

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

Peer-reviewed author version

Pauwels, Sara; Truijen, Ilse; Ghosh, Manosij; Duca, Radu Corneliu; LANGIE, Sabine; Bekaert, Bram; Freson, Kathleen; Huybrechts, Inge; Koppen, Gudrun; Devlieger, Roland & Godderis, Lode (2017) The effect of paternal methyl-group donor intake on offspring DNA methylation and birth weight. In: JOURNAL OF DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE, 8(3), p. 311-321.

DOI: 10.1017/S2040174417000046

Handle: <http://hdl.handle.net/1942/24207>

1 The effect of paternal methyl-group donor intake on offspring DNA methylation and birth weight.
2 Sara Pauwels^{1,2*}, Ilse Truijien¹, Manosij Ghosh¹, Radu Corneliu Duca¹, Sabine Langie², Bram Bekaert^{3,4},
3 Kathleen Freson⁵, Inge Huybrechts⁶, Gudrun Koppen², Roland Devlieger^{7,8}, Lode Godderis^{1,9}

4 1 KU Leuven- University of Leuven, Department of Public Health and Primary Care, Environment and Health,
5 Kapucijnenvoer 35 blok D box 7001, 3000 Leuven, Belgium. Sara.pauwels@med.kuleuven.be,
6 ilse.truijien@student.kuleuven.be, manosij.ghosh@kuleuven.be, radu.duca@kuleuven.be,
7 lode.godderis@kuleuven.be

8 2 Flemish Institute of Technological Research (VITO), Unit Environmental Risk and Health, Boeretang 200, 2400
9 Mol, Belgium. Sabine.langie@vito.be, Gudrun.koppen@vito.be

10 3 KU Leuven - University of Leuven; Department of Imaging & Pathology; 3000 Leuven, Belgium.
11 bram.bekaert@uzleuven.be

12 4 KU Leuven - University of Leuven; University Hospitals Leuven; Department of Forensic Medicine; Laboratory
13 of Forensic Genetics and Molecular Archeology; 3000 Leuven, Belgium

14 5 KU Leuven-University of Leuven, Center for Molecular and Vascular Biology, UZ Herestraat 49 - box 911, 3000
15 Leuven, Belgium. kathleen.freson@kuleuven.be

16 6 International Agency for Research on Cancer, Dietary Exposure Assessment Group, 150 Cours Albert Thomas,
17 69372 Lyon CEDEX 08, France. Huybrechts.I@iarc.fr

18 7 KU Leuven-University of Leuven, Department of Development and Regeneration, 3000 Leuven, Belgium.
19 roland.devlieger@kuleuven.be

20 8 University Hospitals of Leuven, Department of Obstetrics and Gynecology, 3000 Leuven, Belgium

21 9 IDEWE, External Service for Prevention and Protection at Work, Interleuvenlaan 58, 3001 Heverlee, Belgium

22

23 *Corresponding author: sara.pauwels@med.kuleuven.be, tel +3216377768

24 Key words: Methyl-group donor, Global DNA methylation, global DNA hydroxymethylation, IGF2
25 DMR methylation

26

27

28

29

30

31

32

33

34 **Abstract**

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

45 **Results:** Betaine intake of the fathers was positively associated with paternal global DNA
46 hydroxymethylation (0.028 % per 100 mg betaine increase, 95 % CI: 0.003, 0.053, $p = 0.03$) and cord
47 blood global DNA methylation (0.969 % per 100 mg betaine increase, 95% CI: 0.091, 1.302, $p = 0.03$).
48 Paternal methionine intake was positively associated with CpG1 (0.345 % per 100 mg methionine
49 increase, 95% CI: 0.122, 0.586, $p = 0.004$), and mean CpG (0.215 % per 100 mg methionine increase,
50 95% CI: 0.015, 0.415, $p = 0.04$) methylation of the IGF2 DMR in cord blood. Furthermore, when
51 fathers had a high intake of methionine, there was evidence for a positive link between folate and
52 IGF2 DMR CpG3 methylation in cord blood. Further, a negative association between birth weight/
53 birth weight-for-gestational age z-score and paternal betaine intake was found. In addition, a
54 negative association of methionine and a positive association of choline with birth weight were also
55 observed.

56 **Conclusion:** Our data indicate a potential impact of paternal methyl-group donor intake on paternal
57 global DNA hydroxymethylation, offspring global and IGF2 DMR DNA methylation, and prenatal
58 growth.

59 **Background**

60 Parents contribute in many ways to the development of their children. It is well documented that
61 maternal lifestyle and exposures before and during gestation influences health and development of
62 the next generation [1]. In recent years, a significant number of studies on various environmental
63 exposures (nutrition, pesticides, lead, bisphenol A) have also reported an influence of paternal
64 exposures on offspring's future health. Anderson et al. [2] reported that paternal food deprivation
65 before conception leads to an impaired glucose metabolism in offspring. Besides genomic effects
66 (DNA mutations), epigenetic modifications have been suggested to explain these paternally
67 transmitted effects [3]. Epigenetic changes, such as DNA methylation alterations, can occur in the
68 male germ line due to environmental exposures, such as diet, and can be further passed on to the
69 offspring [4]. DNA methylation may result in changes in gene expression and phenotype without
70 altering the DNA sequence itself by adding a methyl-group (CH₃) to the carbon-5 position of the base
71 cytosine in CpG dinucleotides, catalyzed by the enzyme DNA methyltransferase (Dnmt) [5].

72 The One-Carbon (I-C) metabolism plays a central role in DNA methylation since it determines the flux
73 of methyl-groups towards methylation of DNA. Folate, betaine, choline, and methionine are the main
74 sources of methyl-groups in the I-C metabolism. All of them enter the I-C metabolism at different
75 sites and are, in the end, all converted to the universal methyl-group donor S-adenosylmethionine
76 (SAM)[6]. So far, the effect of methyl donor intake (e.g. folic acid supplementation) on offspring DNA
77 methylation has been mainly studied through maternal intake [7, 8]. However, Mejos et al. [9] have
78 shown that both maternal and paternal folate deficiency (4 week folate deficient diet) can decrease
79 hepatic global DNA methylation in rat offspring. Carone et al. [10] found that male mice consuming a
80 low-protein diet fathered offspring with altered DNA methylation at specific liver CpG islands
81 (including a potential enhancer for the key lipid regulator PPAR α) affecting cholesterol and lipid
82 metabolism.

83 Besides DNA methylation, the DNA can be demethylated by oxidizing 5-methylcytosine (5-mC) to 5-
84 hydroxymethylcytosine (5-hmC) by the Ten-eleven translocation (TET) enzymes and further to 5-
85 formylcytosine (5-fC) and 5-carboxycytosine (5-caC) [11]. Increased levels of 5-hmC may inhibit the
86 binding of methyl-CpG binding proteins and thereby counteract transcriptional repression of 5-mC
87 [12]. Changes in DNA methylation have been related to nutritional exposures such as folic acid
88 supplementation [13-16]. To our very best knowledge, no human studies have evaluated the effect
89 of the parental nutrition on global DNA hydroxymethylation. Most studies on hydroxymethylation
90 were focused on prenatal development, especially stem cell differentiation and lineage. For example,
91 some recent studies have examined the influence of dietary factors (e.g. vitamin C) on 5-hmC.
92 Vitamin C not only induces increased levels of 5-hmC, but also of 5-fC and 5-caC in mouse embryonic
93 stem cells [17].

94 First human evidence of epigenetic changes in the offspring being paternally induced came from the
95 Newborn Epigenetics Study (NEST). Soubry et al. observed that paternal periconceptional obesity
96 (over-nutrition) was significantly associated with offspring DNA methylation at differentially
97 methylated regions (DMRs) of several imprinted genes. Hypomethylation at the IGF2 DMR [18],
98 MEST, PEG3, and NNAT DMRs [19] were associated with paternal obesity. In order to affect offspring
99 methylation through paternal environmental exposures, the exposure needs to be transferred to the
100 male gametes and be sustained through developmental processes. During gametogenesis, from
101 primordial germ cells to spermatozoa, epigenetic marks are established in a sex-specific way. This
102 seems to be the only window of susceptibility during the lifespan of the father (from puberty to
103 adulthood) where paternal environmental exposures can affect epigenetics marks in the gametes.
104 Shortly after fertilization the embryo undergoes genome wide demethylation, except for imprinted
105 marks and repeat sequences which retain their methylation status, making the overall epigenome
106 hypomethylated [5]. Imprinted genes are therefore perfect candidate genes to capture and keep the
107 paternal environmental exposure, since they withstand reprogramming [20]. Our study focuses on
108 the paternally expressed imprinted insulin-like growth factor 2 (IGF2) which plays a critical role in

109 embryogenesis and fetal growth. Its imprinting is regulated by two DMR's: H19 en IGF2 DMR. The
110 imprint marks at these DMR's are established during spermatogenesis, so methylation is only present
111 on the paternally inherited allele in the offspring [21]. To date, a handful of animal studies suggest an
112 effect of paternal nutrition on offspring DNA methylation [9, 10]. In humans however, the impact of
113 paternal diet on offspring DNA methylation and demethylation has not yet been studied.

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

119 **Methods**

120 **Study subjects**

121 The MANOE study (Maternal Nutrition and Offspring's Epigenome) is an ongoing prospective,
122 observational study at the Department of Obstetrics and Gynecology of the University Hospital
123 Leuven (Belgium) that investigates the link between parental methyl-group donor intake and
124 offspring DNA methylation. Pregnant women were followed-up at their scheduled ultrasounds and at
125 these time points fathers were asked to participate (figure 1). Of the 178 women included in the
126 MANOE study, 115 Caucasian fathers provided detailed socio-demographic information (e.g. age,
127 marital status, education), as well as multiple lifestyle or health characteristics (smoking behavior,
128 physical activity, allergies). From these 115 fathers, 41 were excluded from analysis due to missing
129 data (no nutritional information), which resulted in 74 fathers for statistical analysis. We were not
130 able to collect a cord blood sample from 16 newborns, which gives a total of 58 father-infant pairs.
131 Further, two children were excluded because the mother developed gestational diabetes, four due to
132 pre-term delivery (<37 weeks gestation), and one mother had a high risk of neural tube defects and
133 was therefore given an extreme high dose of folic acid (4 mg/day). 51 father-infant pairs were

134 included in the statistical analysis. A screening for gestational diabetes was performed at 24-28
135 weeks using a 50 g glucose challenge test. When the test showed a glycaemia ≥ 140 mg/dL (≥ 7.8
136 mmol/L) a 75 g oral glucose tolerance test (OGTT) was also performed. Based on this test two women
137 were diagnosed with gestational diabetes mellitus (153 – 199 mg/dl or 8.5 – 11 mmol/L glucose)[22].

138 This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all
139 procedures involving human subjects were approved by the UZ Leuven-Committee for Medical Ethics
140 (reference number: ML7975). At the start of the study, all participants signed an informed consent.

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

142 **Paternal dietary information**

143 All 74 fathers were seen once at the Department of Obstetrics and Gynecology at the day of a
144 scheduled ultrasound. To assess the paternal intake of dietary methyl-group donors (methionine,
145 folate, betaine, and choline) fathers were asked to complete a 7-day estimated dietary record (7d
146 EDR). The participants were given guidelines to fill out their diary. This food record is an open-entry
147 diary categorized into six eating occasions (breakfast, morning snacks, lunch, afternoon snacks,
148 dinner, and evening snacks) and involves reporting all foods and drinks consumed over seven
149 consecutive days. It is often considered the most accurate measure of intake and has been referred
150 to as the gold standard [23]. Detailed information on the type (including brand names, the food type
151 (e.g. use of whole, semi-skimmed, or skimmed milk, the type of bread used, etc.) and portion size
152 (expressed as household measures, standard units (e.g. a medium sized apple) or units like grams or
153 liters) of the foods consumed was collected using an open entry format. Only complete food diaries,
154 including seven completed record days and containing sufficiently detailed descriptions of the food
155 products and portion sizes consumed, were taken into consideration. The complete EDRs were
156 encoded and entered into a Diet Entry and Storage program (NUBEL Voedingsplanner [24]) using a
157 manual on food portions and household measures [25]. Methionine, choline, betaine, and folate are
158 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 $\mu\text{g}/100\text{ g}$ (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, Birmingham, 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].

180

181

182

183 **Sample collection and DNA extraction**

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

191 **Global DNA (hydroxy)methylation measurements**

192 Paternal and cord blood DNA was analyzed by a fast and sensitive liquid chromatography-tandem
193 mass spectrometry (LC-MS/MS) method for the simultaneous quantification of DNA cytosine
194 methylation (5-mC) and 5-hydroxymethylcytosine (5-hmC) as described previously [33]. Briefly,
195 isolated genomic DNA samples (10 µg) were hydrolyzed to individual deoxyribonucleosides by a
196 simple one-step DNA hydrolysis procedure. For this, a digest mix was prepared by adding
197 phosphodiesterase I, alkaline phosphatase and benzonase® Nuclease to Tris-HCl buffer. 10 µL of
198 digest mix was added to the extracted DNA and incubated at 37°C for at least 8 hours. After
199 hydrolysis, 490 µL of acetonitrile/water was added to each sample. Global DNA methylation and
200 hydroxymethylation was obtained by quantifying 5mdC, 5hmdC and dC using ultra-pressure liquid
201 chromatography (UPLC), in combination with tandem mass spectrometry (MS-MS). Global DNA
202 methylation was expressed as a percentage of 5mdC versus the sum of 5mdC, 5hmdC and dC (%
203 global DNA methylation = $5\text{mdC} / (5\text{mdC} + 5\text{hmdC} + \text{dC})$), while global DNA hydroxymethylation was
204 expressed as a percentage of 5hmdC versus the sum of 5mdC, 5hmdC and dC (% global DNA
205 hydroxymethylation = $5\text{hmdC} / (5\text{mdC} + 5\text{hmdC} + \text{dC})$).

206

207 **IGF2 DMR methylation measurements**

208 *Bisulfite Conversion and PCR*

209 Genomic DNA (200 ng) was bisulfite converted using the EZ-96 DNA Methylation-Gold™ Kit (#D5008,
210 Zymo Research). Converted DNA was eluted with 30 µL of M-elution buffer. Subsequently, 1 µL of
211 converted DNA was amplified by PCR in a total volume of 25 µL containing 0.2 µM of primers and 2x
212 Qiagen PyroMark PCR Master Mix (#978703, Qiagen). Primer sequences for IGF2 DMR were taken
213 from the original paper. The IGF2 DMR is one of the two DMR's that are involved in the imprinting of
214 the IGF2/H19 domain on chromosome 11p15.5. This DMR is located upstream of the imprinted
215 promoters of IGF2 [34]. PCR reactions for IGF2 DMR consisted of an initial hold at 5°C for 15 min
216 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
217 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.

218 *Pyrosequencing*

219 In order to assess CpG methylation levels, 20 µL of biotinylated PCR product was immobilized to
220 Streptavidin Sepharose High Performance beads (#17-5113-01, GE Healthcare) followed by annealing
221 to 25 µL of 0.3 µM sequencing primer at 80°C for 2 min with a subsequent 10 min cooling down
222 period. Pyrosequencing was performed using Pyro Gold reagents (#970802, Qiagen) on the PyroMark
223 Q24 instrument (Qiagen) following the manufacturer's instructions. Pyrosequencing results were
224 analyzed using the PyroMark analysis 2.0.7 software (Qiagen).

225 **Statistical analysis**

226 First, an independent t-test was used to compare the characteristics of fathers with and without
227 dietary data. Next, Pearson correlations were used to display the association between paternal
228 global DNA methylation and global DNA hydroxymethylation. To determine the effect of paternal
229 methyl-group donor intake on paternal global DNA (hydroxy)methylation, cord blood global DNA
230 (hydroxy)methylation, cord blood IGF2 DMR methylation, and birth weight linear regression models

231 were used. Multivariable models were used to correct for possible confounders. Potential
232 confounders were selected based on the association with paternal nutrition and paternal
233 methylation: paternal age, paternal physical activity (yes/no), paternal smoking (never/past/current),
234 and paternal BMI. When assessing the effect of paternal nutrition on offspring methylation; maternal
235 smoking (did not smoke during pregnancy/smoked during pregnancy) and maternal BMI were also
236 selected as potential confounders. Maternal methyl-group donor intake was not selected as a
237 confounder, since there was a significant difference in paternal and maternal methyl donor intake
238 within one household. Model selection was based on the Akaike Information Criterion (AIC): the
239 model with the lowest AIC (indicating the best model fit) was selected among all tested models
240 (every possible combination of the 4 methyl-group donors together with the pairwise interactions).
241 All tests were two-sided, a 5 % significance level was assumed for all tests. Analyses were performed
242 using SAS software (version 9.4 of the SAS System for Windows).

243 **Results**

244 *Paternal characteristics and methyl-group donor intake*

245 Characteristics of the fathers are presented in table 1. From the 115 included fathers, mean paternal
246 age was 31.8 y (range: 24 - 48). BMI of the participating fathers averaged 24.7 ± 2.9 kg/m². Most men
247 (53.9 %, n = 62) never smoked cigarettes and 32 men (27.8 %) smoked in the past. 67 % (n = 77) of
248 the fathers were physically active (yes/no). From the included fathers with dietary data (n = 74) mean
249 paternal age was 32 y (range: 25 - 48). BMI of these fathers averaged 24.6 ± 2.9 kg/m². Most men
250 (55.4 %, n = 41) never smoked cigarettes and 22 men (29.7 %) smoked in the past. 67.6 % (n = 50)
251 were physically active (yes/no). From the excluded fathers without dietary data (n = 41) mean
252 paternal age was 31.2 y (range: 24 - 38). BMI of these fathers averaged 24.9 ± 3.3 kg/m². Most men
253 (56 %, n = 23) never smoked cigarettes and nine men (22 %) smoked in the past. 68.3 % (n = 28) were
254 physically active (yes/no). No significant differences between fathers with and without dietary data
255 were observed.

256 **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 (%)				0.29
Never-smoker		41 (55.4)	23 (56)	62 (53.9)	
Past-smoker		22 (29.7)	9 (22)	32 (27.8)	
Smoker		11 (14.9)	9 (22)	21 (18.3)	
Physically active	N (%)				0.82
Yes		50 (67.6)	28 (68.3)	77 (67)	
No		24 (32.4)	13 (31.7)	38 (33)	

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

259

260 The average daily intake of methyl-group donors of the 74 fathers is shown in table 2. The average
261 intake of choline and folate corresponded with the average requirements (AR) for these nutrients
262 (35, 36). 55.4 % of the fathers had intake below the dietary guideline for folate and 79.7% for choline.

263 The dietary guideline for methionine is 10.4 mg/kg (37). Mean weight of the fathers with dietary data
264 was 81.3 ± 12.0 kg, resulting in a recommended daily intake 845.2 ± 124.8 mg for methionine. The
265 father’s intake of methionine was much higher than the dietary guideline (range: 1234.4 – 3602.1
266 mg). For betaine no guideline for dietary intake exists.

267

268

269

270

271 **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)

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

273 The 74 fathers had a mean global DNA methylation level of 5.92 ± 1.45 % and a mean global DNA
 274 hydroxymethylation level of 0.12 ± 0.08 %. Global DNA methylation and global DNA
 275 hydroxymethylation were highly correlated ($r = 0.88$, $p < 0.0001$) (figure 2).

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

278

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

286

287

288

289

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

	Global DNA methylation	Global DNA hydroxymethylation
	β (95 % CI)	β (95 % CI)
	p-value	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

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

294

295 *The effect of paternal methyl-group donor intake on offspring*

296 Besides the effect of dietary methyl-group donors consumed by the father on paternal methylation,
 297 we were also interested in its effect on offspring methylation and growth. This analysis was
 298 performed on 51 father-infant pairs. Newborn characteristics and methylation profiles are described
 299 in table 4. The newborns, 26 of which were girls (51 %), had a mean birth weight of 3.472 ± 0.392 kg,
 300 and mean gestational age of 39.75 ± 0.92 weeks. Birth weight-for-gestational age z-score was
 301 calculated and a mean z-score of 0.39 ± 0.95 was obtained (range: -1.38 - 2.45). The 51 newborns had
 302 a mean global DNA methylation level of $6.61 \pm 1.66\%$ and a mean global DNA hydroxymethylation
 303 level of $0.24 \pm 0.15\%$. The mean methylation percentage of the three CpG's of the IGF2 DMR was
 304 $51.04 \pm 3.93\%$.

305

306

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

Characteristics	Unit/Category	
Birth weight, kg	Mean ± SD Range	3.472 ± 0.392 (2.8 – 4.32)
Gestational age, weeks	Mean ± SD Range	39.75 ± 0.92 (37.71 - 41.43)
Gender		
Male	N (%)	25 (49)
Female		26 (51)
Birth weight-for-gestational age, z-score	Mean ± SD Range	0.39 ± 0.95 (-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

308

309 To assess the effects of paternal methyl-group donor intake on offspring global DNA methylation, the
 310 best model was the model with betaine as the only predictive value. Higher intakes of betaine was
 311 linked with higher levels of offspring global DNA methylation (0.969 % per 100 mg betaine increase,
 312 95% CI: 0.091, 1.302, p = 0.03) in a model adjusted for paternal age, paternal BMI, paternal smoking
 313 status, and paternal physical activity. We also included maternal BMI and maternal smoking status as
 314 possible confounders. There was no evidence that paternal methyl-group donor intake had any
 315 predictive value for offspring global DNA hydroxymethylation (table 5).

316

317

318

319 **Table 5. Associations between paternal methyl-group donor intake and offspring global DNA**
 320 **(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

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

323

324 We also determined the effect of paternal methyl-group donor intake on offspring IGF2 DMR
 325 methylation. We assessed the effect on each CpG separately (CpG1, CpG2, and CpG3) and on the
 326 mean methylation of the three CpG's. Only significant results are shown in table 6. The best model to
 327 test the effects of paternal methyl-group donor intake on IGF2 DMR CpG1 and mean CpG
 328 methylation was a model with methionine as the only predictive value. Higher intakes of methionine
 329 correlated with higher levels at CpG1 of IGF2 DMR (0.345 % per 100 mg methionine increase, 95 %
 330 CI: 0.122, 0.586, p = 0.004) and mean CpG methylation (0.215 % per 100 mg methionine increase, 95
 331 % CI: 0.015, 0.415, p = 0.04). For the effects of paternal methyl-group donor intake on IGF2 DMR
 332 CpG3 the best model, was a model with choline, folate, methionine, and the interactions
 333 choline*methionine and folate*methionine. This multivariable model showed a significant
 334 interaction between folate and methionine (p = 0.03). When there was a high intake of methionine,
 335 there was evidence for a positive link between folate and IGF2 DMR CpG3 methylation. There was no
 336 evidence that paternal methyl-group donor intake has any predictive value for IGF2 DMR CpG2
 337 methylation.

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

	IGF2 DMR		Mean CpG
	CpG1	CpG3	
	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

340 * Slope folate at high methionine intake
 341 β -estimate is an absolute change in percentage of IGF2 DMR methylation; slope >0 means positive
 342 association; CI: confidence interval.
 343

344 At last, we determined the effect of paternal methyl-group donor intake on fetal growth, using birth
 345 weight (kg) and birth weight-for-gestational age z-scores. For the effects of paternal methyl-group
 346 donor intake on birth weight and birth weight-for-gestational age z-score the best model, was a
 347 model with betaine, choline, and methionine as the predictive values. Table 7 shows the results for
 348 the three methyl-groups in the multivariable model. The results show a negative association between
 349 birth weight/birth weight-for-gestational age z-score and betaine. The negative association of
 350 methionine and the positive association of choline with birth weight were statistically significant and
 351 borderline significant with birth weight-for-gestational age z-score.

352

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

	Birth weight (kg)	Birth weight-for-gestational age z-score
	β (95 % CI) p-value	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

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

357

358 **Discussion**

359 Combining paternal dietary and methylation data, we were able to assess the effect of methyl-group
360 donor intake on global DNA methylation and global DNA hydroxymethylation. Although our sample
361 size was limited, we found a statistically significant positive association between betaine intake and
362 global DNA hydroxymethylation. Betaine, present in foods like wheat, shellfish, spinach, and sugar
363 beets, is the immediate substrate providing methyl-groups to remethylate homocysteine and form
364 methionine [38]. In 30 Gambian women of reproductive age, the methyl-group donor intake was
365 measured through dietary records and blood biomarkers related to the I-C metabolism were
366 determined. Positive correlations between dietary intakes and I-C blood biomarkers (homocysteine
367 and dimethylglycine concentrations) were also found for betaine only [39]. Although little is known
368 about the effect of methyl-group donor intake on hydroxymethylation, a recent study by Takumi et
369 al. [40] found that a methionine-choline-deficient diet for one week significantly up regulated gene
370 expression of several enzymes (TET2 and TET3) involved in the DNA demethylation pathway. We
371 observed a positive association between betaine and global DNA hydroxymethylation, which could
372 be mediated through a change in the I-C metabolism and/or regulation of TET family proteins. In our
373 study, no associations between methyl-group donor intake and global DNA methylation were found.
374 However, the (positive) association between betaine intake and global DNA methylation was
375 borderline significant ($p = 0.08$). The same direction in association of both epigenetic markers was
376 also found by Tellez-Plaza [41] who investigated the relationship between metal exposure and global
377 DNA methylation and hydroxymethylation in 48 participants at two different visits about 10 years
378 apart. They found a correlation of 0.32 ($p = 0.03$) at visit 1 and 0.54 ($p < 0.001$) at visit 2 between
379 global DNA methylation and global DNA hydroxymethylation, which lies in line with our findings ($r =$
380 0.88, $p < 0.0001$).

381 We hypothesized that not only in utero, but also preconceptional exposures through the father may
382 induce epigenetic shifts in global DNA (hydroxy)methylation and at the DMR of IGF2 in the offspring.
383 These epigenetic alterations may provide a plausible link between paternal diet and adverse birth

384 outcomes. We only found a significant positive association between paternal betaine intake and
385 offspring global DNA methylation. To our very best knowledge, this is the first study that examines
386 the association between paternal methyl-group donor intake and global DNA (hydroxy)methylation
387 in the offspring. The association between maternal methyl-group donor intake and offspring LINE-1
388 methylation has been studied. Boeke et al. [7] did not find associations between intake of methyl
389 donor nutrients during pregnancy and LINE-1 methylation. However, in a post hoc sex-specific
390 analysis, they found lower cord blood methylation with higher periconceptional intakes of choline
391 and betaine in male offspring only. We confirmed this in a parallel study where we also didn't find an
392 association between maternal dietary methyl-group donor intake and offspring global DNA
393 (hydroxy)methylation in the MANOE study (in preparation). Suggesting that parental dietary methyl-
394 group donor intake does not affect offspring global DNA (hydroxy)methylation. However, several
395 studies have shown the possibility that parental methyl-group donor intake could induce changes in
396 offspring gene specific DNA methylation [9, 10, 42-44].

397 In this study we selected the paternally expressed IGF2 DMR gene which is important during
398 embryogenesis and fetal growth [21]. Higher intakes of paternal methionine suggested higher levels
399 of IGF2 DMR CpG1 and mean of the three CpG's. For IGF2 DMR CpG3, there was evidence for a
400 positive link with folate when methionine intake was high. Methionine, an essential amino acid, and
401 folate, a water-soluble vitamin, are in the end converted to SAM, which is the universal methyl-group
402 donor. High dietary intake of methionine or folate can influence the I-C metabolism and can
403 therefore induce epigenetic changes [8, 45]. Carone et al. [10] demonstrated that male mice
404 consuming a low protein diet fathered offspring with altered DNA methylation at gene specific CpG
405 islands from the liver (for example, an increase in methylation at a CpG island upstream of PPAR α). In
406 humans, Soubry and colleagues [18, 19] showed that paternal obesity (poor/over-nutrition during
407 spermatogenesis) is associated with altered DNA methylation patterns at imprinted genes
408 (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

435 the same order of accuracy when ranking subjects and the respondent burden is lower [53]. Lastly,
436 we selected the imprinted *IGF2* gene, since it is paternally expressed, so methylation is only present
437 on the paternally inherited allele in the offspring. Isolated leucocytes from cord blood were used as a
438 marker for the newborn's epigenetic status. The use of cord blood, which has different cell types,
439 could be a potential limitation; however the epigenetic profile of imprinted genes is expected to be
440 similar across all cell types, given the establishment of the epigenetic profile prior to conception [54,
441 55]. Murphy et al. found no difference in *IGF2* DMR methylation profiles in DNA from different cell
442 fractions from cord blood [34].

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

457 **Conclusion**

458 We found a positive association between paternal betaine intake and paternal global DNA
459 hydroxymethylation and offspring global DNA methylation, and a negative association with birth
460 weight-for-gestational age z-score. A positive association was also found between paternal

461 methionine intake and offspring IGF2 DMR methylation. These results suggest that preconceptional
462 paternal methyl-group donor intake may cause epigenetics effects in the next generation. The
463 MANOE children will be followed-up to see if paternally induced epigenetic changes may increase the
464 susceptibility for chronic diseases, like obesity, at a later age.

465 **Declarations**

466 *Ethical approval:* This study was conducted according to the guidelines laid down in the Declaration
467 of Helsinki and all procedures involving human subjects were approved by the UZ Leuven-Committee
468 for Medical Ethics (reference number: ML7975). At the start of the study, all participants signed an
469 informed consent.

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

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

473 *Funding:* Funding for the present study was provided by a PhD grant (grant number 11B1812N) from
474 The Research Foundation-Flanders (FWO) and the Flemish Institute of Technological Research (VITO).

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

480 *Acknowledgements:* We acknowledge the men who volunteered to take part in this study. Also the
481 Unit Leuven Biostatistics and Statistical Bioinformatics Centre (L-BioStat) and in particular
482 Annouschka Laenen who did the statistical analysis.

483

- 485 [1] Painter RC, Roseboom TJ, Bleker OP. Prenatal exposure to the Dutch famine and disease in later
486 life: an overview. *Reprod Toxicol.* 2005;20(3):345-52.
- 487 [2] Anderson LM, Riffle L, Wilson R, Travlos GS, Lubomirski MS, Alvord WG. Preconceptional fasting of
488 fathers alters serum glucose in offspring of mice. *Nutrition.* 2006;22(3):327-31.
- 489 [3] Soubry A, Hoyo C, Jirtle RL, Murphy SK. A paternal environmental legacy: evidence for epigenetic
490 inheritance through the male germ line. *Bioessays.* 2014;36(4):359-71.
- 491 [4] Marques CJ, João Pinho M, Carvalho F, Bièche I, Barros A, Sousa M. DNA methylation imprinting
492 marks and DNA methyltransferase expression in human spermatogenic cell stages. *Epigenetics.*
493 2011;6(11):1354-61.
- 494 [5] Chen ZX, Riggs AD. DNA methylation and demethylation in mammals. *J Biol Chem.*
495 2011;286(21):18347-53.
- 496 [6] McKay JA, Mathers JC. Diet induced epigenetic changes and their implications for health. *Acta*
497 *Physiol (Oxf).* 2011;202(2):103-18.
- 498 [7] Boeke CE, Baccarelli A, Kleinman KP, Burris HH, Litonjua AA, Rifas-Shiman SL, et al. Gestational
499 intake of methyl donors and global LINE-1 DNA methylation in maternal and cord blood: prospective
500 results from a folate-replete population. *Epigenetics.* 2012;7(3):253-60.
- 501 [8] Steegers-Theunissen RP, Obermann-Borst SA, Kremer D, Lindemans J, Siebel C, Steegers EA, et al.
502 Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of
503 the IGF2 gene in the very young child. *PLoS One.* 2009;4(11):e7845.
- 504 [9] Mejos KK, Kim HW, Lim EM, Chang N. Effects of parental folate deficiency on the folate content,
505 global DNA methylation, and expressions of FR α , IGF-2 and IGF-1R in the postnatal rat liver. *Nutr Res*
506 *Pract.* 2013;7(4):281-6.
- 507 [10] Carone BR, Fauquier L, Habib N, Shea JM, Hart CE, Li R, et al. Paternally induced
508 transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell.*
509 2010;143(7):1084-96.
- 510 [11] Dao T, Cheng RY, Revelo MP, Mitzner W, Tang W. Hydroxymethylation as a Novel Environmental
511 Biosensor. *Curr Environ Health Rep.* 2014;1(1):1-10.
- 512 [12] Langie SA, Achterfeldt S, Gorniak JP, Halley-Hogg KJ, Oxley D, van Schooten FJ, et al. Maternal
513 folate depletion and high-fat feeding from weaning affects DNA methylation and DNA repair in brain
514 of adult offspring. *FASEB J.* 2013;27(8):3323-34.
- 515 [13] Bae S, Ulrich CM, Bailey LB, Malysheva O, Brown EC, Maneval DR, et al. Impact of folic acid
516 fortification on global DNA methylation and one-carbon biomarkers in the Women's Health Initiative
517 Observational Study cohort. *Epigenetics.* 2014;9(3):396-403.
- 518 [14] Crider KS, Quinlivan EP, Berry RJ, Hao L, Li Z, Maneval D, et al. Genomic DNA methylation
519 changes in response to folic acid supplementation in a population-based intervention study among
520 women of reproductive age. *PLoS One.* 2011;6(12):e28144.
- 521 [15] Pufulete M, Al-Ghnam R, Khushal A, Appleby P, Harris N, Gout S, et al. Effect of folic acid
522 supplementation on genomic DNA methylation in patients with colorectal adenoma. *Gut.*
523 2005;54(5):648-53.
- 524 [16] Axume J, Smith SS, Pogribny IP, Moriarty DJ, Caudill MA. The MTHFR 677TT genotype and folate
525 intake interact to lower global leukocyte DNA methylation in young Mexican American women. *Nutr*
526 *Res.* 2007;27(1):1365-17.
- 527 [17] Yin R, Mao SQ, Zhao B, Chong Z, Yang Y, Zhao C, et al. Ascorbic acid enhances Tet-mediated 5-
528 methylcytosine oxidation and promotes DNA demethylation in mammals. *J Am Chem Soc.*
529 2013;135(28):10396-403.
- 530 [18] Soubry A, Schildkraut JM, Murtha A, Wang F, Huang Z, Bernal A, et al. Paternal obesity is
531 associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study
532 (NEST) cohort. *BMC Med.* 2013;11:29.

533 [19] Soubry A, Murphy SK, Wang F, Huang Z, Vidal AC, Fuemmeler BF, et al. Newborns of obese
534 parents have altered DNA methylation patterns at imprinted genes. *Int J Obes (Lond)*. 2013.
535 [20] Soubry A, Guo L, Huang Z, Hoyo C, Romanus S, Price T, et al. Obesity-related DNA methylation at
536 imprinted genes in human sperm: Results from the TIEGER study. *Clin Epigenetics*. 2016;8:51.
537 [21] Chao W, D'Amore PA. IGF2: epigenetic regulation and role in development and disease. *Cytokine*
538 *Growth Factor Rev*. 2008;19(2):111-20.
539 [22] World Health Organization W. Diagnostic Criteria and Classification of Hyperglycaemia First
540 Detected in Pregnancy WHO/NMH/MND/13.2. WHO Geneva; 2013.
541 [23] Willett W. *Nutritional Epidemiology*. 3 ed: Oxford University Press; 2012. 529 p.
542 [24] Nubel VoedingsPlanner. v.z.w. NUBEL; 2010.
543 [25] Maten en gewichten. Handleiding voor gestandaardiseerde kwantificering van
544 voedingsmiddelen in België: revisie januari 2005. Hoge Gezondheidsraad. 2nd Ed ed. Brussels,
545 Belgium2005.
546 [26] NUBEL *Belgain Food Composition Table*, Ministry of Public Health. 5th Ed ed. Brussels,
547 Belgium2010.
548 [27] NEVO *Dutch Food Composition table* , NEVO Foundation, Zeist, Netherlands. In: NEVO
549 Foundation Z, Netherlands, editor. 2011.
550 [28] USDA Database for the Choline Content of Common Foods, U.S. Department of Agriculture,
551 Agricultural Research Service. Release 2 ed2008.
552 [29] Dehne LI, Klemm C, Henseler G, Hermann-Kunz E. The German Food Code and Nutrient Data
553 Base (BLS II.2). *Eur J Epidemiol*. 1999;15(4):355-9.
554 [30] Pexsters A, Daemen A, Bottomley C, Van Schoubroeck D, De Catte L, De Moor B, et al. New
555 crown-rump length curve based on over 3500 pregnancies. *Ultrasound Obstet Gynecol*.
556 2010;35(6):650-5.
557 [31] Villar J, Cheikh Ismail L, Victora CG, Ohuma EO, Bertino E, Altman DG, et al. International
558 standards for newborn weight, length, and head circumference by gestational age and sex: the
559 Newborn Cross-Sectional Study of the INTERGROWTH-21st Project. *Lancet*. 2014;384(9946):857-68.
560 [32] J S, DW R. *Molecular Cloning: a laboratory manual*: Cold spring harbor laboratory press; 2001.
561 [33] Godderis L, Schouteden C, Tabish A, Poels K, Hoet P, Baccarelli AA, et al. Global Methylation and
562 Hydroxymethylation in DNA from Blood and Saliva in Healthy Volunteers. *Biomed Res Int*.
563 2015;2015:845041.
564 [34] Murphy SK, Huang Z, Hoyo C. Differentially methylated regions of imprinted genes in prenatal,
565 perinatal and postnatal human tissues. *PLoS One*. 2012;7(7):e40924.
566 [35] EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2014. Scientific
567 Opinion on Dietary Reference Values for folate. *EFSA Journal* 2014;12(11):3893, 59 pp.
568 doi:10.2903/j.efsa.2014.3893.
569 [36] Institute of Medicine Standing Committee on the Scientific Evaluation of Dietary Reference
570 Intakes and its Panel on Folate OBVaC. The National Academies Collection: Reports funded by
571 National Institutes of Health. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6,
572 Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. Washington (DC): National Academies
573 Press (US)
574 National Academy of Sciences.; 1998.
575 [37] World Health Organization FaAOotUN, United Nations University. Protein and amino acid
576 requirements in human nutrition. Report of a joint FAO/WHO/UNU expert consultation (WHO
577 Technical Report, Series 935). 2007.
578 [38] Craig SAS. Betaine in human nutrition. *The American journal of clinical nutrition*. 2004;80(3):539.
579 [39] Dominguez-Salas P, Moore SE, Cole D, da Costa KA, Cox SE, Dyer RA, et al. DNA methylation
580 potential: dietary intake and blood concentrations of one-carbon metabolites and cofactors in rural
581 African women. *Am J Clin Nutr*. 2013;97(6):1217-27.

582 [40] Takumi S, Okamura K, Yanagisawa H, Sano T, Kobayashi Y, Nohara K. The effect of a methyl-
583 deficient diet on the global DNA methylation and the DNA methylation regulatory pathways. *J Appl*
584 *Toxicol.* 2015;35(12):1550-6.

585 [41] Tellez-Plaza M, Tang WY, Shang Y, Umans JG, Francesconi KA, Goessler W, et al. Association of
586 global DNA methylation and global DNA hydroxymethylation with metals and other exposures in
587 human blood DNA samples. *Environ Health Perspect.* 2014;122(9):946-54.

588 [42] Hoyo C, Murtha AP, Schildkraut JM, Jirtle RL, Demark-Wahnefried W, Forman MR, et al.
589 Methylation variation at IGF2 differentially methylated regions and maternal folic acid use before
590 and during pregnancy. *Epigenetics.* 2011;6(7):928-36.

591 [43] Ba Y, Yu H, Liu F, Geng X, Zhu C, Zhu Q, et al. Relationship of folate, vitamin B12 and methylation
592 of insulin-like growth factor-II in maternal and cord blood. *Eur J Clin Nutr.* 2011;65(4):480-5.

593 [44] Jiang X, Yan J, West AA, Perry CA, Malysheva OV, Devapatla S, et al. Maternal choline intake
594 alters the epigenetic state of fetal cortisol-regulating genes in humans. *FASEB J.* 2012;26(8):3563-74.

595 [45] Waterland RA. Assessing the effects of high methionine intake on DNA methylation. *J Nutr.*
596 2006;136(6 Suppl):1706S-10S.

597 [46] Fan C, Huang T, Cui F, Gao M, Song L, Wang S. Paternal factors to the offspring birth weight: the
598 829 birth cohort study. *Int J Clin Exp Med.* 2015;8(7):11370-8.

599 [47] Lambrot R, Xu C, Saint-Phar S, Chountalos G, Cohen T, Paquet M, et al. Low paternal dietary
600 folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. *Nat*
601 *Commun.* 2013;4:2889.

602 [48] Adkins RM, Krushkal J, Tylavsky FA, Thomas F. Racial Differences in Gene- Specific DNA
603 Methylation Levels are Present at Birth. *Birth Defects Research Part A-Clinical And Molecular*
604 *Teratology.*91(8):728-36.

605 [49] He J, Zhang A, Fang M, Fang R, Ge J, Jiang Y, et al. Methylation levels at IGF2 and GNAS DMRs in
606 infants born to preeclamptic pregnancies. *BMC Genomics.*14:472-.

607 [50] Finer S, Mathews C, Lowe R, Smart M, Hillman S, Foo L, et al. Maternal gestational diabetes is
608 associated with genome-wide DNA methylation variation in placenta and cord blood of exposed
609 offspring. *Hum Mol Genet.* 2015;24(11):3021-9.

610 [51] Cade J, Thompson R, Burley V, Warm D. Development, validation and utilisation of food-
611 frequency questionnaires - a review. *Public Health Nutr.* p. 567-87.

612 [52] Beaton GH, Milner J, Corey P, McGuire V, Cousins M, Stewart E, et al. Sources of variance in 24-
613 hour dietary recall data: implications for nutrition study design and interpretation. *The American*
614 *journal of clinical nutrition.*32(12):2546.

615 [53] Chinnock A. Validation of an estimated food record. *Public Health Nutrition.*9(7):934-41.

616 [54] Murphy SK, Huang Z, Hoyo C. Differentially Methylated Regions of Imprinted Genes in Prenatal,
617 Perinatal and Postnatal Human Tissues (Imprinted Gene Methylation in Early Development). *PLoS*
618 *ONE.*7(7):e40924.

619 [55] Soubry A, Schildkraut Joellen M, Murtha A, Wang F, Huang Z, Bernal A, et al. Paternal obesity is
620 associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study
621 (NEST) cohort. *BMC Medicine.*11(1):29.

622

623

624