG2 and *DNMT1* CpG4). In the last trimester of pregnancy, a high intake of choline and folate was associated with higher methylation levels of *DNMT1* CpG2 (0.29% per 100 mg increase, 95% CI: 0.1 - 0.84, P = 0.022) and lower methylation levels of *RXRA* CpG2 (-1.001% per 100 µg increase, 95% CI: -1.96 - -0.04, P = 0.041), respectively. Finally, no significant associations between maternal dietary methyl-group donor intake before and during pregnancy and cord blood global DNA (hydroxy)methylation, *IGF2* DMR methylation (CpG1, CpG2, CpG3, and mean CpG), and birth weight were found (data not shown). In addition, no associations between maternal dietary methyl-group intake in the first trimester and cord blood methylation were found.

Impact of folic acid intake before conception on cord blood DNA methylation

We found statistically significant differences in cord blood *LEP* methylation (CpG1, CpG3, and mean CpG) by duration of maternal folic acid intake before conception (no folic acid use before conception/ 1 - 3 months prior to conception/ 3 - 6 months prior to conception /> 6 months prior to conception). The results are shown in figure 1. For *LEP* CpG1, we found a statistically significant difference between the four groups (P = 0.029). A post-hoc test revealed that the methylation percentage was significantly higher when the mother used a folic acid supplement more than 6 months before conception (34.6 ± 6.3%, P = 0.011) compared to 3 to 6 months before conception (30.1 ± 3.6%). Also *LEP* CpG3 (P = 0.037) and mean *LEP* CpG (P = 0.024) methylation percentages showed significant differences: significantly higher methylation levels were seen when women took a folic acid supplement more than 6 months prior to conception compared to no folic acid use [16.2 ± 4.4% vs. 13.9 ± 3% (P = 0.036) for LEP CpG3 and 24.5 ± 3.5 vs. 22.2 ± 3.5% (P = 0.045) for mean *LEP* CpG].

Impact of folic acid intake during pregnancy on cord blood DNA methylation

We found statistically significant differences in cord blood *RXRA* methylation (CpG1, CpG2, CpG3, CpG4, CpG5, and mean CpG) by duration of maternal supplemental folic acid intake during pregnancy (stop folic acid supplement intake at the end of the first trimester; stop in the second trimester; stop at the end of the third trimester). The results are shown in figure 2. For all five CpGs and the mean CpG, we found statistically significant differences between the 3 groups (CpG1, P = 0.027; CpG2, P = 0.012; CpG3, P = 0.009; CpG4, P = 0.024; CpG5, P = 0.037; mean CpG, P = 0.01). Post-hoc tests revealed that *RXRA* methylation percentages---in all CpGs (except CpG5) and mean CpG---were significantly higher in mothers using a folic acid supplement during the whole pregnancy compared to stopping the supplementation after the first or second trimester. The mean %, standard error of the mean, and *P*-value for CpG1, CpG2, CpG3, CpG4, and mean CpG were 8.1 ± 1.3% vs. 7.3 ± 1.6% ($P = 0.02$); 27.1 ± 4.1% vs. 24.4 ± 4.7% ($P = 0.009$); 8.2 ± 1.2% vs. 7.4 ± 1.5% ($P = 0.008$); 8.6 ± 1.8% vs. 7.7 ± 1.9% ($P = 0.05$); 12.3 ± 1.9% vs. 11.1 ± 2% ($P = 0.008$), respectively.

Discussion

This study supports the hypothesis that maternal methyl-group donor intake before and during pregnancy can induce epigenetic modifications in offspring genes related to metabolism.

We first studied the effect of supplemental folic acid intake before conception on cord blood methylation (global DNA methylation and hydroxymethylation, and gene specific methylation at *IGF2* DMR, *LEP*, *RXRA*, and *DNMT1*). It is recommended that women, who desire to become pregnant, use a folic acid supplement of 400 µg/day starting 4 weeks before conception until 12 weeks of pregnancy for the prevention of spina bifida.[7] In reality, women often start the folic acid supplementation months before conception, exposing the fetus to high levels of circulating folic acid during early embryonic development. We found statistically significant differences in cord blood *LEP* methylation depending on the start of the folic acid supplementation before conception. Specifically, a higher *LEP* methylation was observed when folic acid supplementation started more than 6 months prior to conception (24.5 ± 3.5%) compared to no pre-conceptional folic acid use (22.2 ± 3.5%). *LEP* is primarily expressed in white adipose tissue and its product, the hormone leptin, has several functions including regulation of food intake (inhibition), body weight, energy homeostasis, and it is expressed and secreted by the placenta during pregnancy.[17] It has been shown that the *LEP* promoter is subject to epigenetic programming and that the expression of leptin can be modulated by DNA methylation.[18] For example, *in utero* exposure to famine and gestational diabetes has been associated with offspring *LEP* promoter hypermethylation in blood of adults[11] and placental *LEP* hypermethylation[19], respectively. According to Lesseur et al.[12], cord blood *LEP* methylation was higher in small for gestational age infants and lower in infants born to pre-pregnancy obese mothers. Modifications in the profile of leptin in early life may contribute to the lower expression of appetite regulators, alter fetal neural development, and, in the end, alter the susceptibility to obesity and metabolic disorders in adulthood.[18]

We also studied the effect of dietary methyl-group donor intake before conception on cord blood DNA methylation. Higher intake of methionine and betaine before conception were associated with higher methylation levels at *LEP* CpG4 and at *DNMT1* CpG4, respectively. *DNMT1* produces the enzyme DNA methyltransferase, which maintains DNA methylation in newly synthesized DNA strands.[5] Animal studies[20-22] have shown that maternal diet can influence *DNMT1* methylation/expression. For example, a choline deficiency in pregnant rats (hypo)methylates the regulatory CpGs within the *DNMT1* gene, leading to its overexpression; this results in an increase of global and gene specific (*IGF2*) DNA methylation.[20]

The periconceptional period may be particularly susceptible to methyl-group donor intake due to global de- and re-methylation of the embryonal DNA in early development (between fertilization and implantation). However, our and other's results show that there are different windows of susceptibility (organogenesis and tissue differentiation) to epigenetic modifications by gestational methyl-group donor intake; therefore, the focus should not be solely on the periconceptional period.[23]

Next, we studied the effect of supplemental folic acid intake during each trimester of pregnancy on cord blood DNA methylation. We found statistically significant differences in cord blood *RXRA* methylation depending on the duration of folic acid intake during pregnancy. *RXRA* methylation was significantly higher (12.3 ± 1.9%) when the mother used a folic acid supplement during the whole pregnancy compared to stopping the supplementation in the second trimester (11.1 ± 2%). The *RXRA* gene is known to be involved in insulin sensitivity, adipogenesis, and fat metabolism. In two independent cohorts, Godfrey et al. found that higher *RXRA* methylation in umbilical cord tissue at birth, was highly correlated with adiposity (fat mass and percentage fat mass) in 9-year-old children. In one of these cohorts, low maternal carbohydrate intake in early pregnancy was associated with higher *RXRA* methylation.[13] This study showed that *RXRA* DNA methylation levels at birth could provide information about prenatal environmental influences, and later phenotype (adiposity). In the current cohort, folic acid supplementation during the entire pregnancy resulted in higher cord blood *RXRA* methylation. Children from the MANOE cohort will be further followed-up in the context of high vitamin intake by mothers, epigenetic modifications in cord blood, and obesity/metabolic disorders in childhood (BMI, fat content). Although it is widely known that folate intake reduces the risk of neural tube defects[7], the potential long-term consequences of an increased folate intake are largely unknown in humans. One study in humans found no effect of supplement use up to 12 weeks of pregnancy (current recommendations) on cord blood methylation. However, supplement use after 12 weeks of gestation was previously associated with higher methylation in the gene *IGF2*, and lower methylation in the *PEG3* gene and LINE-1 total DNA methylation in cord blood.[24] Our data suggest significant epigenetic modifications in the examined metabolic genes when taking a folic acid supplement beyond the current advice.

Finally, we studied the effect of dietary methyl-group donor intake during each trimester of pregnancy on cord blood DNA methylation. The intake of dietary methyl-group donors during pregnancy was found to be associated with *LEP*, *RXRA*, and *DNMT1* cord blood methylation, but not with global DNA (hydroxy)methylation and *IGF2 DMR* methylation. Only negative associations between dietary methyl-group donor intake and cord blood methylation were found in the second trimester of pregnancy, positive associations were observed for the other time points. A possible explanation for this shift could be a change in the I-C metabolism during gestation. A higher rate of transsulfuration was previously reported in the first trimester of pregnancy and a higher rate of transmethylation in the third trimester.[25] At each time point, we found that the intake of methyl-group donors was associated with *DNMT1* methylation. One possible mechanism that leads to changes in *LEP* and *RXRA* methylation could be via alterations in the methylation and, thus, gene expression of *DNMT1*.[20] In this study, positive, negative, and no associations between maternal methyl-group donor intake and offspring DNA methylation levels were found. It seems that there is no simplistic correlation between maternal methyl-group donor intake and offspring DNA methylation. Other studies also did not find a linear relationship; for example, undernutrition, which correlates with reduced methyl-group donor availability, resulted in a decrease and increase in the methylation of different site-specific genes.[4, 11, 26-28]

There are some strengths and limitations in the present study we need to address. The strengths of the present study include a unique study design that allowed us to collect longitudinal maternal data (starting before pregnancy and during each trimester of pregnancy), and offspring global and gene specific DNA methylation data in cord blood. The use of a validated food-frequency questionnaire designed to assess the intake of the nutrients under study. In addition, at each study time point, detailed information about supplement use was obtained. We have detailed covariate data allowing for adjustment for potential confounding variables. Another advantage was the use bisulfite pyrosequencing for DNA methylation analysis in candidate genes. It enabled the determination of DNA methylation levels at individual CpG sites and the calculation of the average methylation percentage of that region. Single CpG site methylation in the promoter region of a gene can be involved in the regulation of transcription, especially when it lies in a relevant transcription factor binding site, and could be associated with diseases. From example, the loss of DNA methylation in one CpG site in the promoter region of *TET1* was associated with air pollution and childhood asthma and could possibly be a potential biomarker for childhood asthma.[29] CpG methylation patterns within the same CpG island in promoter regions have been shown to be highly correlated; these methylation patterns differed from methylation patterns elsewhere, indicating that they have a specific biological role.[30]

The first limitation of our study is that we measured offspring methylation using cord blood, which is composed of different cell types, each with a different DNA methylation profile. Cord blood might not be the target tissue of interest for long-term metabolic outcomes, but is most often used in epidemiological studies because it is easy to obtain. In addition, cord blood consists primarily of infant blood[31] and can be considered as a good surrogate for the newborn's blood epigenome. Another limitation is that the Belgian food composition database NUBEL[32] does not contain information about the four methyl-group donors under study. Databases of neighboring countries or the USDA database for choline and betaine[33] content were used in the validation of the food frequency questionnaire (FFQ).[34, 35] For folate, the Dutch NEVO food composition database was used[36] and the German BLS Nutrient database for methionine.[37] The USDA database was also used for the nutrient content of folate and methionine if not found in NEVO and BSL databases respectively.

To conclude, this study shows that maternal methyl-group donor intake (through diet and supplement use) before and during each trimester of pregnancy can influence offspring DNA methylation in genes related to metabolism. Especially, long-term folic acid use before or during pregnancy was associated with higher *LEP* and *RXRA* cord blood methylation levels. Our results suggest significant epigenetic alterations in the genes under study when not following the current advice for pregnant women on folic acid supplementation between 4 weeks before until 12 weeks pregnancy. However, the impact these methylation changes may have on (later) health are yet to be determined.

Methods

Study subjects

We studied participants enrolled in the MAternal Nutrition and Offspring's Epigenome (MANOE) study, an ongoing prospective, observational cohort study initiated in April 2012. We enrolled 150 women (34 women before pregnancy and 116 in the first trimester of pregnancy) between April 2012 and January 2015 at the Department of Obstetrics and Gynecology of the University Hospitals Leuven (Belgium). The last delivery of the cohort took place in September 2015. Of the 150 enrolled women, 35 mother-infant pairs were excluded from analysis due to either missing nutritional data (n = 2), a missing cord blood sample (n = 14), development of pregnancy complications [gestational diabetes (n = 8) and preeclampsia (n = 1)], pre-term delivery (n = 6), extreme high intake of folic acid (≥ 4 mg/day) (n = 2), or birth defects (n = 2). After these exclusions, 115 mother-infant pairs were available for statistical analysis. The recruitment process has been described in more detail in a previous study [38].

Maternal and Neonatal Measurements

All 115 women were followed-up during pregnancy at their scheduled ultrasounds (11 - 13 weeks, 18 - 22 weeks, and 30 - 34 weeks of gestation) and at delivery. From the women recruited before pregnancy (n = 27) we obtained extra measurements before conception. A food-frequency questionnaire (FFQ) was developed and validated to assess maternal intake of dietary methyl-group donors (methionine, folate, betaine, and choline) before and during each trimester of pregnancy.[34, 35] Twenty-four FFQs were obtained before pregnancy, 96 FFQs at 11 - 13 weeks, 89 FFQs at 18 - 22 weeks, and 83 at 30 - 34 weeks of pregnancy. To assess the intake of methyl-group donors through supplement use, questions were asked about the use of nutritional supplements (frequency, brand/type, dosage) before and during each trimester of pregnancy. Only the intake of folic acid (synthetic form of folate) was registered, since there was no report on the supplemental intake of methionine, betaine, and choline. Furthermore, using a combination of questionnaires and interviews, we collected information about a range of socio-demographic factors, life style habits, and physical activity. Information on mothers' smoking status before and during pregnancy was obtained at each consultation. Questions were asked about smoking before and in each trimester of pregnancy and the number of cigarettes smoked on average per day. From these data, a dichotomous variable for maternal smoking before and during pregnancy was derived (smoked/did not smoke). Height and pre-pregnancy weight was used to calculate the Body Mass Index (BMI, kg/m²). Maternal measurements have been described in more detail in a previous paper.[38]

Determination of gestational age was based on the crown rump length measured between 7 and 14 weeks of gestation in all patients.[39] We obtained birth weight and length from the hospital clinical record. Gender specific z-scores for birth weight for gestational age were generated using the INTERGROWTH-21st tool.[40]

Sample collection and DNA extraction

At delivery, we collected umbilical cord blood in 4.5-mL tubes containing EDTA (BD Vacutainer Systems) via venipuncture. Umbilical cord blood samples were put in the freezer (-20°C) immediately after collection.

DNA from whole blood was extracted with the Salting out method[41], the quantity and purity of DNA were determined by a NanoDrop spectrophotometer. Samples were stored at -80°C until analysis.

Global DNA methylation and hydroxymethylation measurements

Cord blood DNA of 115 infants was analyzed by fast and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), as described previously.[42] Briefly, isolated genomic DNA samples (10 µg) were hydrolyzed to individual deoxyribonucleosides by a simple one-step DNA hydrolysis procedure. For this, a digest mix was prepared by adding phosphodiesterase I, alkaline phosphatase and benzonase nuclease to Tris-HCl buffer. Extracted DNA was then hydrolyzed by adding 10 µL digest mix and incubating at 37°C for at least 8 h. After hydrolysis, 490 µL of acetonitrile/water was added to each sample. Global DNA methylation and hydroxymethylation was obtained by quantifying 5mC, 5hmC, and C using ultra-pressure liquid chromatography (UPLC) in combination with tandem mass spectrometry (MS-MS). Global DNA methylation was expressed as a percentage of 5mC over the sum of 5mC, 5hmC, and C {% Global DNA Methylation = [5mC / (5mC + 5hmC+ C)*100]}. Global DNA hydroxymethylation was expressed as a percentage of 5hmC over the sum of 5mC, 5hmC, and C {% Global DNA Hydroxymethylation = [5hmC / (5mC + 5hmC + C)*100]}.

Gene specific DNA methylation measurements

Gene and region selection

We adopted a candidate gene approach and consulted previously published EWAS data, thus selecting candidate genes based on a literature study. We selected 12 genes that are known to be involved in the onset of obesity, genes of which the DNA methylation state is nutrient sensitive, or genes involved in DNA (de)methylation reactions. In a first phase, we analyzed offspring DNA methylation of the 12 selected genes on a subsample (n = 30). The subsample was selected based on maternal methyl-group donor intake (low vs.

high intake). After statistical analysis, we selected 4 genes (*DNMT1*, with role in maintenance of DNA methylation patters; *LEP*, with role in appetite control; *RXRA*, with role in insulin sensitivity, adipogenesis, and fat metabolism; and *IGF2* DMR, with role growth) to test our hypothesis on the entire cohort. For *IGF2* DMR, DNA methylation was measured at CpGs in the DMR that regulates parental imprinting of the *IGF2* gene in early development. For the other three genes, we have selected CpGs within the promoter region, since epigenetic changes in these regulatory regions can influence gene expression.

Bisulfite Conversion and PCR

Genomic DNA (200 ng) was bisulfite converted using the EZ-96 DNA Methylation-Gold™ Kit (#D5008, Zymo Research). Converted DNA was eluted with 30 µL of M-elution buffer. Subsequently, 1 µL of converted DNA was amplified by PCR in a total volume of 25 µL containing 0.2 µM of primers and 2x Qiagen PyroMark PCR Master Mix (#978703, Qiagen). Primers for *DNMT1, RXRA*, and *LEP* were ordered from Qiagen (#PM00075761, #PM00144431, #PM00129724 PyroMark CpG Assays).

Primer sequences for *IGF2* DMR used in the current study were taken from the original paper.[43] PCR reactions for *DNMT1*, *RXRA*, and *LEP* consisted of an initial hold at 95°C for 15 min followed by 45 cycles of 30s at 94°C, 30s at 54°C, and 30s at 72°C. PCR amplification ended with a final extension step at 72°C for 10 min. PCR reactions for *IGF2* DMR consisted of an initial hold at 5°C for 15 min followed by 5 cycles of 30s at 94°C, 30s at 68°C, and 30s at 72°C. This was followed by 50 cycles of 30s at 94°C, 30s at 64°C, and 30s at 72°C and ended with a final extension step at 72°C for 10 min. Primer information can be found in supplementary tables 1 and 2.

Pyrosequencing

In order to assess CpG methylation levels, 20 µL of biotinylated PCR product was immobilized to Streptavidin Sepharose High Performance beads (#17-5113-01, GE Healthcare) followed by annealing to 25 µL of 0.3 µM sequencing primer at 80°C for 2 min with a subsequent 10 min cooling down period.

Pyrosequencing was performed using Pyro Gold reagents (#970802, Qiagen) on the PyroMark Q24 instrument (Qiagen) following the manufacturer's instructions. Pyrosequencing results were analyzed using the PyroMark analysis 2.0.7 software (Qiagen). Pyrosequencing provides information in about the methylation status of individual CpG sites and the average CpG methylation can be calculated.

Statistical analysis

First, we assessed the intake of dietary and supplemental maternal methyl-group donors before and during pregnancy using a multivariate regression model for longitudinal measurements with methyl-group donor intake as a response variable and time point as a factor (LSD post-hoc test).

Next, we determined the effect of maternal dietary methyl-group donor intake on cord blood global DNA (hydroxy)methylation and gene specific methylation using linear regression models. Multivariable models were used to correct for possible covariates. Potential covariates were selected based on the association with DNA methylation and maternal nutrition: maternal age, maternal BMI, maternal smoking before and during each trimester of pregnancy (did not smoke /smoked), gestational weight gain. Analyses were performed separately per time point (pre-pregnancy, 11 - 13 weeks pregnancy, 18 - 22 weeks pregnancy, 30 - 34 weeks pregnancy). As high correlations were observed between methyl-group donor intakes at the different time points, it was less appropriate to model the intake levels jointly in a multivariable model, given that highly correlated variables induce multicollinearity. Proportional odds models for ordinal data were used in case the response variable showed less than five levels. This was the case for the methylation percentage of *DNMT1* CpG1, 2, 3, and 5.

Finally, we assessed whether there were differences in cord blood gene specific DNA methylation (*RXRA, IGF2 DMR, LEP, and DNMT1*) depending on the duration of maternal supplemental folic acid intake before and during pregnancy using one-way ANOVA. Post-hoc tests [Tukey test and Games Howell test (when the data did not meet the homogeneity of variances assumption)] were run when an overall significant difference in-group means was shown. For pre-conceptional supplemental folic acid intake, women were divided into 4 categories: no folic acid use before conception; start folic acid use 1 - 3 months prior to conception; 3 - 6 months prior to conception; or more than 6 months prior to conception. To test the effect of duration of supplemental folic acid use during pregnancy, women were divided into 3 categories: stop folic acid intake after the first trimester; stop after the second trimester; stop at the end of the third trimester.

All tests were two-sided, a 5% significance level was assumed for all tests. Analyses were performed using SAS software (version 9.4 of the SAS System for Windows).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Ethics

The current study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the UZ Leuven-Committee for Medical Ethics (reference number: ML7975). Written informed consent was obtained from all subjects.

References

1. Lumey LH, Stein AD, Kahn HS, van der Pal-de Bruin KM, Blauw GJ, Zybert PA, Susser ES. Cohort profile: the Dutch Hunger Winter families study. Int J Epidemiol 2007; 36:1196-204.

2. Roseboom TJ, van der Meulen JH, Ravelli AC, Osmond C, Barker DJ, Bleker OP. Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. Twin Res 2001; 4:293-8.

3. Chmurzynska A. Fetal programming: link between early nutrition, DNA methylation, and complex diseases. Nutr Rev 2010; 68:87-98.

4. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, Slagboom PE, Lumey LH. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A 2008; 105:17046-9.

5. Chen ZX, Riggs AD. DNA methylation and demethylation in mammals. J Biol Chem 2011; 286:18347-53.

6. Dao T, Cheng RY, Revelo MP, Mitzner W, Tang W. Hydroxymethylation as a Novel Environmental Biosensor. Curr Environ Health Rep 2014; 1:1-10.

7. Scholl TO, Johnson WG. Folic acid: influence on the outcome of pregnancy. Am J Clin Nutr 2000; 71:1295S-303S.

8. McKay JA, Mathers JC. Diet induced epigenetic changes and their implications for health. Acta Physiol (Oxf) 2011; 202:103-18.

9. Faulk C, Dolinoy DC. Timing is everything: the when and how of environmentally induced changes in the epigenome of animals. Epigenetics 2011; 6:791-7.

10. Chao W, D'Amore PA. IGF2: epigenetic regulation and role in development and disease. Cytokine Growth Factor Rev 2008; 19:111-20.

11. Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, Stein AD, Slagboom PE, Heijmans BT. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum Mol Genet 2009; 18:4046-53.

12. Lesseur C, Armstrong DA, Paquette AG, Koestler DC, Padbury JF, Marsit CJ. Tissue-specific Leptin promoter DNA methylation is associated with maternal and infant perinatal factors. Mol Cell Endocrinol 2013; 381:160-7.

13. Godfrey KM, Sheppard A, Gluckman PD, Lillycrop KA, Burdge GC, McLean C, Rodford J, Slater-Jefferies JL, Garratt E, Crozier SR, et al. Epigenetic gene promoter methylation at birth is associated with child's later adiposity. Diabetes 2011; 60:1528-34.

14. Steegers-Theunissen RP, Obermann-Borst SA, Kremer D, Lindemans J, Siebel C, Steegers EA, Slagboom PE, Heijmans BT. Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child. PLoS One 2009; 4:e7845.

15. Hoyo C, Murtha AP, Schildkraut JM, Jirtle RL, Demark-Wahnefried W, Forman MR, Iversen ES, Kurtzberg J, Overcash F, Huang Z, et al. Methylation variation at IGF2 differentially methylated regions and maternal folic acid use before and during pregnancy. Epigenetics 2011; 6:928-36.

16. Jiang X, Yan J, West AA, Perry CA, Malysheva OV, Devapatla S, Pressman E, Vermeylen F, Caudill MA. Maternal choline intake alters the epigenetic state of fetal cortisol-regulating genes in humans. FASEB J 2012; 26:3563-74.

17. Klok MD, Jakobsdottir S, Drent ML. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. Obes Rev 2007; 8:21-34.

18. Vickers MH, Sloboda DM. Leptin as mediator of the effects of developmental programming. Best Pract Res Clin Endocrinol Metab 2012; 26:677-87.

19. Lesseur C, Armstrong DA, Paquette AG, Li Z, Padbury JF, Marsit CJ. Maternal obesity and gestational diabetes are associated with placental leptin DNA methylation. Am J Obstet Gynecol 2014; 211:654.e1-9.

20. Kovacheva VP, Mellott TJ, Davison JM, Wagner N, Lopez-Coviella I, Schnitzler AC, Blusztajn JK. Gestational choline deficiency causes global and Igf2 gene DNA hypermethylation by up-regulation of Dnmt1 expression. J Biol Chem 2007; 282:31777-88.

21. Gong L, Pan YX, Chen H. Gestational low protein diet in the rat mediates Igf2 gene expression in male offspring via altered hepatic DNA methylation. Epigenetics 2010; 5:619-26.

22. Lan X, Cretney EC, Kropp J, Khateeb K, Berg MA, Peñagaricano F, Magness R, Radunz AE, Khatib H. Maternal Diet during Pregnancy Induces Gene Expression and DNA Methylation Changes in Fetal Tissues in Sheep. Front Genet 2013; 4:49.

23. Jiménez-Chillarón JC, Díaz R, Martínez D, Pentinat T, Ramón-Krauel M, Ribó S, Plösch T. The role of nutrition on epigenetic modifications and their implications on health. Biochimie 2012; 94:2242-63.

24. Haggarty P, Hoad G, Campbell DM, Horgan GW, Piyathilake C, McNeill G. Folate in pregnancy and imprinted gene and repeat element methylation in the offspring. Am J Clin Nutr 2013; 97:94-9.

25. Kalhan SC, Marczewski SE. Methionine, homocysteine, one carbon metabolism and fetal growth. Rev Endocr Metab Disord 2012; 13:109-19.

26. Waterland RA, Kellermayer R, Laritsky E, Rayco-Solon P, Harris RA, Travisano M, Zhang W, Torskaya MS, Zhang J, Shen L, et al. Season of conception in rural gambia affects DNA methylation at putative human metastable epialleles. PLoS Genet 2010; 6:e1001252.

27. Dominguez-Salas P, Moore SE, Baker MS, Bergen AW, Cox SE, Dyer RA, Fulford AJ, Guan Y, Laritsky E, Silver MJ, et al. Maternal nutrition at conception modulates DNA methylation of human metastable epialleles. Nat Commun 2014; 5:3746.

28. Tobi EW, Slagboom PE, van Dongen J, Kremer D, Stein AD, Putter H, Heijmans BT, Lumey LH. Prenatal famine and genetic variation are independently and additively associated with DNA methylation at regulatory loci within IGF2/H19. PLoS One 2012; 7:e37933.

29. Somineni HK, Zhang X, Biagini Myers JM, Kovacic MB, Ulm A, Jurcak N, Ryan PH, Khurana Hershey GK, Ji H. Ten-eleven translocation 1 (TET1) methylation is associated with childhood asthma and traffic-related air pollution. J Allergy Clin Immunol 2016; 137:797-805.e5.

30. Zhang W, Spector TD, Deloukas P, Bell JT, Engelhardt BE. Predicting genome-wide DNA methylation using methylation marks, genomic position, and DNA regulatory elements. Genome Biol 2015; 16:14.

31. Hall JM, Lingenfelter P, Adams SL, Lasser D, Hansen JA, Bean MA. Detection of maternal cells in human umbilical cord blood using fluorescence in situ hybridization. Blood 1995; 86:2829-32.

32. NUBEL *Belgain Food Composition Table,* Ministry of Public Health. Brussels, Belgium, 2010.

33. USDA Database for the Choline Content of Common Foods, U.S. Department of Agriculture, Agricultural Research Service. 2008.

34. Pauwels S, Doperé I, Huybrechts I, Godderis L, Koppen G, Vansant G. Validation of a food-frequency questionnaire assessment of methyl-group donors using estimated diet records and plasma biomarkers: the method of triads. Int J Food Sci Nutr 2014; 65:768-73.

35. Pauwels S, Doperé I, Huybrechts I, Godderis L, Koppen G, Vansant G. Reproducibility and validity of an FFQ to assess usual intake of methyl-group donors. Public Health Nutr 2015:1-10.

36. NEVO *Dutch Food Composition table , NEVO Foundation, Zeist, Netherlands*. In: NEVO Foundation Z, Netherlands, ed., 2011.

37. Dehne LI, Klemm C, Henseler G, Hermann-Kunz E. The German Food Code and Nutrient Data Base (BLS II.2). Eur J Epidemiol 1999; 15:355-9.

38. Pauwels S, Duca RC, Devlieger R, Freson K, Straetmans D, Van Herck E, Huybrechts I, Koppen G, Godderis L. Maternal Methyl-Group Donor Intake and Global DNA (Hydroxy)Methylation before and during Pregnancy. Nutrients 2016; 8.

39. Pexsters A, Daemen A, Bottomley C, Van Schoubroeck D, De Catte L, De Moor B, D'Hooghe T, Lees C, Timmerman D, Bourne T. New crown-rump length curve based on over 3500 pregnancies. Ultrasound Obstet Gynecol 2010; 35:650-5.

40. Villar J, Cheikh Ismail L, Victora CG, Ohuma EO, Bertino E, Altman DG, Lambert A, Papageorghiou AT, Carvalho M, Jaffer YA, et al. International standards for newborn weight, length, and head

circumference by gestational age and sex: the Newborn Cross-Sectional Study of the INTERGROWTH-21st Project. Lancet 2014; 384:857-68.

41. J S, DW R. Molecular Cloning: a laboratory manual. Cold spring harbor laboratory press, 2001.

42. Godderis L, Schouteden C, Tabish A, Poels K, Hoet P, Baccarelli AA, Van Landuyt K. Global Methylation and Hydroxymethylation in DNA from Blood and Saliva in Healthy Volunteers. Biomed Res Int 2015; 2015:845041.

43. Murphy SK, Huang Z, Hoyo C. Differentially methylated regions of imprinted genes in prenatal, perinatal and postnatal human tissues. PLoS One 2012; 7:e40924.

Table 1. Characteristics of the mother-infant pairs included in the statistical analysis (n = 115)

Characteristics	Mean (SD)	Range
Mother		
Maternal age (y)	31 (3.6)	25 -- 41
BMI (kg/m^2)	23.1 (3.4)	17.9 -- 33
Gestational weight gain (kg)	14.8 (4.2)	1.9 -- 28.9
Neonate		
Birth weight (gram)		
Gestational age (weeks)	3518 (405.4)	2720 -- 4750
Birth weight z-score	39.6 (0.9)	37.1 -- 41.4
	0.57 (0.93)	1.53
	%	**n**
Maternal smoking (yes)		
Before pregnancy	4.3	5
First trimester	4.3	5
Second trimester	2.6	3
Third trimester	2.6	3

Gender newborn		
Boy	52.2	60
Girl	47.8	55

Table 2. Mean maternal intake of dietary methyl-group donors before and during pregnancy.

Methyl-group donor	Before pregnancy Mean (SE) n = 24	First trimester (11 - 13w) Mean (SE) n = 96	Second trimester (18 - 22w) Mean (SE) n = 85	Third trimester (30 - 34w) Mean (SE) n = 83
Methionine (mg)	1665.9 (468.2)	1662.8 (476.4)	1609.4 (450.8)	1625.9 (481.8)
Folate (µg)	271.4 (89.4)	275.4 (89.5)	263.2 (92.3)	273.4 (102.6)
Choline (mg)	285.9 (73.7)	278.9 (74.2)	271.4 (74.8)	273 (84.8)
Betaine (mg)	172.1 (63.7)	167.9 (59.5)	168.9 (61.6)	171.4 (62.5)

Multivariate regression model for longitudinal measurements

Table 3. Supplemental folic acid intake before and during pregnancy (n = 115).

	Mean (SE)	**Range**
Mean daily intake of folic acid (µg)		
Before pregnancy	371.5 (21.5)	0 -- 1000
First trimester (11 - 13 weeks)	504.6 (14.1)*	171 -- 1000
Second trimester (18 - 22 weeks)	386.9 (24.2)	0 -- 1000
Third trimester (30 - 34 weeks)	354 (26.3)	0 -- 1100
	%	**N**
Start folic acid use before pregnancy		
No	25.2	29
1 - 3 months	28.7	33
4 - 6 months	20	23
> 6 months	26.1	30
Folic acid use during pregnancy		

No	0	0
First trimester	18.3	21
Until second trimester	43.5	50
Whole pregnancy	38.3	44

*Significant higher folic acid intake in the first trimester of pregnancy (multivariate regression model for longitudinal measurements)

Table 4. Associations between maternal dietary methyl-group donor intake (before and during pregnancy) and offspring gene-specific (*LEP*, *DNMT1*, *RXRA*) methylation measured in cord blood. Significant results are presented in bold.

Time point	**Before pregnancy n = 24** β (95% CI) *P*-value		**Second trimester n = 89** β (95% CI) *P*-value		**Third trimester n = 83** β (95% CI) *P*-value	
Gene / Nutrient	*LEP* *CpG4*	*DNMT1* *CpG4*	*LEP* *CpG2*	*DNMT1* *CpG4*	*RXRA* *CpG2*	*DNMT1* *CpG2*
Betaine	-0.13	**0.675**	**-0.575**	-0.25	0.35	0.97
	(-3.45;3.19)	**(0.04;1.31)**	**(-1.16;0.01)**	(-0.58;0.09)	(-1.24;1.94)	(0.26;3.67)
	0.94	**0.039**	**0.05**	0.15	0.66	0.96
Choline	1.48	0.13	-0.47	**-0.301**	-0.935	**0.291**
	(-1.48;4.45)	(-0.52;0.78)	(-0.95;0.02)	**(-0.57;-0.03)**	(-2.08;0.21)	**(0.1;0.84)**
	0.31	0.68	0.058	**0.031**	0.11	**0.022**
Folate	-0.33	0.21	**-0.507**	**-0.226**	**-1.001**	0.48
	(-2.75;2.09)	(-0.3;0.72)	**(-0.89;-0.13)**	**(-0.45;-0.01)**	**(-1.96;-0.04)**	(0.22;1.06)
	0.78	0.4	**0.009**	**0.045**	**0.041**	0.07
Methionine	**0.427**	0.04	-0.06	-0.04	-0.15	0.87
	(0.01;0.85)	(-0.06;0.14)	(-0.14;0.02)	(-0.08;0.009)	(-0.35;0.06)	(0.74;1.04)
	0.048	0.37	0.15	0.12	0.16	0.12

β-estimate is an absolute change in percentage of gene specific methylation; slope >(<) 0 means positive (negative) association; CI: confidence interval. Only the statistically significant associations are shown in this table.

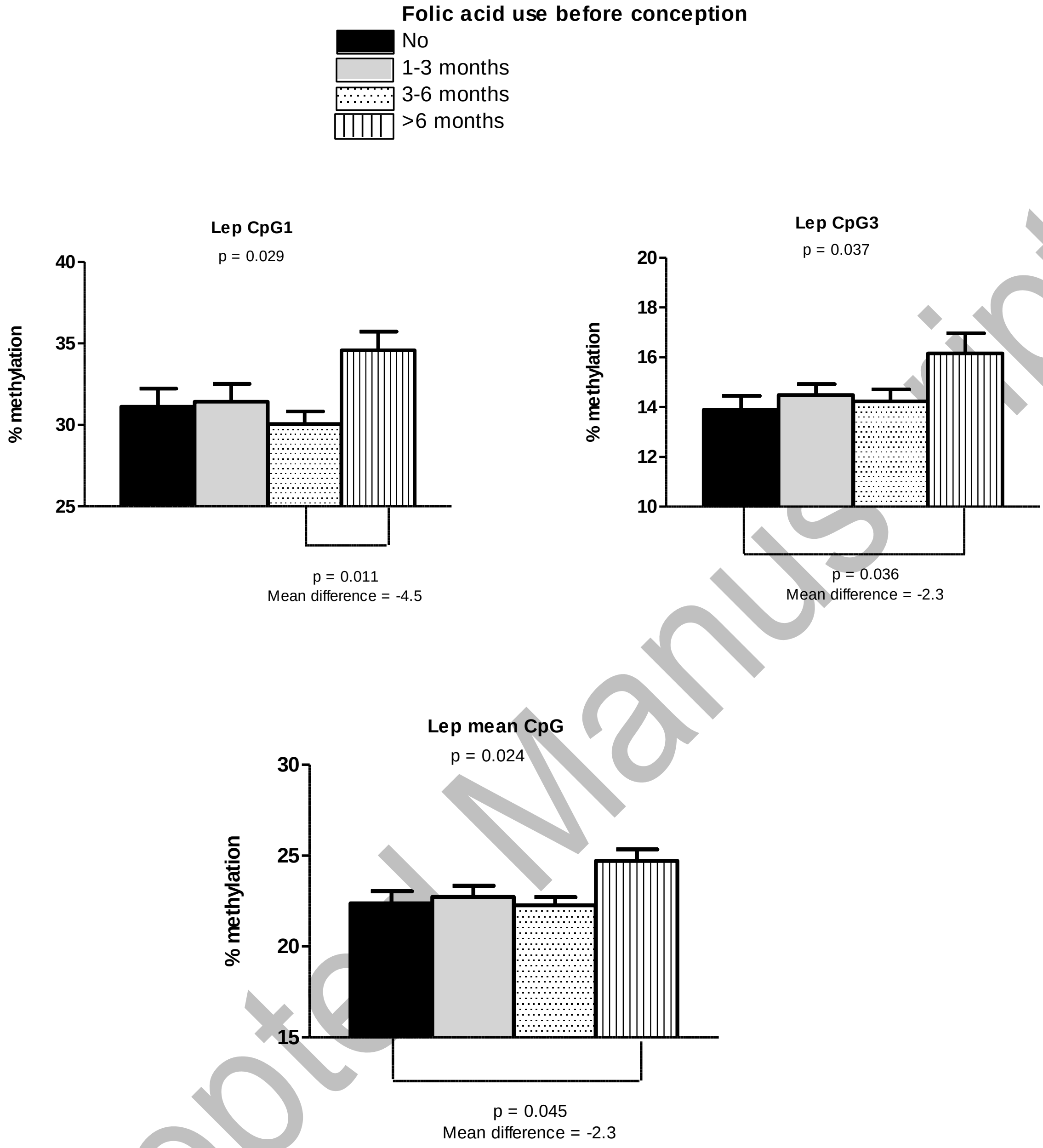

Figure 1. Cord blood *LEP* methylation by duration of maternal supplemental folic acid intake before conception. Bars represent the mean methylation values and standard errors of the mean of the 115 newborns. The results are based on the duration (4 categories) of maternal supplemental folic acid intake before conception. The overall *P*-values (one-way ANOVA) and significant *P*-values with mean differences from post-hoc tests are shown.

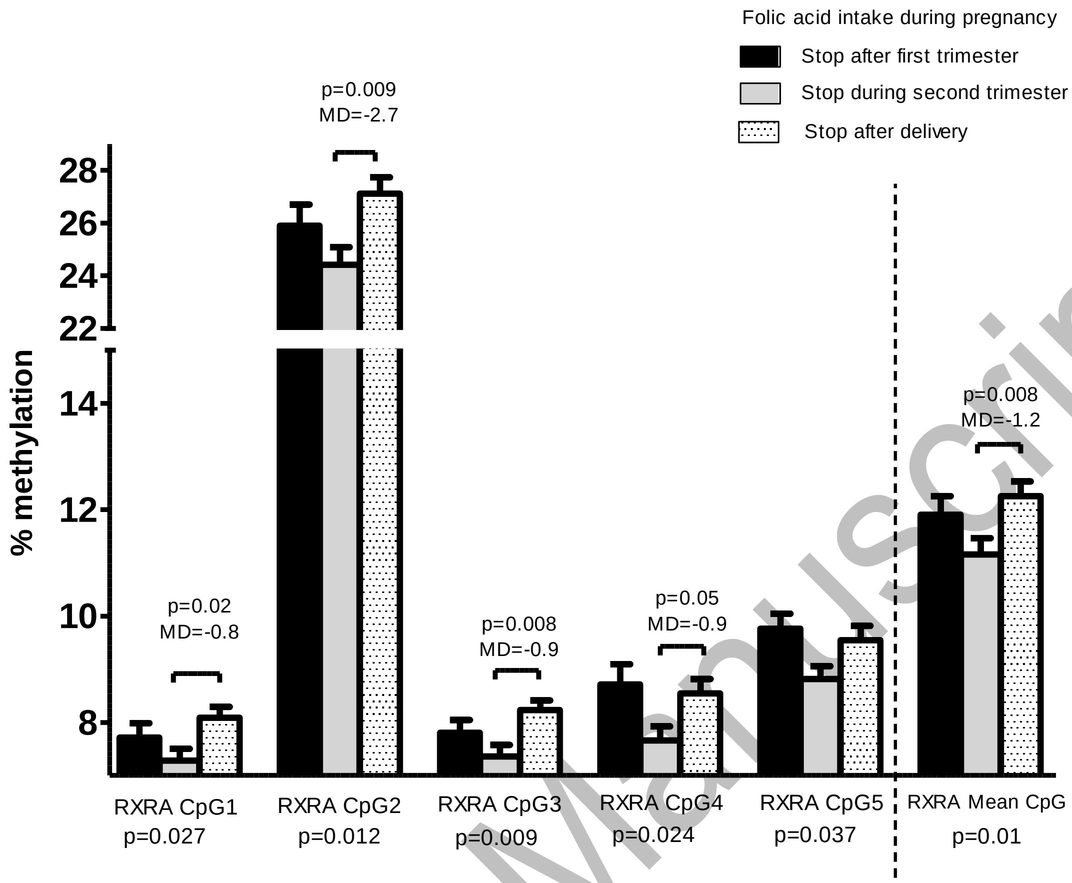

Figure 2. Cord blood *RXRA* methylation by duration of supplemental folic acid intake during pregnancy. The bars represent the mean methylation values and standard errors of the mean of 115 newborns. The results are based on the duration (3 categories) of maternal folic acid supplement intake during pregnancy. The overall *P*-values (one-way ANOVA) and significant *P*-values with mean differences from post-hoc tests are shown. MD, mean difference