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

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Abstract

Background: Most nutritional studies on the development of children focus on mother-infant interactions. Indeed, maternal nutrition is critically involved in the growth and development of the fetus, birth weight and future disease risk, but what is the contribution of the father's diet? The aim of this study is to investigate the effects of paternal methyl-group donor intake (methionine, folate, betaine, and choline) on paternal and offspring global DNA (hydroxy)methylation, offspring IGF2 DMR DNA methylation, and birth weight. Questionnaires, 7-day estimated dietary records (7d EDRs), and anthropometric measurements from 74 fathers were obtained. 51 cord blood samples were collected and their birth weight was obtained. In cord blood and paternal blood, DNA methylation status was measured using LC-MS/MS (global DNA methylation and global DNA hydroxymethylation) and pyrosequencing (IGF2 DMR methylation).

Results: Betaine intake of the fathers 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 (0.969 % per 100 mg betaine increase, 95% CI: 0.091, 1.302, p = 0.03). Paternal methionine intake was positively associated with CpG1 (0.345 % per 100 mg methionine increase, 95% CI: 0.122, 0.586, p = 0.004), and mean CpG (0.215 % per 100 mg methionine increase, 95% CI: 0.015, 0.415, p = 0.04) methylation of the IGF2 DMR in cord blood. Furthermore, when fathers had a high intake of methionine, there was evidence for a positive link between folate and IGF2 DMR CpG3 methylation in cord blood. Further, a negative association between birth weight/ birth weight-for-gestational age z-score and paternal betaine intake was found. In addition, a negative association of methionine and a positive association of choline with birth weight were also observed.

Conclusion: 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.

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 paternal food deprivation 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]. 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 since it determines the flux of methyl-groups towards 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 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 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 (NEST). 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 epigenetics 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, since 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 DMR's: H19 en IGF2 DMR. The imprint marks at these DMR's 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 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 MANOE study (Maternal Nutrition and Offspring's Epigenome) 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 (figure 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 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). 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 glycaemia ≥ 140 mg/dL (≥ 7.8 mmol/L) a 75 g oral glucose tolerance test (OGTT) 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.

Figure 1 Flowchart of fathers enrolled in the MANOE study and included in the statistical analysis.

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 donors (methionine, folate, betaine, and choline) fathers were asked to complete a 7-day estimated dietary record (7d 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

159 food composition databases from other countries. The Dutch NEVO food composition database [27]
160 was used for folate, the USDA database for the Choline Content of Common Foods [28] for choline
161 and betaine, and the German BLS Nutrient database [29] for methionine. The nutritional values of
162 the food products in the four databases were quantified in mg/100 g (methionine, choline, and
163 betaine) or µg/100 g (folate). The methyl-group donor intake was calculated by multiplying these
164 nutritional values of each consumed product during the seven recorded days with the portion size
165 (grams) of the product and dividing it by 100. For each methyl-group donor, the intakes of the
166 products consumed in one day were added up. Finally, the average methyl-group donor intake of the
167 seven recorded days was calculated.

168 **Paternal and neonatal measurements**

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

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

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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 (LC-MS/MS) method for the simultaneous quantification of DNA cytosine methylation (5-mC) and 5-hydroxymethylcytosine (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 hours. After hydrolysis, 490 µL of acetonitrile/water was added to each sample. Global DNA methylation and hydroxymethylation was obtained by quantifying 5mdC, 5hmdC and dC using ultra-pressure liquid chromatography (UPLC), in combination with tandem mass spectrometry (MS-MS). Global DNA methylation was expressed as a percentage of 5mdC versus the sum of 5mdC, 5hmdC and dC (% global DNA methylation = 5mdC / (5mdC + 5hmdC+ dC)), while global DNA hydroxymethylation was expressed as a percentage of 5hmdC versus the sum of 5mdC, 5hmdC and dC (% global DNA hydroxymethylation = 5hmdC / (5mdC + 5hmdC + dC)).

IGF2 DMR methylation measurements

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). Primer sequences for IGF2 DMR were taken from the original paper. The IGF2 DMR is one of the two DMR's 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 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.

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 idependent t-test was used to compare the characteristics of fathers with and without dietary data. Next, pearson 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) and maternal BMI were also selected as potential confounders. Maternal methyl-group donor intake was not selected as a confounder, since there was a significant difference in paternal and maternal methyl donor intake within one household. 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 4 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 y (range: 24 - 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 y (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 31.2 y (range: 24 - 38). BMI of these fathers averaged 24.9 ± 3.3 kg/m2. 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.

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, y	Mean ± SD Range	32 ± 4.4 25-48	31.2 ± 3.5 24-38	31.8 ± 4.2 24-48	0.26
Weight, kg	Mean ± SD	81.3 ± 12	83.5 ± 14.3	81.6 ± 12.7	0.33
BMI, kg/m^2	Mean ± SD	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)	0.82
No		24 (32.4)	13 (31.7)	38 (33)	

Independent sample t-test was performed to compare characteristics of fathers with and without dietary data.

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 (AR) for these nutrients (35, 36). 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.

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

Methyl-group donors	Mean ± SD	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)

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 level of 0.12 ± 0.08 %. Global DNA methylation and global DNA hydroxymethylation were highly correlated ($r = 0.88$, $p < 0.0001$) (figure 2).

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

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).

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

β-estimate is an absolute change in percentage of global DNA (hydroxy)methylation; slope >(<) 0 means positive (negative) association; CI: confidence interval.

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 - 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 CpG's of the IGF2 DMR was 51.04 ± 3.93%.

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

Characteristics	**Unit/Category**	
Birth weight, kg	Mean ± SD	3.472 ± 0.392
	Range	(2.8 – 4.32)
Gestational age, weeks	Mean ± SD	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 ± SD	0.39 ± 0.95
	Range	(-1.38 – 2.45)
Methylation profile	**Unit/Category**	
Global	Mean ± SD	
Methylation, %		6.61 ± 1.66
Hydroxymethylation, %		0.24 ± 0.15
IGF2 DMR Methylation, %	Mean ± SD	
CpG1		49.06 ± 4.72
CpG2		53.14 ± 4.02
CpG3		50.92 ± 3.92
Mean		51.04 ± 3.93

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 (0.969 % per 100 mg betaine increase, 95% CI: 0.091, 1.302, p = 0.03) in a model adjusted for paternal age, paternal BMI, paternal smoking status, and paternal physical activity. We also included maternal BMI and maternal smoking status as possible confounders. There was no evidence that paternal methyl-group donor intake had any predictive value for offspring global DNA hydroxymethylation (table 5).

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	**0.696 (0.091, 1.302)** **0.03**	0.015 (-0.041, 0.072) 0.58
Choline	0.241 (-0.364, 0.846) 0.43	-0.003 (-0.056, 0.051) 0.92
Folate	0.486 (-0.219, 1.191) 0.17	0.012 (-0.051, 0.075) 0.70
Methionine	-0.038 (-0.128, 0.052) 0.40	-0.006 (-0.014, 0.001) 0.11

β-estimate is an absolute change in percentage of global DNA (hydroxymethylation); slope >(<) 0 means positive (negative) association; CI: confidence interval.

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 CpG's. 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 (0.345 % per 100 mg methionine increase, 95 % CI: 0.122, 0.586, p = 0.004) and mean CpG methylation (0.215 % per 100 mg methionine increase, 95 % CI: 0.015, 0.415, p = 0.04). For the effects of paternal methyl-group donor intake on IGF2 DMR CpG3 the best model, was a model with choline, folate, methionine, and the interactions choline*methionine and folate*methionine. This multivariable model showed a significant interaction between folate and methionine (p = 0.03). When there was a high intake of methionine, there was evidence for a positive link between folate and IGF2 DMR CpG3 methylation. There was no evidence that paternal methyl-group donor intake has any predictive value for IGF2 DMR CpG2 methylation.

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

	IGF2 DMR		
	CpG1	**CpG3**	**Mean CpG**
	β (95 % CI) p-value	β (95 % CI) p-value	β (95 % CI) p-value
Methionine	0.354 (0.122, 0.586) 0.004	3.092 (0.827, 5.356)* 0.009	0.215 (0.015, 0.415) 0.04

* Slope folate at high methionine intake

β-estimate is an absolute change in percentage of IGF2 DMR methylation; slope >0 means positive association; CI: confidence interval.

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-groups in the multivariable model. The results show a negative association between birth weight/birth weight-for-gestational age z-score and betaine. The negative association of methionine and the positive association of choline with birth weight were statistically significant and borderline significant with birth weight-for-gestational age z-score.

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	**-0.206 (-0.368, -0.043)** **0.01**	**-0.548 (-0.935, -0.160)** **0.007**
Choline	**0.208 (0.013, 0.403)** **0.04**	0.457 (-0.007, 0.922) 0.05
Methionine	**-0.027 (-0.053, -0.002)** **0.04**	-0.059 (-0.120, 0.003) 0.06

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

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 [38]. 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 [39]. Although little is known about the effect of methyl-group donor intake on hydroxymethylation, a recent study by Takumi et al. [40] found that a methionine-choline-deficient diet for one week significantly up regulated gene expression of several enzymes (TET2 and TET3) involved in the DNA demethylation pathway. We observed a positive association between betaine and global DNA hydroxymethylation, which could be mediated through a change in the I-C metabolism and/or regulation of TET family proteins. In our study, no associations between methyl-group donor intake and global DNA methylation were found. However, the (positive) association between betaine intake and global DNA methylation was borderline significant ($p = 0.08$). The same direction in association of both epigenetic markers was also found by Tellez-Plaza [41] 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 ($r = 0.88$, $p < 0.0001$).

We hypothesized that not only in utero, 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 between paternal methyl-group donor intake and global DNA (hydroxy)methylation in the offspring. 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 were we also didn't find an association between maternal dietary methyl-group donor intake and offspring global DNA (hydroxy)methylation in the MANOE study (in preparation). 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, 42-44].

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 CpG's. For IGF2 DMR CpG3, there was evidence for a positive link with folate when methionine intake was high. Methionine, an essential amino acid, and folate, a water-soluble vitamin, are in the end converted to SAM, which is the universal methyl-group donor. High dietary intake of methionine or folate can influence the I-C metabolism and can therefore induce epigenetic changes [8, 45]. Carone et al. [10] demonstrated that male mice consuming a low protein diet fathered offspring with altered DNA methylation at gene specific CpG islands from the liver (for example, an increase in methylation at a CpG island upstream of PPARα). In humans, Soubry and colleagues [18, 19] showed that paternal obesity (poor/over-nutrition during spermatogenesis) is associated with altered DNA methylation patterns at imprinted genes (hypomethylation at IGF2 DMR, MEST, PEG3, and NNAT DMR's). Based on these results we could

409 conclude that the availability of paternal dietary methyl-group donors during the preconceptional
410 period may affect offspring IGF2 DMR methylation.
411
412 We also investigated the paternal contribution through the preconceptional diet on offspring birth
413 weight. Paternal as well as maternal factors can influence offspring birth weight, although maternal
414 factors make bigger contributions [46]. In this study however, we did find a negative association
415 between paternal betaine intake and birth weight/birth weight-for-gestational age z-score. In
416 addition, choline was positively and methionine negatively associated with birth weight. The possible
417 mechanism behind this could be that methyl-group donor intake alters the level of DNA methylation
418 in spermatogenesis with consequences for the sperm epigenome and pregnancy outcomes. Lambrot
419 and colleagues [47] showed that folate status of male mice alters gene specific sperm DNA
420 methylation and was associated with birth defects (for example musculoskeletal malformations).
421 Genes affected were implicated in development and chronic disease (Aff3, Nkx2-2, and Uts2, which
422 are implicated in diabetes).
423
424 Some strengths and limitations need to be addressed. Good inclusion and exclusion criteria were set
425 up. One of the strengths is that only Caucasian men were enrolled in the study as there can be
426 biogeographic differences in DNA methylation levels [48]. Furthermore, infants from mothers who
427 developed pregnancy complications (gestational diabetes and pre-eclampsia) or delivered pre-term
428 were excluded because these disorders can cause differences in offspring DNA methylation levels
429 [49, 50]. A 7d EDR was used instead of a food-frequency questionnaire to calculate methyl-group
430 donor intake, since there is no validated questionnaire available to assess methyl-group donor intake
431 in men. A 7d EDR is completed in a prospective manner, so it does not depend on memory, is open-
432 ended, and involves a direct estimation of portion size [51]. The 7d EDR also takes into account the
433 within-person variability in food intake, which is necessary because there is a strong day-of-the-week
434 effect [52]. 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 [53]. Lastly, we selected the imprinted *IGF2* gene, since it is paternally expressed, so methylation is only present on the paternally inherited allele in the offspring. Isolated leucocytes 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 prior to conception [54, 55]. Murphy et al. found no difference in IGF2 DMR methylation profiles in DNA from different cell fractions from cord blood [34].

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. showed that the maternal intake of methyl-group donors during pregnancy is stable, except the folate intake was significantly higher before conception (in preparation). 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 since 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.

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 epigenetics 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.

Declarations

Ethical approval: 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.

Availability of data and material: The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: The study was designed by LG. The nutritional data was analyzed by SP, IT, and IH. Data were processed by SP and IT. SP, LD, and RD participated in the conduction and coordination of the study. The paper was written by SP. The samples were collected by SP and IT. Samples were analyzed by SP, IT, RD, MG, BB, KF, SL, and GK. All authors read and approved the final manuscript.

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