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1 ***Maternal folate deficiency and metabolic dysfunction in offspring***

2
3 by

4
5 **Jill A McKay and John C Mathers**

6 **Human Nutrition Research Centre**

7 **Institute of Cellular Medicine**

8 **Newcastle University**

9 **Campus for Ageing & Vitality**

10 **Newcastle upon Tyne**

11 **NE4 5PL**

12 **UK**

13
14
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19 **Corresponding author: Professor John C. Mathers**

20 **Telephone: +44 (0) 191 2081133**

21 **Fax: +44 (0) 191 2081101**

22 **E-mail: john.mathers@ncl.ac.uk**

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24 **Abstract**

25 The importance of folate during pregnancy was established more than 80 years ago
26 by Lucy Wills' ground-breaking studies of tropical macrocytic anaemia. More recently,
27 it has become apparent that the adverse consequences of inadequate nutrient
28 supply during early developmental may be exacerbated by over-nutrition post-natally.

29 This paper aims to recent evidence that maternal methyl donor (notably folate)
30 supply peri-conceptually and during pregnancy has long-term effects on offspring
31 (metabolic) health. In addition, we propose the hypothesis that epigenetic
32 mechanisms, especially DNA methylation, may mediate the effects of these early life
33 nutritional insults. We discuss evidence from a natural experiment in humans which
34 provides proof of principle for the hypothesis. We describe an attempt to test this
35 hypothesis using a mouse model in which female C57Bl/6 mice were randomized to
36 low or normal folate diets (0.4 or 2 mg folic acid/kg diet) prior to, and during,
37 pregnancy and lactation. ~~At 4 weeks of age, offspring were randomized to high- or~~
38 ~~low-fat diets.~~ Low maternal folate supply resulted in offspring that were more
39 susceptible to detrimental metabolic effects of a high-fat diet **fed from weaning**,
40 manifested as increased circulating triacylglycerols (TAG) concentration.
41 Interestingly, this metabolic phenotype in adult offspring occurred without any
42 detectable change in adiposity, suggesting a different etiological origin from the more
43 commonly reported observation that maternal under-nutrition leads to increased
44 offspring adiposity and to symptoms of the Metabolic Syndrome. The widespread
45 prevalence of overweight and obesity and of folate deficiency among women of
46 child-bearing age highlights the possibility that this double nutritional insult may
47 exacerbate the risk of metabolic disease in their offspring.

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51 In 1928, Lucy Wills, a young English doctor, travelled to India to investigate an
52 unusual form of anaemia (later called tropical macrocytic anaemia) which was
53 common during pregnancy in women working in the textile mills. At first, she
54 investigated intestinal infections as a possible cause of the anaemia but later
55 became convinced that the disease had a nutritional aetiology. Following successful
56 studies with a rat model of anaemia in pregnancy which could be prevented by
57 adding yeast to the diet, Wills began human studies in which she gave anaemic
58 women yeast or a yeast extract and examined the haematological responses⁽¹⁾. As
59 she reported in her classical paper in 1931, yeast extract (supplied by the Marmite
60 Food Extract Company) was highly effective in resolving the anaemia⁽²⁾. On her
61 return to the Pathology Department at the Royal Free Hospital in London, Wills
62 continued to investigate the curative factor in yeast using rhesus monkeys as
63 models⁽³⁾ but it was not until 1945 that other researchers in the USA isolated and
64 identified a new B vitamin which we know as folate⁽⁴⁾. The availability of a synthetic
65 form of the vitamin, folic acid, facilitated further studies of the biochemistry of folate
66 and the discovery of its central role in one-carbon metabolism.

67 In the 1960s, low maternal folate status was linked with increased risk of neural tube
68 defects (NTDs)⁽⁵⁾ and this association led, eventually, to randomised controlled trials
69 (RCTs) which demonstrated the efficacy of supplementation with relatively large
70 doses of folic acid in reducing both NTD recurrence⁽⁶⁾ and occurrence⁽⁷⁾. These
71 ground-breaking RCTs provided the evidence base for national-scale public health
72 interventions, initially in the USA and Canada, which require the mandatory

73 fortification of flour with 140 µg of folic acid per 100 g flour. This fortification
74 programme, which began in 1996 and became mandatory from January 1998, has
75 been highly effective in raising the folate status of the whole population⁽⁸⁾ and has
76 been associated with a substantial fall in NTD prevalence⁽⁹⁾. Several other countries,
77 excluding those in the European Union, have implemented similar folic acid
78 fortification policies.

79 **Early life origins of metabolic diseases**

80 Folate and one-carbon metabolism is now seen as an integrator of nutrient status
81 through which changes in nutrient inputs have multiple health effects via multiple
82 changes in cell processes⁽¹⁰⁾ (Fig.1). For example, because its role as a source of
83 methyl groups for the re-methylation of homocysteine to methionine (Fig 2), low
84 folate supply leads to elevated concentrations of homocysteine⁽¹¹⁾ which are
85 associated with increased risk of NTDs, cardiovascular disease, cancers, dementias
86 and osteoporosis⁽¹²⁾. In India and other Asian countries, the prevalence of type 2
87 diabetes and other metabolic diseases is rising rapidly, apparently as a consequence
88 of the double nutritional insults of poor maternal nutrition followed by over-nutrition
89 associated with urbanisation and the adoption of higher fat diets⁽¹³⁾. More specifically,
90 the observation that raised maternal homocysteine concentration in pregnancy is
91 associated with low birthweight has been confirmed by Mendelian randomisation
92 analysis supports a causal role for dysregulated one-carbon metabolism in poor fetal
93 growth⁽¹⁴⁾. In addition, abnormal folate and homocysteine concentrations in mothers
94 during pregnancy associate with both small birth size and increased likelihood of
95 childhood insulin resistance in the offspring, emphasising the potential for
96 nutritionally-driven disturbances in one-carbon metabolism to enhance fetal
97 programming of diabetes and other metabolic diseases⁽¹⁵⁾. These observations fit

98 with the “predictive adaptive response” hypothesis which proposes that mismatch
99 between the environment anticipated by the fetus, based on early (in utero)
100 environmental exposures, and the environment encountered post-natally may
101 predispose to the early development of metabolic, and other, diseases⁽¹⁶⁾. Therefore
102 the adverse consequences of inadequate nutrient (folate) supply during early
103 developmental may be exacerbated by over-nutrition post-natally. **Whilst the**
104 **mechanisms responsible for the lifelong consequences of adverse nutritional**
105 **exposures in early life are poorly understood, it seems probable that they include**
106 **epigenetic processes** ⁽¹⁷⁾ .

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108 **Epigenetics as a mechanism linking early life nutrition with later health**

109 **Epigenetics describes an integrated system of marks (DNA methylation and post-**
110 **translational modification of histones) and molecules (including small non-coding**
111 **RNAs) which is responsible for regulating the transcriptional state of individual cells.**
112 **Importantly, epigenetic mechanisms are responsive to the cell’s environment and so**
113 **are modulated by dietary and other exposures**⁽¹⁸⁾. **The importance of the maternal**
114 **intake of folate and other methyl donors during pregnancy on DNA methylation**
115 **patterns and offspring phenotype was established more than a decade ago using the**
116 **agouti (A(vy)) mice which have a transposable element in the agouti gene**⁽¹⁹⁾.
117 **Maternal supplementation with folic acid, vitamin B₁₂, choline, and betaine increased**
118 **CpG methylation at the A(vy) locus in the offspring and increased the proportion of**
119 **leaner offspring with darker coats**⁽¹⁹⁾. **The agouti locus is an example of a metastable**
120 **epiallele (ME) that is variably expressed in genetically identical individuals due to**
121 **epigenetic modifications that were established during early development**⁽²⁰⁾. **In**

122 humans, Waterland and colleagues were the first to establish that the maternal
123 environment around the time of conception influences the methylation status of
124 MEs in the offspring⁽²¹⁾. This work was undertaken in rural Gambia where seasonal
125 differences in work patterns, food availability and other environmental factors provide
126 a natural experiment for testing the effects of maternal exposures on pregnancy
127 outcomes. Although in such natural experiments it is impossible to determine which
128 of the many factors is responsible for a given outcome, it seems likely that nutrition is
129 important. For example, seasonal differences in maternal consumption of methyl-
130 donor nutrients influenced the maternal plasma concentrations of multiple substrates
131 for one-carbon metabolism during pregnancy and these changes were reflected in
132 altered methylation of MEs in DNA extracted from lymphocytes and from hair follicles
133 in their infant offspring⁽²²⁾. More recent study of these Gambian children revealed that
134 methylation of the tumour suppressor gene *VTRNA2-1* differed according to season
135 of conception⁽²³⁾. In addition, once altered *in utero*, the methylation status of
136 *VTRNA2-1* was stable over at least 10 years⁽²³⁾. *VTRNA2-1* is a ME which appears
137 to influence both cancer risk and immune function and the authors argued that this
138 season of birth-related epigenetic change is a plausible candidate pathway to explain
139 their earlier observation that season of birth predicts adult mortality from infection-
140 related causes in rural Gambians⁽²⁴⁾. Whilst these exciting findings should stimulate
141 further investigations of the impact of peri-conceptual nutrition on health outcomes
142 and the possible mediating effects of epigenetic mechanisms, the results should be
143 interpreted with caution since they demonstrate associations but not causality.

144 **Towards a mouse model for testing the effects of maternal folate inadequacy**
145 **on the metabolic health of the offspring**

146 Direct testing in humans of the effects of maternal folate inadequacy on the
147 metabolic health of the offspring is fraught with obvious ethical and practical
148 difficulties. In addition, attempts to investigate the epigenetic mechanisms through
149 which such nutritional insults during early development produce their long-term
150 effects on health⁽²⁵⁾ in humans would require access to tissue such as liver and
151 placenta. This is because the patterns of epigenetic marks, notably DNA methylation,
152 are cell and tissue specific so that methylation marks assessed in a surrogate tissue
153 e.g. blood or buccal cells may not reflect those in the cell or tissue of interest⁽²⁶⁾. For
154 example, we quantified DNA methylation by pyrosequencing® for a panel of genes
155 including *Esr1*, *Igf2* and *Slc39a4* using DNA extracted from blood, liver, and kidney
156 from female mice and observed tissue-specific differences in methylation at all loci⁽²⁶⁾.
157 Such inter-tissue differences in DNA methylation patterns in humans were confirmed
158 for *IGF2*, *GNASAS* and *IL10* by Waterland and colleagues who examined
159 methylation patterns in post-mortem samples of brain, liver and kidney obtained from
160 Vietnamese motor vehicle accident victims⁽²¹⁾. In addition, we observed that folate
161 depletion during pregnancy altered *Igf2* methylation in a tissue-specific manner
162 ($p < 0.05$)⁽²⁶⁾. In this context, MEs are a special case since their methylation status is
163 very similar in all tissues investigated and methylation changes induced in early
164 development appear to be stable indefinitely⁽²³⁾. As a consequence, suitable animal
165 models are necessary to test for causality, to undertake investigations of possible
166 epigenetic mechanisms and to augment the epidemiological evidence available from
167 human studies.

168 In recently completed work, we set out to test 3 hypotheses:

- 169 1. Maternal folate depletion during pregnancy and lactation may contribute to the
170 development of obesity and the Metabolic Syndrome (MS);

- 171 2. These effects may be exacerbated by provision of a high fat diet post-weaning
172 (double nutritional insult);
- 173 3. The altered phenotype may be due to altered gene expression through
174 epigenetic mechanisms.

175 On the basis that better models facilitate better hypothesis testing, we developed a
176 mouse model of maternal folate depletion which was uncomplicated by other factors.
177 In addition, because epigenetic processes may be particularly plastic in early
178 embryonic/ fetal life, we considered it important that the mouse dams were folate
179 depleted at mating. To minimise potential confounding by other factors, the degree of
180 folate depletion should be sufficient to impose a nutritional stress but not be so
181 severe as to limit reproduction. Finally, by feeding a high-fat diet from weaning, we
182 aimed to simulate a common secondary nutrition stress. Such a model may yield
183 data which may be more readily interpretable mechanistically and which are likely to
184 be of greater relevance to human nutrition i.e. more potential for translation.

185 Our first question was how long would it take to induce folate-depletion in young
186 female mice? We addressed this question by feeding female mice a folate-free diet
187 for up to 7 weeks⁽²⁵⁾. Blood was collected from pairs of culled mice weekly and red
188 blood cell (RBC) folate concentration measured. As shown in Fig 3, there were 2
189 distinct phases in RBC folate kinetics; an initial phase lasting approximately 2 weeks
190 during which RBC folate concentration remained largely unchanged followed by
191 exponential “decay” in RBC folate concentration toward a new equilibrium after 4-5
192 weeks⁽²⁵⁾. For comparison, Leamon and colleagues reported that RBC folate
193 reached a new (lower) plateau in BALB/c mice approximately 6 weeks after transfer to
194 a low folate diet⁽²⁷⁾. Further studies showed that a milder nutritional insult (feeding

195 0.4 mg folic acid/ kg diet) reduced maternal and offspring RBC folate concentrations
196 (measured at weaning) by about 50%⁽²⁵⁾.

197 For the remaining studies described in this report, we adopted a 2*2 factorial design
198 in which female C57/Bl6 mice were fed a folate-normal or folate-depleted diet (2 and
199 0.4 mg folic acid/ kg diet respectively) for 4-5 weeks before mating and throughout
200 pregnancy and lactation. At weaning, offspring were randomised to either a control
201 (CONT) diet or a high fat (HF) diet (50 and 200g fat/kg diet respectively) from
202 weaning until aged 6 months (Fig 4)^(28,29). For dams with successful pregnancies,
203 duration of dietary exposure did not differ between those fed the normal and low-
204 folate diets ($p=0.42$). However, dams fed the low-folate diet were more likely to
205 experience reproductive failure due to miscarriage or postpartum litter death⁽²⁹⁾. At
206 weaning, maternal serum folate concentration was reduced by approximately two
207 thirds ($P < 0.001$) in the dams fed the low-folate diet confirming the magnitude of the
208 maternal dietary insult⁽²⁹⁾. Whilst the low-folate diet had no effect on litter size, body
209 weight at weaning was 6% lower for offspring of mothers fed the low-folate diet⁽²⁹⁾.

210 As expected, offspring randomised to the high-fat (HF) diet were heavier and
211 contained more body fat at ages 3 and 6 months than those randomised to the
212 lower-fat (CONT) diet but there was no effect of maternal folate depletion on
213 offspring adult weight or adiposity⁽²⁹⁾. In addition, there was no effect of maternal
214 folate supply on the gross anatomy of the 6 month old offspring as gauged by organ
215 weights and gut lengths⁽²⁹⁾. However, adult offspring from dams fed the folate-
216 depleted diet had significantly raised plasma triacylglycerol (TAG) concentrations
217 when given the HF diet from weaning whereas the HF diet had no effect on TAG
218 concentrations in the offspring of mothers with adequate folate supply before mating
219 and during pregnancy and lactation ($P_{\text{interaction}} = 0.005$)⁽²⁹⁾. This provided evidence

220 that the early life nutritional insult (maternal folate depletion during pregnancy and
221 lactation) had long-term adverse metabolic consequences for the offspring which
222 were revealed only when the offspring were exposed to a second nutritional insult i.e.
223 HF feeding from weaning. This adverse metabolic phenotype in the adult offspring
224 occurred in the absence of a detectable effect on adiposity which suggests a
225 different aetiological origin from the frequently reported phenotype characterised by
226 enhanced adiposity and other symptoms of the Metabolic Syndrome (MS) in the
227 adult offspring of dams exposed to malnutrition during pregnancy⁽³⁰⁾.

228 **Investigation of possible mechanism(s) for long-term metabolic effect on**
229 **offspring of folate depletion during pregnancy and lactation**

230 We hypothesised that maternal folate depletion alters folate supply to the embryo
231 and the developing fetus with potential widespread effects because of its impact on
232 one-carbon metabolism. In particular, inadequate folate limits the availability of
233 methyl groups for S-adenosylmethionine (SAM) synthesis and this generates
234 competition between cellular processes which use SAM, notably the methylation of
235 macromolecules including lipids, proteins and DNA. Further, we hypothesised that
236 reduced SAM availability would alter the pattern of DNA methylation which, because
237 of its role in regulating transcription, would change the repertoire of expressed
238 genes⁽³¹⁾. Finally, the altered gene expression would reduce the capacity of adult
239 offspring to cope metabolically with a second nutritional stress, e.g. HF feeding,
240 leading to elevated plasma TAG concentrations. If these putative effects were
241 induced in early life, we anticipated that they might be detectable in the fetus late in
242 pregnancy and that this might be an informative life-stage at which to test our
243 hypothesis.

244 Using time-mated dams, we collected fetuses and their placentae at pregnancy day
245 17.5. Both tissues were used for investigation of genome-wide gene expression and,
246 in addition, genome-wide promoter methylation was investigated in the fetal livers.
247 The outcomes of those mechanistic studies will be published elsewhere. ~~We~~
248 ~~observed no effect of maternal folate depletion on placental mass or placental~~
249 ~~efficiency (McKay, Ford & Mathers, unpublished) suggesting that the maternal~~
250 ~~nutritional insult did not disadvantage the offspring through a gross effect on~~
251 ~~placental size or function. Similarly, there was no effect on fetal liver mass. Using~~
252 ~~genome-wide microarray approaches, we investigated gene expression in both~~
253 ~~tissues and found that approximately twice as many genes were differentially~~
254 ~~expressed in the fetal liver (nearly 1000) than in the placenta (<500) in response to~~
255 ~~maternal folate depletion. In both tissues, approximately equal proportions of genes~~
256 ~~were up- and down-regulated. However, the particular genes which were~~
257 ~~differentially expressed in the two tissues were almost totally different and only 4~~
258 ~~genes showed concordant expression changes in both liver and placenta. These~~
259 ~~observations support the hypothesis that when folate supply is limiting during fetal~~
260 ~~development, gene expression changes are tissue specific. Such specificity in~~
261 ~~response to a nutritional challenge may have survival advantage with the “short-lived”~~
262 ~~placenta responding very differently from the liver where changes in phenotype may~~
263 ~~have lifelong implications.~~

264 Genome-wide DNA methylation analysis by microarray showed that >300 genes
265 were differentially methylated in the fetal liver from folate-depleted dams and,
266 curiously, most of these genes were hyper-methylated. There was limited overlap
267 between the genes which were differentially methylated and those which were
268 differentially expressed which suggests that these epigenetic changes were not

269 responsible for most the observed transcriptional changes. This may be because
270 changes in transcription factors, microRNA or other regulatory mechanisms caused
271 the changes in gene expression when folate supply was inadequate or that the
272 nutritional insult altered methylation of CpG sites which were not assayed by the
273 microarray used in this study.

274 **Public health implications**

275 Adequate folate intake pre-conception and during pregnancy and lactation is
276 essential for good maternal and child health. Recent data from the National Diet and
277 Nutrition Survey Rolling programme shows widespread folate inadequacy among
278 women of child-bearing age in the UK (Table 1)⁽³²⁾. Biochemical folate deficiency is
279 more prevalent in younger, than in older, women and in Scotland and Northern
280 Ireland than in the UK as a whole. In addition, overweight and obesity are common
281 among women of child-bearing age⁽³³⁾. The Health Survey for England reported that
282 14% of females aged 16 - 24 years were obese and that this rose to almost 25% for
283 women aged 35-44 years⁽³³⁾. If the observations from our mouse study⁽²⁹⁾ apply in
284 humans, then the combination of folate deficiency and excess adiposity among
285 women of child-bearing age may disadvantage the health of their offspring by
286 increasing the risk of metabolic diseases exemplified by raised TAG concentrations.
287 This risk may be exacerbated if the folate needs of obese women are greater than
288 those of normal weight women. A study of short-term folate pharmacokinetics in
289 women of child-bearing age showed that the-area-under-the-curve (AUC) for the
290 absorption phase (0-3h) and the peak serum folate concentration were both
291 significantly lower in obese women following consumption of 400µg folic acid – the
292 recommended supplemental dose to lower NTD risk (Fig. 4)⁽³⁴⁾. In a previous study
293 in which the folic acid dose administered was calculated per total body weight, the

294 AUC was higher in obese women and the authors suggested that it would be
295 preferable to define the folate dose in relation to lean body weight⁽³⁵⁾. In summary,
296 folate recommendations for obese women of reproductive age may need to be
297 reconsidered to ensure adequacy.

298 In conclusion, **our mouse studies showed that** uncomplicated folate inadequacy peri-
299 conceptually and during pregnancy and lactation predisposed the offspring to
300 metabolic derangements when fed a high fat diet. The widespread prevalence of
301 folate deficiency and of overweight and obesity among women of child-bearing age
302 highlights the possibility that this double nutritional insult may exacerbate the risk of
303 metabolic disease in their offspring and points to the need for appropriate
304 interventions.

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409

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416 **Conflicts of interest**

417 None

418 **Authorship**

419 JCM drafted this manuscript based on the lecture that he gave at the Nutrition
420 Society Spring conference on the 25th and 26th of March, 2015 in Aberdeen. Both
421 authors approved the final manuscript.

422

423 Table 1. Percentages of women of child-bearing age with evidence of biochemical
424 folate deficiency based on serum and red blood cell (RBC) folate concentrations
425 (NDNS Rolling Programme 2015)

Age range	16-24 years	25-34 years	34-49 years
Serum folate*	22.1	17.7	13.1
RBC folate†	15.6	9.5	10.1

426 * Percentage of women with serum folate concentration < 10nM⁽³⁶⁾.

427 † Percentage of women with RBC folate concentration < 340nM⁽³⁶⁾.

428

429 **Figure legends**

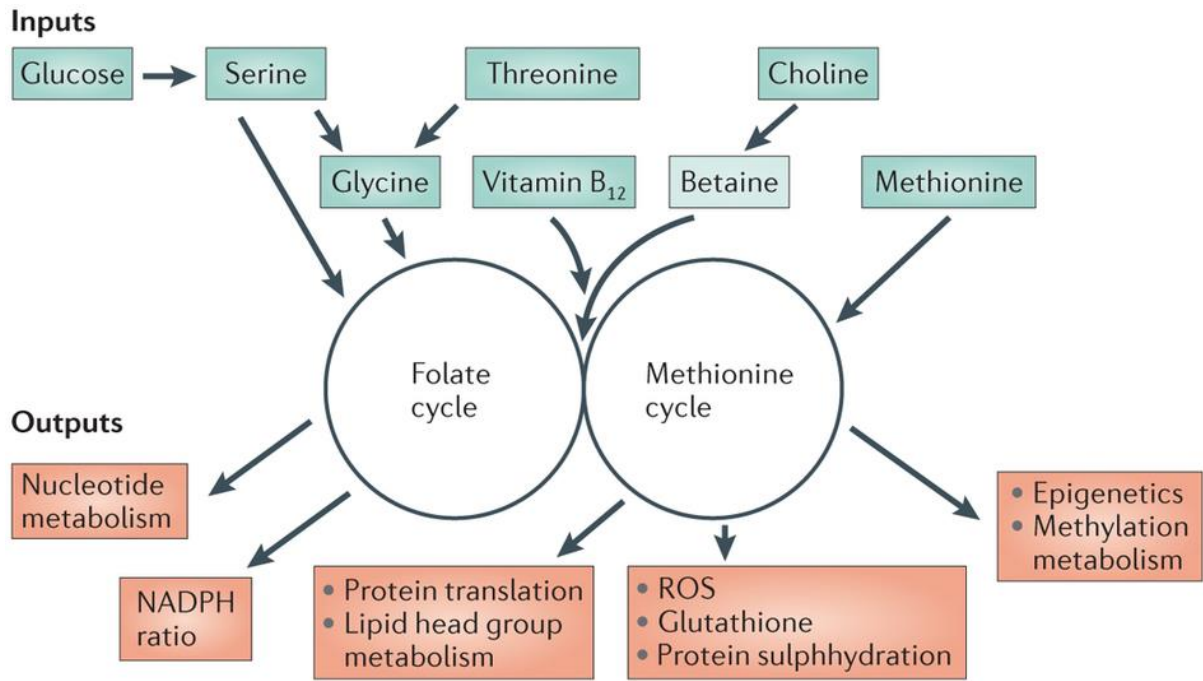
430 **Fig. 1. Overview of one-carbon metabolism indicating its role as an integrator**
431 **of nutrient status (from Reference 10)**

432 **Fig. 2. Role of folate in one-carbon metabolism supplying methyl groups for**
433 **multiple purposes (from Reference 11)**

434 **Fig. 3. Study design investigating effects of maternal folate depletion and high**
435 **fat feeding from weaning (from Reference 28)**

436 **Fig. 4. Serum folate responses following ingestion of 400µg folic acid in obese**
437 **and normal weight women (from Reference 34)**

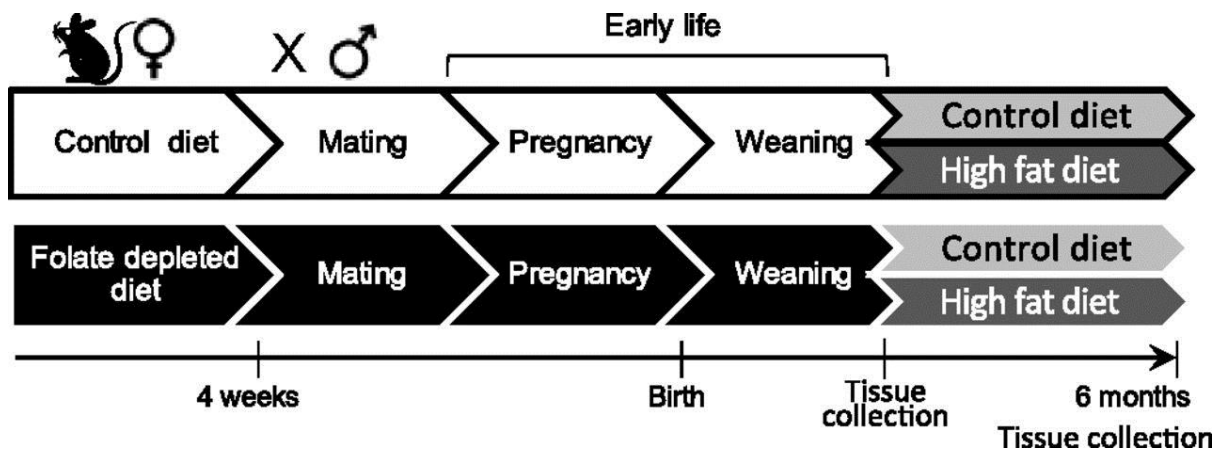
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439

440 **Fig. 1.**

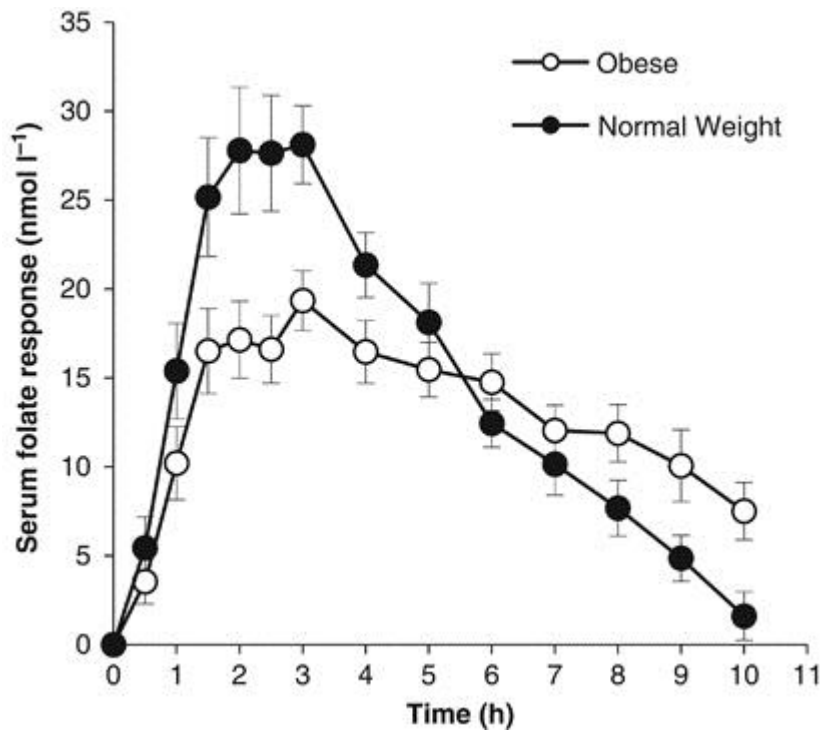
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445

446 **Fig. 3.**

447



448

449 **Fig. 4.**