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ORIGINAL ARTICLE

The effect of paternal methyl-group donor intake on offspring DNA methylation and birth weight

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Most nutritional studies on the development of children focus on mother–infant interactions. Maternal nutrition is critically involved in the growth and development of the fetus, but what about the father? The aim is to investigate the effects of paternal methyl-group donor intake (methionine, folate, betaine, choline) on paternal and offspring global DNA (hydroxy)methylation, offspring *IGF2* DMR DNA methylation, and birth weight. Questionnaires, 7-day estimated dietary records, whole blood samples, and anthropometric measurements from 74 fathers were obtained. A total of 51 cord blood samples were collected and birth weight was obtained. DNA methylation status was measured using liquid chromatography-tandem mass spectrometry (global DNA (hydroxy)methylation) and pyrosequencing (*IGF2* DMR methylation). Paternal betaine intake was positively associated with paternal global DNA hydroxymethylation (0.028% per 100 mg betaine increase, 95% CI: 0.003, 0.053, $P = 0.03$) and cord blood global DNA methylation (0.679% per 100 mg betaine increase, 95% CI: 0.057, 1.302, $P = 0.03$). Paternal methionine intake was positively associated with CpG1 (0.336% per 100 mg methionine increase, 95% CI: 0.103, 0.569, $P = 0.006$), and mean CpG (0.201% per 100 mg methionine increase, 95% CI: 0.001, 0.402, $P = 0.049$) methylation of the *IGF2* DMR in cord blood. Further, a negative association between birth weight/birth weight-for-gestational age *z*-score and paternal betaine/methionine intake was found. In addition, a positive association between choline and birth weight/birth weight-for-gestational age *z*-score was also observed. Our data indicate a potential impact of paternal methyl-group donor intake on paternal global DNA hydroxymethylation, offspring global and *IGF2* DMR DNA methylation, and prenatal growth.

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Key words: global DNA methylation, global DNA hydroxymethylation, *IGF2* DMR methylation, methyl-group donor

Background

Parents contribute in many ways to the development of their children. It is well documented that maternal lifestyle and exposures before and during gestation influences health and development of the next generation.[1] In recent years, a significant number of studies on various environmental exposures (nutrition, pesticides, lead, bisphenol A) have also reported an influence of paternal exposures on offspring's future health. Anderson *et al.*[2] reported that food deprivation of male mice before conception leads to an impaired glucose metabolism in offspring. Besides genomic effects (DNA mutations), epigenetic modifications have been suggested to explain these paternally transmitted effects.[3] Epigenetic changes, such as DNA methylation alterations, can occur in the male germ line due to environmental exposures, such as diet, and can be further passed on to the offspring.[4] In humans, DNA methylation may result in changes in gene expression and phenotype without altering the DNA sequence itself by adding a methyl-group (CH_3) to the carbon-5 position of the base cytosine in CpG dinucleotides, catalyzed by the enzyme DNA methyltransferase (Dnmt).[5]

The One-Carbon (I-C) metabolism plays a central role in DNA methylation as it determines the flux of methyl-groups toward methylation of DNA. Folate, betaine, choline and methionine are the main sources of methyl-groups in the I-C metabolism. All of them enter the I-C metabolism at different sites and are, in the end, all converted to the universal methyl-group donor *S*-adenosylmethionine (SAM).[6] So far, the effect of methyl donor intake (e.g. folic acid supplementation) on offspring DNA methylation has been mainly studied

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through maternal intake.[7,8] However, Mejos *et al.*[9] have shown that both maternal and paternal folate deficiency (4-week folate deficient diet) can decrease hepatic global DNA methylation in rat offspring. Carone *et al.*[10] found that male mice consuming a low-protein diet fathered offspring with altered DNA methylation at specific liver CpG islands [including a potential enhancer for the key lipid regulator peroxisome proliferator-activated receptor alpha (PPARα)] affecting cholesterol and lipid metabolism.

Besides DNA methylation, the DNA can be demethylated by oxidizing 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) by the Ten-eleven translocation (TET) enzymes and further to 5-formylcytosine (5-fC) and 5-carboxycytosine (5-caC).[11] Increased levels of 5-hmC may inhibit the binding of methyl-CpG binding proteins and thereby counteract transcriptional repression of 5-mC.[12] Changes in DNA methylation have been related to nutritional exposures such as folic acid supplementation.[13–16] To our very best knowledge, no human studies have evaluated the effect of the parental nutrition on global DNA hydroxymethylation. Most studies on hydroxymethylation were focused on prenatal development, especially stem cell differentiation and lineage. For example, some recent studies have examined the influence of dietary factors (e.g. vitamin C) on 5-hmC. Vitamin C not only induces increased levels of 5-hmC, but also of 5-fC and 5-caC in mouse embryonic stem cells.[17]

First human evidence of epigenetic changes in the offspring being paternally induced came from the Newborn Epigenetics Study. Soubry *et al.* observed that paternal periconceptional obesity (over-nutrition) was significantly associated with offspring DNA methylation at differentially methylated regions (DMRs) of several imprinted genes. Hypomethylation at the *IGF2* DMR,[18] *MEST*, *PEG3* and *NNAT* DMRs[19] were associated with paternal obesity. In order to affect offspring methylation through paternal environmental exposures, the exposure needs to be transferred to the male gametes and be sustained through developmental processes. During gametogenesis, from primordial germ cells to spermatozoa, epigenetic marks are established in a sex-specific way. This seems to be the only window of susceptibility during the lifespan of the father (from puberty to adulthood) where paternal environmental exposures can affect epigenetic marks in the gametes. Shortly after fertilization the embryo undergoes genome wide demethylation, except for imprinted marks and repeat sequences which retain their methylation status, making the overall epigenome hypomethylated.[5] Imprinted genes are therefore perfect candidate genes to capture and keep the paternal environmental exposure, as they withstand reprogramming.[20] Our study focuses on the paternally expressed imprinted insulin-like growth factor 2 (*IGF2*) which plays a critical role in embryogenesis and fetal growth. Its imprinting is regulated by two DMRs: *H19* and *IGF2* DMR. The imprint marks at these DMRs are established during spermatogenesis, so methylation is only present on the paternally inherited allele in the offspring.[21] To date, a handful of animal studies suggest an effect of paternal nutrition on offspring DNA methylation.[9,10] In humans however, the impact of paternal diet on offspring DNA methylation and demethylation has not yet been studied.

In this study, we first aimed to determine the effect of paternal dietary methyl-group donor intake (methionine, folate, choline and betaine) on paternal whole blood global DNA methylation and hydroxymethylation. Next, we assessed the effect of paternal methyl donor intake on cord blood global DNA methylation and hydroxymethylation, *IGF2* DMR methylation, and investigated a possible link with offspring birth weight.

Methods

Study subjects

The Maternal Nutrition and Offspring's Epigenome (MANOE) study is an ongoing prospective, observational study at the Department of Obstetrics and Gynecology of the University Hospital Leuven (Belgium) that investigates the link between parental methyl-group donor intake and offspring DNA methylation. Pregnant women were followed-up at their scheduled ultrasounds and at these time points fathers were asked to participate (Fig. 1). Of the 178 women included in the MANOE study, 115 Caucasian fathers provided detailed socio-demographic information (e.g. age, marital status, education), as well as multiple lifestyle or health characteristics (smoking behavior, physical activity, allergies). From these 115 fathers, 41 were excluded from analysis due to missing data (no nutritional information), which resulted in 74 fathers for statistical analysis. We were not able to collect a cord blood sample from 16 newborns, which gives a total of 58 father–infant pairs. Further, two children were excluded because the mother developed gestational diabetes, four due to pre-term delivery (<37 weeks of gestation), and one mother had a high risk of neural tube defects and was therefore given an extreme high dose of folic acid (4 mg/day). A total of 51 father–infant pairs were included in the statistical analysis. A screening for gestational diabetes was performed at 24–28 weeks using a 50 g glucose challenge test. When the test showed a glycemia ⩾140 mg/dl (⩾7.8 mmol/l) a 75 g oral glucose tolerance test was also performed. Based on this test two women were diagnosed with gestational diabetes mellitus (153–199 mg/dl or 8.5–11 mmol/l glucose).[22]

This 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). At the start of the study, all participants signed an informed consent.

Paternal dietary information

All 74 fathers were seen once at the Department of Obstetrics and Gynecology at the day of a scheduled ultrasound. To assess the paternal intake of dietary methyl-group

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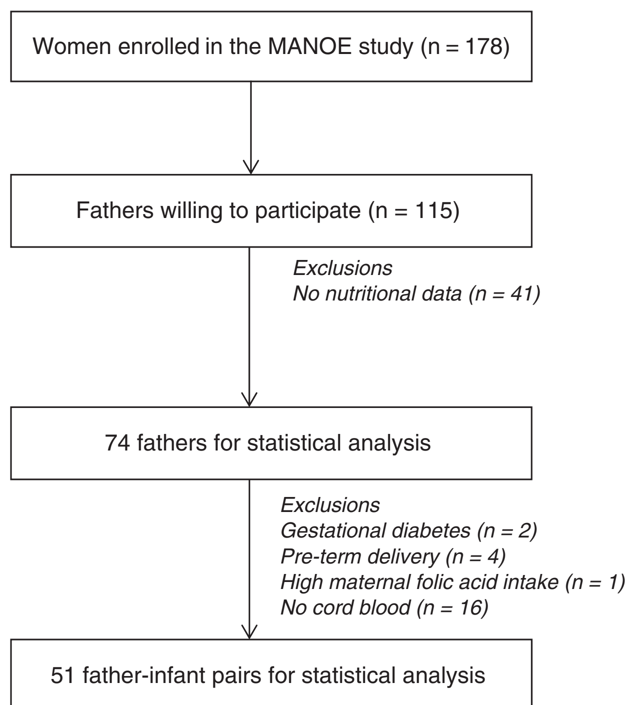

Fig. 1. Flowchart of fathers enrolled in the Maternal Nutrition and Offspring's Epigenome (MANOE) study and included in the statistical analysis.

donors (methionine, folate, betaine and choline) fathers were asked to complete a 7-day estimated dietary record (EDR). The participants were given guidelines to fill out their diary. This food record is an open-entry diary categorized into six eating occasions (breakfast, morning snacks, lunch, afternoon snacks, dinner and evening snacks) and involves reporting all foods and drinks consumed over seven consecutive days. It is often considered the most accurate measure of intake and has been referred to as the gold standard.[23] Detailed information on the type including brand names, the food type (e.g. use of whole, semi-skimmed, or skimmed milk, the type of bread used, etc.) and portion size [expressed as household measures, standard units (e.g. a medium sized apple) or units like grams or liters] of the foods consumed was collected using an open entry format. Only complete food diaries, including seven completed record days and containing sufficiently detailed descriptions of the food products and portion sizes consumed, were taken into consideration. The complete EDRs were encoded and entered into a Diet Entry and Storage program (NUBEL Voedingsplanner)[24] using a manual on food portions and household measures.[25] Methionine, choline, betaine and folate are not included in the Belgian food composition table Nubel,[26] so the diet records were linked to food composition databases from other countries. The Dutch NEVO food composition database[27] was used for folate, the USDA database for the Choline Content of Common Foods[28] for choline and betaine, and the German BLS Nutrient database[29] for methionine. The nutritional values of the food products in the four databases were quantified in mg/100 g (methionine, choline and betaine) or µg/100 g (folate). The methyl-group donor intake was calculated by multiplying these nutritional values of each consumed product during the 7-recorded days with the portion size (grams) of the product and dividing it by 100. For each methyl-group donor, the intakes of the products consumed in 1 day were added up. Finally, the average methyl-group donor intake of the 7-recorded days was calculated.

Paternal and neonatal measurements

Through an interview, we collected information about a range of socio-demographic factors, lifestyle habits (e.g. smoking: never smoked/past smoker/current smoker), and physical activity (yes/no). Body mass index (BMI) was calculated from the father's height and weight. Fathers were weighed at the consultation on a standard weighing scale (SECA Alpha model 888 or 877, Teleflex, Belgium) with indoor clothes (no shoes) to the nearest 0.1 kg. The height was measured with a microtoise to the nearest 0.5 cm (SECA model 206; Leicester Height Measure, Burmingham, UK) without shoes.

Gestational age was determined by measuring crown rump length between 7 and 14 weeks of gestation.[30] At delivery, we collected umbilical cord blood in 4.5 ml tubes containing ethylenediaminetetraacetic acid (EDTA; BD Vacutainer Systems). We obtained birth weight and length from the hospital clinical records. Gender-specific *z*-scores for birth weight-for-gestational age were generated using the INTERGROWTH-21st tool.[31]

Sample collection and DNA extraction

Blood samples from fathers were collected using 4.5 ml tubes with EDTA (BD Vacutainer® Blood Collection System). Blood samples were put in the freezer (−20°C) immediately after collection. At delivery, umbilical cord blood was collected via umbilical vein puncture into 4.5 ml tubes containing EDTA (BD Vacutainer® Blood Collection System), followed by storage at −20°C. DNA extraction from whole blood samples was done using the Salting out method.[32] The quantity and purity of DNA was determined by a Nano Drop spectrophotometer. Extracted DNA was further stored in TE-buffer at −80°C until further analysis.

Global DNA (hydroxy)methylation measurements

Paternal and cord blood DNA was analyzed by a fast and sensitive liquid chromatography-tandem mass spectrometry method for the simultaneous quantification of DNA 5-mC and 5-hmC as described previously.[33] 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; 10 µl of digest mix was added to the extracted DNA and incubated at 37°C for at least 8 h. After hydrolysis,

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490 μl of acetonitrile/water was added to each sample. Global DNA methylation and hydroxymethylation was obtained by quantifying 5-mdC, 5-hmdC and dC using ultra-pressure liquid chromatography, in combination with tandem mass spectrometry (MS-MS). Global DNA methylation was expressed as a percentage of 5-mdC *v.* the sum of 5-mdC, 5-hmdC and dC [%global DNA methylation =5-mdC/(5-mdC + 5-hmdC + dC)], while global DNA hydroxymethylation was expressed as a percentage of 5-hmdC *v.* the sum of 5-mdC, 5-hmdC and dC [%global DNA hydroxymethylation = 5-hmdC/(5-mdC + 5-hmdC + dC)].

IGF2 *DMR methylation measurements*

Bisulfite conversion and polymerase chain reaction (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 2 × Qiagen PyroMark PCR Master Mix (#978703; Qiagen). Primer sequences for *IGF2* DMR were taken from the original paper. The *IGF2* DMR is one of the two DMRs that are involved in the imprinting of the *IGF2/H19* domain on chromosome 11p15.5. This DMR is located upstream of the imprinted promoters of *IGF2*.[34] PCR reactions for *IGF2* DMR consisted of an initial hold at 5°C for 15 min followed by five cycles of 30 s at 94°C, 30 s at 68°C and 30 s at 72°C. This was followed by 50 cycles of 30 s at 94°C, 30 s at 64°C and 30 s at 72°C and ended with a final extension step at 72°C for 10 min.

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).

Statistical analysis

First, an independent *t*-test was used to compare the characteristics of fathers with and without dietary data. Next, Pearson's correlations were used to display the association between paternal global DNA methylation and global DNA hydroxymethylation. To determine the effect of paternal methyl-group donor intake on paternal global DNA (hydroxy) methylation, cord blood global DNA (hydroxy)methylation, cord blood *IGF2* DMR methylation, and birth weight linear regression models were used. Multivariable models were used to correct for possible confounders. Potential confounders were selected based on the association with paternal nutrition and paternal methylation: paternal age, paternal physical activity (yes/no), paternal smoking (never/past/current), and paternal BMI. When assessing the effect of paternal nutrition on offspring methylation; maternal smoking (did not smoke during pregnancy/smoked during pregnancy), maternal BMI, and maternal methyl-group donor intake (methionine, betaine, choline and folate) were also selected as potential confounders. Model selection was based on the Akaike Information Criterion (AIC): the model with the lowest AIC (indicating the best model fit) was selected among all tested models (every possible combination of the four methyl-group donors together with the pairwise interactions). 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).

Results

Paternal characteristics and methyl-group donor intake

Characteristics of the fathers are presented in Table 1. From the 115 included fathers, mean paternal age was 31.8 years (range: 2–48). BMI of the participating fathers averaged 24.7 ± 2.9 kg/m^2. Most men (53.9%, $n = 62$) never smoked cigarettes and 32 men (27.8%) smoked in the past; 67% ($n = 77$) of the fathers were physically active (yes/no). From the included fathers with dietary data ($n = 74$) mean paternal age was 32 years (range: 25–48). BMI of these fathers averaged 24.6 ± 2.9 kg/m^2. Most men (55.4%, $n = 41$) never smoked cigarettes and 22 men (29.7%) smoked in the past; 67.6% ($n = 50$) were physically active (yes/no). From the excluded fathers without dietary data ($n = 41$) mean paternal age was 31.2y (range: 24–38). BMI of these fathers averaged 24.9 ± 3.3 kg/m^2. Most men (56%, $n = 23$) never smoked cigarettes and nine men (22%) smoked in the past; 68.3% ($n = 28$) were physically active (yes/no). No significant differences between fathers with and without dietary data were observed.

The average daily intake of methyl-group donors of the 74 fathers is shown in Table 2. The average intake of choline and folate corresponded with the average requirements for these nutrients.[35,36] A total of 55.4% of the fathers had intake below the dietary guideline for folate and 79.7% for choline. The dietary guideline for methionine is 10.4 mg/kg.[37] Mean weight of the fathers with dietary data was 81.3 ± 12.0 kg, resulting in a recommended daily intake 845.2 ± 124.8 mg for methionine. The father's intake of methionine was much higher than the dietary guideline (range: 1234.4–3602.1 mg). For betaine no guideline for dietary intake exists.

The effect of methyl-group donor intake on paternal DNA methylation

The 74 fathers had a mean global DNA methylation level of 5.92 ± 1.45% and a mean global DNA hydroxymethylation

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Table 1. *Paternal characteristics*

Characteristics	Unit/category	Fathers with dietary information (n = 74)	Fathers without dietary information (n = 41)	All recruited fathers (n = 115)	P-value
Age (years)	Mean ± S.D.	32 ± 4.4	31.2 ± 3.5	31.8 ± 4.2	0.26
	Range	25–48	24–38	24–48	
Weight (kg)	Mean ± S.D.	81.3 ± 12	83.5 ± 14.3	81.6 ± 12.7	0.33
BMI (kg/m^2)	Mean ± S.D.	24.6 ± 2.9	24.9 ± 3.3	24.7 ± 2.9	0.62
Smoking	n (%)				
Never smoker		41 (55.4)	23 (56)	62 (53.9)	0.29
Past smoker		22 (29.7)	9 (22)	32 (27.8)	
Smoker		11 (14.9)	9 (22)	21 (18.3)	
Physically active	n (%)				
Yes		50 (67.6)	28 (68.3)	77 (67)	
No		24 (32.4)	13 (31.7)	38 (33)	0.82

BMI, body mass index.
Independent sample t-test was performed to compare characteristics of fathers with and without dietary data.

Table 2. *Paternal average daily intake of methyl-group donors (*n = *74)*

Methyl-group donors	Mean ± S.D.	Range	Dietary guideline	Fathers with intake below the guideline [n (%)]
Betaine (mg)	174.8 ± 66.3	57.7–456.7	/	/
Choline (mg)	334.3 ± 77.5	191.6–556.3	400	59 (79.7)
Folate (µg)	243.6 ± 63.7	137.5–414.5	250	41 (55.4)
Methionine (mg)	2188.9 ± 508.8	1234.4–3602.1	845.2	0 (0)

level of 0.12 ± 0.08%. The inter-individual variability of the global DNA methylation and global DNA hydroxymethylation levels ranged 3.17–8.37% and 0.02–0.28%, respectively. These results are in line with some studies that reported similar inter-individual variability.[38,39] Nevertheless, lower variability were previously reported while studying a limited number of volunteers[33] or on other tissue then whole blood[40] Global DNA methylation and global DNA hydroxymethylation were highly correlated (r = 0.88, P < 0.0001) (Fig. 2).

The best model explaining paternal hydroxymethylation via paternal methyl-group donor intake was a model with betaine as the only predictive value. Higher intakes of betaine was associated with higher levels of paternal global DNA hydroxymethylation in a model adjusted for age, BMI, smoking status, and physical activity (0.028% per 100 mg betaine increase, 95% CI: 0.003, 0.053, P = 0.03). There was no evidence that paternal methyl-group donor intake had any predictive value for paternal global DNA methylation, although the association between paternal betaine intake and paternal global DNA methylation was borderline significant (P = 0.08) (Table 3).

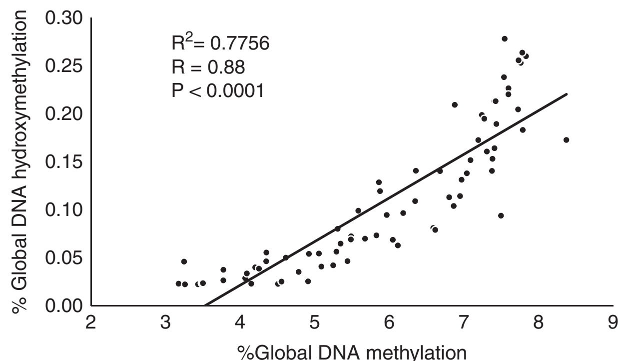

Fig. 2. Relationship between paternal global DNA methylation and global DNA hydroxymethylation percentages in blood.

The effect of paternal methyl-group donor intake on offspring

Besides the effect of dietary methyl-group donors consumed by the father on paternal methylation, we were also interested in its effect on offspring methylation and growth. This analysis was performed on 51 father–infant pairs. Newborn characteristics and methylation profiles are described in Table 4. The newborns, 26 of which were girls (51%), had a mean birth weight of 3.472 ± 0.392 kg, and mean gestational age of 39.75 ± 0.92 weeks. Birth weight-for-gestational age z-score was calculated and a mean z-score of 0.39 ± 0.95 was obtained (range: −1.38 to 2.45). The 51 newborns had a mean global DNA methylation level of 6.61 ± 1.66% and a mean global DNA hydroxymethylation level of 0.24 ± 0.15%. The mean methylation percentage of the three CpGs of the *IGF2* DMR

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Table 3. *Associations between paternal methyl-group donor intake and paternal global DNA (hydroxy)methylation (n = 74)*

	Global DNA methylation [β (95% CI) *P*-value]	Global DNA hydroxymethylation [β (95% CI) *P*-value]
Betaine	0.430 (−0.058, 0.919) 0.08	**0.028 (0.003, 0.053) 0.03**
Choline	0.328 (−0.094, 0.750) 0.13	0.013 (−0.009, 0.035) 0.23
Folate	0.296 (−0.237, 0.828) 0.27	0.015 (−0.013, 0.043) 0.29
Methionine	0.029 (−0.036, 0.094) 0.38	0.001 (−0.002, 0.004) 0.55

CI, confidence interval.
β-estimate is an absolute change in percentage of global DNA (hydroxy) methylation; slope > (<) 0 means positive (negative) association.
Bold values are the statistically significant results ($p < 0.05$).

Table 4. *Newborn characteristics and methylation profiles (n = 51)*

	Unit/category	
Characteristics		
Birth weight (kg)	Mean ± S.D.	3.472 ± 0.392
	Range	(2.8–4.32)
Gestational age (weeks)	Mean ± S.D.	39.75 ± 0.92
	Range	(37.71–41.43)
Gender		
Male	*n* (%)	25 (49)
Female		26 (51)
Birth weight-for-gestational age *z*-score	Mean ± S.D.	0.39 ± 0.95
	Range	(−1.38–2.45)
Methylation profile		
Global	Mean ± S.D.	
Methylation (%)		6.61 ± 1.66
Hydroxymethylation (%)		0.24 ± 0.15
IGF2 DMR methylation (%)	Mean ± S.D.	
CpG1		49.06 ± 4.72
CpG2		53.14 ± 4.02
CpG3		50.92 ± 3.92
Mean		51.04 ± 3.93

IGF2, insulin-like growth factor 2; DMR, differentially methylated region.

was 51.04 ± 3.93%. Offspring cord blood global DNA methylation and global DNA hydroxymethylation were highly correlated ($r = 0.86$, $P < 0.0001$) (Fig. 3).

To assess the effects of paternal methyl-group donor intake on offspring global DNA methylation, the best model was the model with betaine as the only predictive value. Higher intakes of betaine was linked with higher levels of offspring global DNA methylation (0.679% per 100 mg betaine increase, 95% CI: 0.057, 1.302, $P = 0.03$) in a model adjusted for

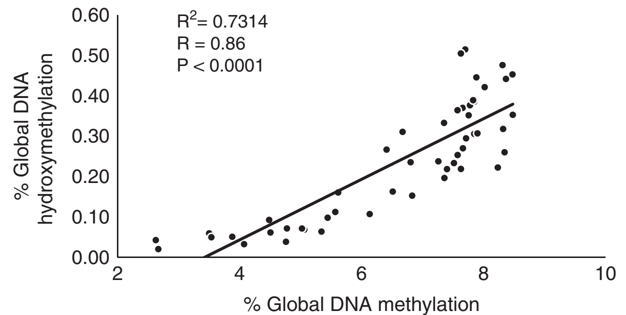

Fig. 3. Relationship between offspring global DNA methylation and global DNA hydroxymethylation percentages in cord blood.

Table 5. *Associations between paternal methyl-group donor intake and offspring global DNA (hydroxy)methylation (n = 51)*

	Global DNA methylation [β (95% CI) *P*-value]	Global DNA hydroxymethylation [β (95% CI) *P*-value]
Betaine	**0.679 (0.057, 1.302) 0.03**	0.013 (−0.044, 0.071) 0.64
Choline	0.236 (−0.375, 0.848) 0.44	0.003 (−0.051, 0.056) 0.92
Folate	0.489 (−0.345, 1.323) 0.24	0.040 (−0.034, 0.113) 0.28
Methionine	−0.035 (−0.126, 0.056) 0.44	−0.005 (−0.013, 0.002) 0.17

CI, confidence interval.
β-estimate is an absolute change in percentage of global DNA (hydroxymethylation); slope > (<) 0 means positive (negative) association.
Bold values are the statistically significant results ($p < 0.05$).

paternal age, paternal BMI, paternal smoking status, and paternal physical activity. We also included maternal BMI, maternal smoking status and maternal methyl-group donor intake as possible confounders. There was no evidence that paternal methyl-group donor intake had any predictive value for offspring global DNA hydroxymethylation (Table 5).

We also determined the effect of paternal methyl-group donor intake on offspring *IGF2* DMR methylation. We assessed the effect on each CpG separately (CpG1, CpG2 and CpG3) and on the mean methylation of the three CpGs. Only significant results are shown in Table 6. The best model to test the effects of paternal methyl-group donor intake on *IGF2* DMR CpG1 and mean CpG methylation was a model with methionine as the only predictive value. Higher intakes of methionine correlated with higher levels at CpG1 of *IGF2* DMR (0.336% per 100 mg methionine increase, 95% CI: 0.103, 0.569, $P = 0.006$) and mean CpG methylation (0.201% per 100 mg methionine increase, 95% CI: 0.001, 0.402, $P = 0.049$). There was no evidence that paternal

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Table 6. *Associations between paternal methyl-group donor intake and offspring* IGF2 *DMR methylation in cord blood (*n = *51)*

	IGF2 DMR	
	CpG1	Mean CpG
	β (95% CI) *P*-value	β (95% CI) *P*-value
Methionine	0.336 (0.103, 0.569) 0.006	0.201 (0.001, 0.402) 0.049

IGF2, insulin-like growth factor 2; DMR, differentially methylated region; CI, confidence interval.
β-estimate is an absolute change in percentage of IGF2 DMR methylation; slope >0 means positive association.

Table 7. *Associations between paternal methyl-group donor intake and offspring birth weight (kg) and birth weight-for-gestational age* z*-score (*n = *51)*

	Birth weight (kg) [β (95% CI) *P*-value]	Birth weight-for-gestational age *z*-score [β (95% CI) *P*-value]
Betaine	−0.269 (−0.447, −0.091) 0.004	−0.726 (−1.143, −0.309) 0.001
Choline	0.262 (0.054, 0.471) 0.02	0.591 (0.102, 1.080) 0.02
Methionine	−0.032 (−0.058, −0.006) 0.02	−0.069 (−0.129, −0.006) 0.03

CI, confidence interval.
β-estimate is an absolute change in z-score of birth weight; slope > (<) 0 means positive (negative) association.

methyl-group donor intake has any predictive value for *IGF2* DMR CpG2 and CpG3 methylation.

At last, we determined the effect of paternal methyl-group donor intake on fetal growth, using birth weight (kg) and birth weight-for-gestational age *z*-scores. For the effects of paternal methyl-group donor intake on birth weight and birth weight-for-gestational age *z*-score the best model, was a model with betaine, choline and methionine as the predictive values. Table 7 shows the results for the three methyl-group donors in the multivariable model. The results show a negative association between birth weight/birth weight-for-gestational age *z*-score and betaine/methionine. In addition, a positive association between choline and birth weight/birth weight-for-gestational age *z*-score was found.

Discussion

Combining paternal dietary and methylation data, we were able to assess the effect of methyl-group donor intake on global DNA methylation and global DNA hydroxymethylation. Although our sample size was limited, we found a statistically significant positive association between betaine intake and global DNA hydroxymethylation. Betaine, present in foods like wheat, shellfish, spinach and sugar beets, is the immediate substrate providing methyl-groups to remethylate homocysteine and form methionine.[41] In 30 Gambian women of reproductive age, the methyl-group donor intake was measured through dietary records and blood biomarkers related to the I-C metabolism were determined. Positive correlations between dietary intakes and I-C blood biomarkers (homocysteine and dimethylglycine concentrations) were also found for betaine only.[42] We observed a positive association between betaine and global DNA hydroxymethylation. No associations between methyl-group donor intake and global DNA methylation was found, however the (positive) association between betaine intake and global DNA methylation was borderline significant ($P = 0.08$); 5-hmC has been shown to be a relatively stable epigenetic mark, likely to play an independent regulatory function.[43–45] DNA methylation is a mechanism that is essential to maintain genomic stability and for the regulation of gene expression, so the methylation level may be more strictly regulated.[46] It is shown that even when dietary methyl-group donors are restricted, there is still an increased gene expression of *DNMT1* and *DNMT3A*.[47] Although little is known about the effect of methyl-group donor intake on hydroxymethylation, a recent study by Takumi *et al.*[47] found that a methionine-choline-deficient diet for 1 week activated the active DNA demethylation pathway starting with oxidation of 5-mC by *TET* enzymes (*TET 2* and *TET 3* gene expression was significantly upregulated). Based on ours and other results,[11,46,48] we could hypothesize that 5-hmC might be a more sensitive biomarker of environmental exposures. We did not observe an association between betaine intake and global DNA methylation, which was borderline significant, but the direction of the association between betaine and global DNA methylation/global DNA hydroxymethylation was both positive. The same direction in association of both epigenetic markers was also found by Tellez-Plaza[46] who investigated the relationship between metal exposure and global DNA methylation and hydroxymethylation in 48 participants at two different visits about 10 years apart. They found a correlation of 0.32 ($P = 0.03$) at visit 1 and 0.54 ($P < 0.001$) at visit 2 between global DNA methylation and global DNA hydroxymethylation, which lies in line with our findings ($r = 0.88$, $P < 0.0001$ for whole blood paternal samples and $r = 0.86$, $P < 0.0001$ for cord blood samples).

We hypothesized that not only in utero exposures through the mother, but also preconceptional exposures through the father may induce epigenetic shifts in global DNA (hydroxy) methylation and at the DMR of *IGF2* in the offspring. These epigenetic alterations may provide a plausible link between paternal diet and adverse birth outcomes. We only found a significant positive association between paternal betaine intake and offspring global DNA methylation. To our very best knowledge, this is the first study that examines the association

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between paternal methyl-group donor intake and global DNA (hydroxy)methylation in the offspring of humans. The association between maternal methyl-group donor intake and offspring LINE-1 methylation has been studied. Boeke *et al.*[7] did not find associations between intake of methyl donor nutrients during pregnancy and LINE-1 methylation. However, in a *post-hoc* sex-specific analysis, they found lower cord blood methylation with higher periconceptional intakes of choline and betaine in male offspring only. We confirmed this in a parallel study where we also did not find an association between maternal dietary methyl-group donor intake and offspring global DNA (hydroxy)methylation in the MANOE study,[49] suggesting that parental dietary methyl-group donor intake does not affect offspring global DNA (hydroxy)methylation. However, several studies have shown the possibility that parental methyl-group donor intake could induce changes in offspring gene specific DNA methylation.[9,10,50–52]

In this study we selected the paternally expressed *IGF2* DMR gene which is important during embryogenesis and fetal growth.[21] Higher intakes of paternal methionine suggested higher levels of *IGF2* DMR CpG1 and mean of the three CpGs. Methionine, an essential amino acid, is in the end converted to SAM, which is the universal methyl-group donor. High dietary intake of methionine can influence the I-C metabolism and can therefore induce epigenetic changes.[8,53] Carone *et al.*[10] demonstrated that male mice consuming a low-protein diet fathered offspring with altered DNA methylation at gene specific CpG islands in the liver (e.g. an increase in methylation at a CpG island upstream of *PPARα*). In humans, Soubry *et al.*[18,19] showed that paternal obesity (poor/overnutrition during spermatogenesis) is associated with altered DNA methylation patterns at imprinted genes (hypomethylation at *IGF2* DMR, *MEST*, *PEG3* and *NNAT* DMRs). Based on these results we could conclude that the availability of paternal dietary methyl-group donors after conception, which stands as a proxy for intake before conception, may affect offspring *IGF2* DMR methylation.

We also investigated the paternal contribution through the diet on offspring birth weight. Paternal as well as maternal factors can influence offspring birth weight, although maternal factors make bigger contributions.[54] In this study however, we did find a negative association between paternal betaine/methionine intake and birth weight/birth weight-for-gestational age *z*-score. In addition, choline was positively associated with birth weight and birth weight-for-gestational age *z*-score. The possible mechanism behind this could be that methyl-group donor intake alters the level of DNA methylation in spermatogenesis with consequences for the sperm epigenome and pregnancy outcomes. As there is no simple correlation between methyl-group donor intake and DNA methylation, the paternal intake of methyl-group donors seems to differently influence offspring DNA methylation, the metabolic status of the offspring, and affect phenotypic outcomes.[55] Lambrot *et al.*[56] showed that folate status of male mice alters gene specific sperm DNA methylation and was associated with birth defects (e.g. musculoskeletal malformations). Genes affected were implicated in development and chronic disease (*Aff3, Nkx2-2 and Uts2*, which are implicated in diabetes).

Some strengths and limitations need to be addressed. Good inclusion and exclusion criteria were set up. One of the strengths is that only Caucasian men were enrolled in the study as there can be biogeographic differences in DNA methylation levels.[57] Furthermore, infants from mothers who developed pregnancy complications (gestational diabetes and pre-eclampsia) or delivered pre-term were excluded because these disorders can cause differences in offspring DNA methylation levels.[58,59] A 7-day EDR was used instead of a food-frequency questionnaire to calculate methyl-group donor intake, as there is no validated questionnaire available to assess methyl-group donor intake in men. A 7-day EDR is completed in a prospective manner, so it does not depend on memory, is open-ended, and involves a direct estimation of portion size.[60] The 7-day EDR also takes into account the within-person variability in food intake, which is necessary because there is a strong day-of-the-week effect.[61] Estimated diet records (instead of weighed diet records) were used because they have the same order of accuracy when ranking subjects and the respondent burden is lower.[62] Lastly, we selected the imprinted *IGF2* gene, as it is paternally expressed, so methylation is only present on the paternally inherited allele in the offspring. Isolated leukocytes from cord blood were used as a marker for the newborn's epigenetic status. The use of cord blood, which has different cell types, could be a potential limitation; however, the epigenetic profile of imprinted genes is expected to be similar across all cell types, given the establishment of the epigenetic profile before conception.[63,64] Murphy *et al.*[34] found no difference in *IGF2* DMR methylation profiles in DNA from different cell fractions from cord blood.

The main limitation of our study is its small sample size. However Soubry *et al.*[18] also described an effect of paternal obesity on *IGF2* DMR methylation in offspring from a small sample size ($n = 79$), suggesting that the paternal impact may be strong enough to be detected in a small population. Another potential concern is proof of paternity. Paternal methyl-group donor intake information was collected after conception. However, Pauwels *et al.*[65] showed that the maternal intake of methyl-group donors during pregnancy is stable, except the folate intake was significantly higher before conception. These results give us an indication that paternal methyl-group intake at the moment of conception is similar to the intake at the contact moment, assuming that also the paternal intake is stable over time. It should also be noted that food composition data for methyl-group donors is still scarce (mainly for betaine and choline as the database has only recently became available), therefore a direct match with the foods consumed was not always possible as no local (Belgian) data were available for these methyl-group donors. Finally, a multitude of statistical tests were performed without correction for multiple testing. Therefore, the results of the linear regression model should be considered exploratory and considered hypothesis generating.

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Conclusion

We found a positive association between paternal betaine intake and paternal global DNA hydroxymethylation and offspring global DNA methylation, and a negative association with birth weight-for-gestational age *z*-score. A positive association was also found between paternal methionine intake and offspring *IGF2* DMR methylation. These results suggest that preconceptional paternal methyl-group donor intake may cause epigenetic effects in the next generation. The MANOE children will be followed-up to see if paternally induced epigenetic changes may increase the susceptibility for chronic diseases, like obesity, at a later age.

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Conflicts of Interest

None.

Ethical Standards

This 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). At the start of the study, all participants signed an informed consent.

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