# *Maternal folate deficiency and metabolic dysfunction in offspring*

by

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## Abstract

The importance of folate during pregnancy was established more than 80 years ago by Lucy Wills' ground-breaking studies of tropical macrocytic anaemia. More recently, it has become apparent that the adverse consequences of inadequate nutrient supply during early developmental may be exacerbated by over-nutrition post-natally. This paper aims to recent evidence that maternal methyl donor (notably folate) supply peri-conceptually and during pregnancy has long-term effects on offspring (metabolic) health. In addition, we propose the hypothesis that epigenetic mechanisms, especially DNA methylation, may mediate the effects of these early life nutritional insults. We discuss evidence from a natural experiment in humans which provides proof of principle for the hypothesis. We describe an attempt to test this hypothesis using a mouse model in which female C57Bl/6 mice were randomized to low or normal folate diets ~~(0.4 or 2 mg folic acid/kg diet)~~ prior to, and during, pregnancy and lactation. ~~At 4 weeks of age, offspring were randomized to high- or low-fat diets.~~ Low maternal folate supply resulted in offspring that were more susceptible to detrimental metabolic effects of a high-fat diet fed from weaning, manifested as increased circulating triacylglycerols (TAG) concentration. Interestingly, this metabolic phenotype in adult offspring occurred without any detectable change in adiposity, suggesting a different etiological origin from the more commonly reported observation that maternal under-nutrition leads to increased offspring adiposity and to symptoms of the Metabolic Syndrome. The widespread prevalence of overweight and obesity and of folate deficiency among women of child-bearing age highlights the possibility that this double nutritional insult may exacerbate the risk of metabolic disease in their offspring.

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

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

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

## Early life origins of metabolic diseases

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

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

## Epigenetics as a mechanism linking early life nutrition with later health

Epigenetics describes an integrated system of marks (DNA methylation and post-translational modification of histones) and molecules (including small non-coding RNAs) which is responsible for regulating the transcriptional state of individual cells. Importantly, epigenetic mechanisms are responsive to the cell’s environment and so are modulated by dietary and other exposures[18]. The importance of the maternal intake of folate and other methyl donors during pregnancy on DNA methylation patterns and offspring phenotype was established more than a decade ago using the agouti (A(vy)) mice which have a transposable element in the agouti gene[19]. Maternal supplementation with folic acid, vitamin $B_{12}$, choline, and betaine increased CpG methylation at the A(vy) locus in the offspring and increased the proportion of leaner offspring with darker coats[19]. The agouti locus is an example of a metastable epiallele (ME) that is variably expressed in genetically identical individuals due to epigenetic modifications that were established during early development[20]. In

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

## Public health implications

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

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

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

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## Conflicts of interest

None

## Authorship

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

Table 1. Percentages of women of child-bearing age with evidence of biochemical folate deficiency based on serum and red blood cell (RBC) folate concentrations (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 |

* Percentage of women with serum folate concentration < 10nM[36].

† Percentage of women with RBC folate concentration < 340nM[36].

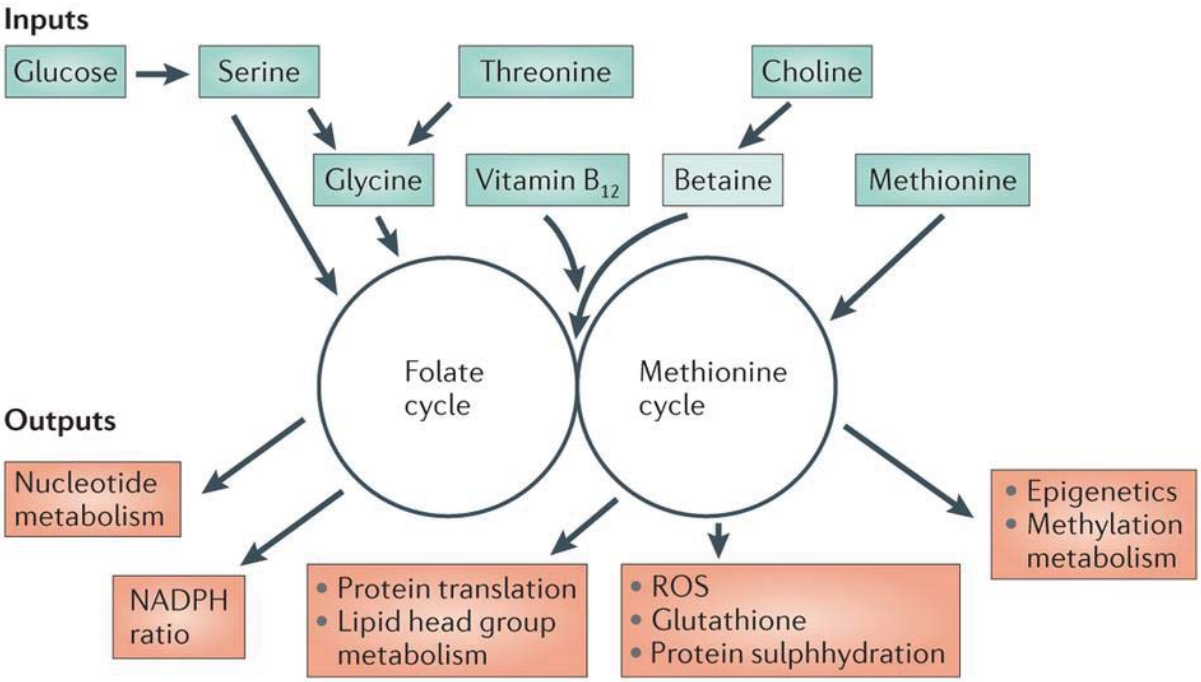
## Figure legends

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

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

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

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

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440 **Fig. 1.**

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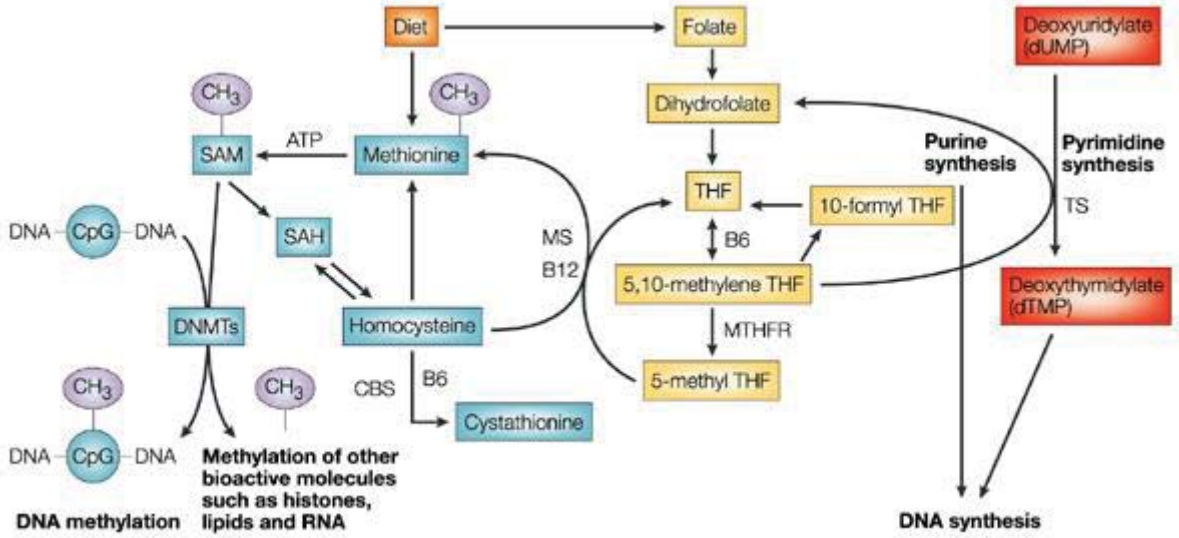


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443 **Fig. 2.**

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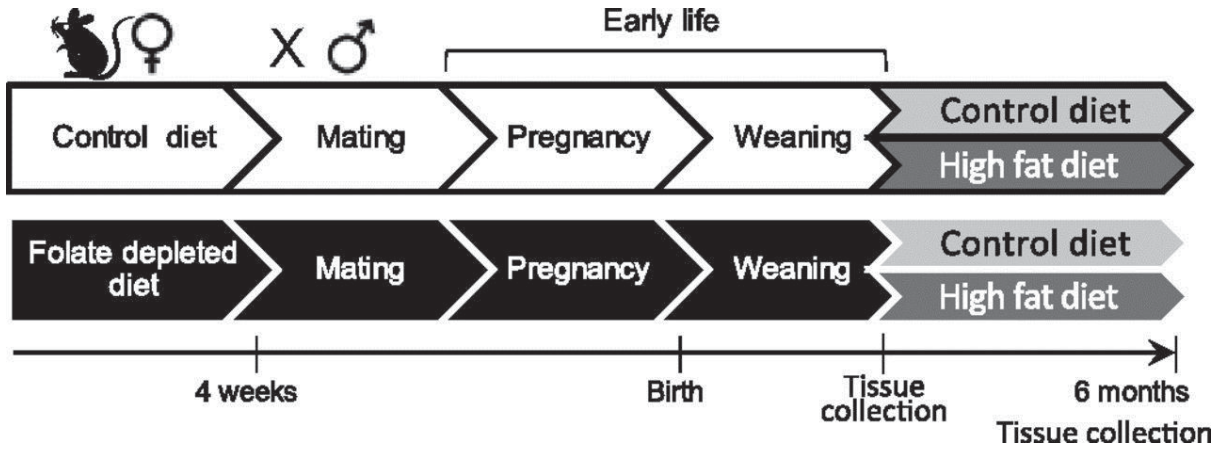


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446 **Fig. 3.**

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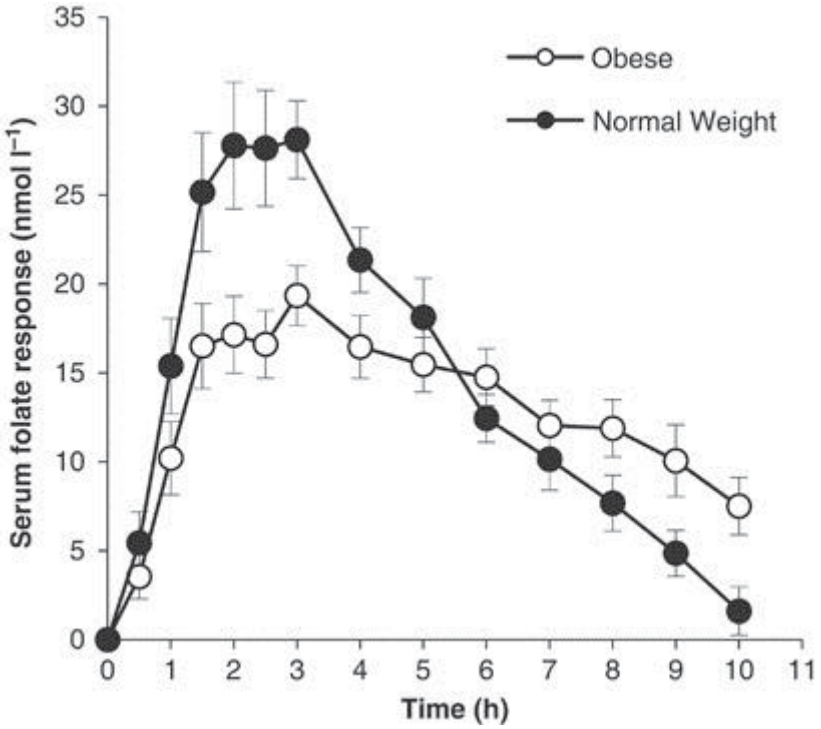
Obese
Normal Weight
Serum folate response (nmol l⁻¹)
Time (h)
0
5
10
15
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25
30
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0
1
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449 **Fig. 4.**