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Symposium 3: Nutrition, epigenetics and the fetal origins of metabolic disease

Maternal folate deficiency and metabolic dysfunction in offspring

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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 postnatally. The present paper aims to review 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 human subjects 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 randomised to low or normal folate diets prior to, and during, pregnancy and lactation. 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 TAG concentration. Interestingly, this metabolic phenotype in adult offspring occurred without any detectable change in adiposity, suggesting a different aetiological origin from the more commonly reported observation that maternal undernutrition 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.

Peri-conceptual: Folate: TAG: Liver: Placenta: Gene expression: DNA methylation

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 C_1 metabolism.

In the 1960s, low maternal folate status was linked with increased risk of neural tube defects (NTD)[5] and this association led, eventually, to randomised controlled trials which demonstrated the efficacy of supplementation with
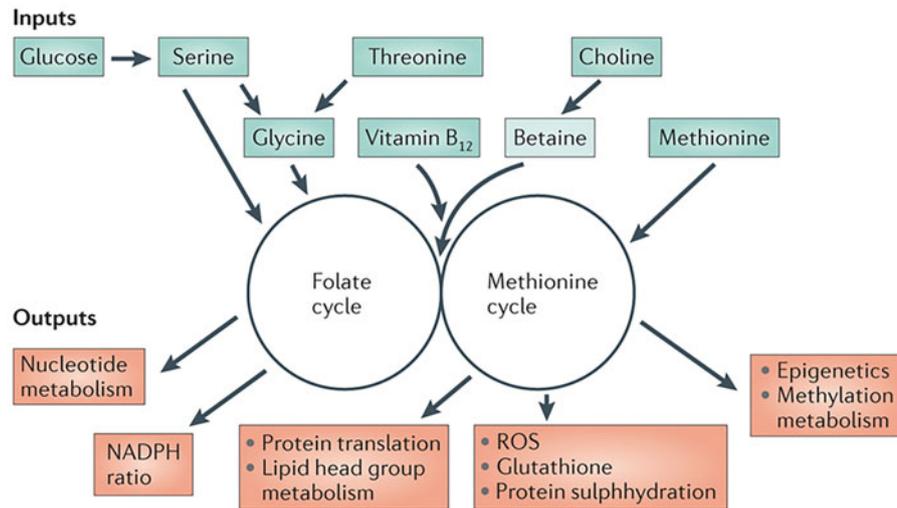

Fig. 1. (Colour online) Overview of C_1 metabolism indicating its role as an integrator of nutrient status (from [10]). ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase.
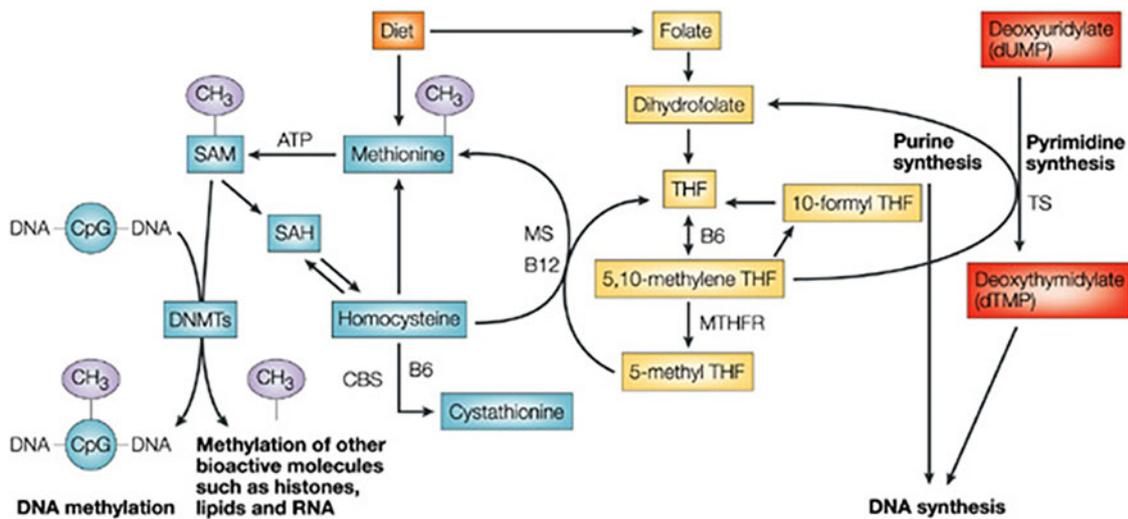

Fig. 2. (Colour online) Role of folate in C_1 metabolism supplying methyl groups for multiple purposes (from [11]). SAM, S-adenosyl methionine; THF, tetrahydrofolate; DNMT, DNA methyl transferase; SAH, S-adenosyl homocysteine; CBS, cystathionine-beta-synthase; MS, methionine synthase; MTHFR, methylene tetrahydrofolate reductase; TS, thymidylate synthase.

relatively large doses of folic acid in reducing both NTD recurrence[6] and occurrence[7]. These ground-breaking randomised controlled trials 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 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 C_1 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 NTD, CVD, 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 overnutrition 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 birth weight has been confirmed by Mendelian randomisation analysis supports a causal role for dysregulated C_1 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 C_1 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 postnatally 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 overnutrition postnatally. 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 RNA) 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 human subjects, Waterland et al. were the first to establish that the maternal environment around the time of conception influences the methylation status of ME 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 C_1 metabolism during pregnancy and these changes were reflected in altered methylation of ME 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 an 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 human subjects 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 human subjects 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 human subjects were confirmed for IGF2, GNASAS and IL10 by Waterland et al. who examined methylation patterns in post-mortem samples of brain, liver and kidney obtained from Vietnamese 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, ME 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
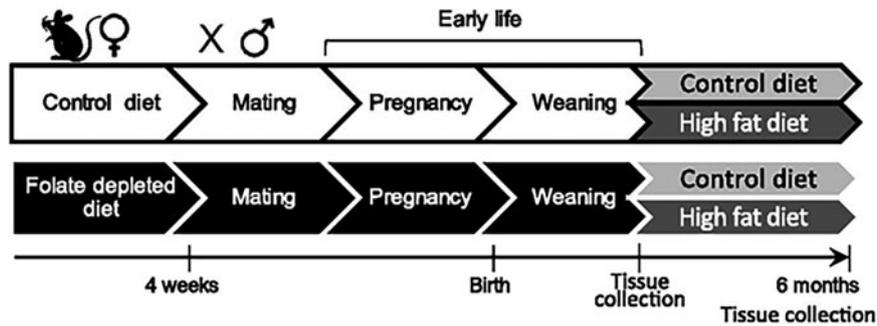
Fig. 3. Study design investigating effects of maternal folate depletion and high-fat feeding from weaning (from [28]).

of possible epigenetic mechanisms and to augment the epidemiological evidence available from human studies.

In recently completed work, we set out to test three hypotheses: Maternal folate depletion during pregnancy and lactation may contribute to the development of obesity and the Metabolic Syndrome; These effects may be exacerbated by provision of a high-fat (HF) diet post-weaning (double nutritional insult); 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 particularly 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 an HF 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 erythrocyte folate concentration measured. There were two distinct phases in erythrocyte folate kinetics; an initial phase lasting approximately 2 weeks during which erythrocyte folate concentration remained largely unchanged followed by exponential 'decay' in erythrocyte folate concentration towards a new equilibrium after 4–5 weeks[25]. For comparison, Leamon et al. reported that erythrocyte 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 erythrocyte 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
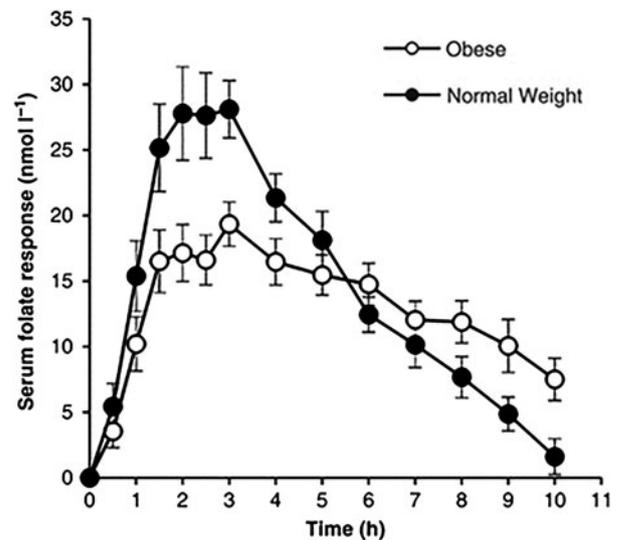

Fig. 4. Serum folate responses following ingestion of 400 μg folic acid in obese and normal weight women (from [34]).

(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 diet or an HF diet (50 and 200 g fat/kg diet, respectively) from weaning until age 6 months (Fig. 3)[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 HF diet were heavier and contained more body fat at ages 3 and 6 months than those randomised to the lower-fat (control) 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 off-spring from dams fed the folate-depleted diet had significantly raised plasma 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 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 C_1 metabolism. In particular, inadequate folate limits the availability of methyl groups for S-adenosylmethionine synthesis and this generates competition between cellular processes which use S-adenosylmethionine, notably the methylation of macromolecules including lipids, proteins and DNA. Further, we hypothesised that reduced S-adenosylmethionine 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, then 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.

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

Table 1. Percentages of women of child-bearing age with evidence of biochemical folate deficiency based on serum and erythrocyte folate concentrations (National Diet and Nutrition Survey rolling programme 2015)

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

* Percentage of women with serum folate concentration <10 nM[36].
† Percentage of women with erythrocyte folate concentration <340 nM[36].

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 human subjects, 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 for the absorption phase (0–3 h) and the peak serum folate concentration were both significantly lower in obese women following consumption of 400 μg 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 area-under-the-curve 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 an HF 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.

Conflicts of Interest

None.

Authorship

J. C. M. drafted this manuscript based on the lecture that he gave at the Nutrition Society Spring Conference on the 25–26 March, 2015 in Aberdeen. Both authors approved the final manuscript.

References

1. Roe DA (1978) Lucy Wills (1888–1964) A biographical sketch. *J Nutr* **108**, 1378–1383.
2. Wills L (1931) Treatment of 'pernicious anaemia' of pregnancy and 'tropical anaemia', with special reference to yeast extract as a curative agent. *BMJ* **1**, 1059–1064.
3. Wills L, Clutterbuck PW & Evans BDF (1937) A new factor in the production and cure of macrocytic anaemias and its relation to other haemopoietic principles curative in pernicious anaemia. *Biochem J* **31**, 2136–2147.
4. Angier RB, Boothe JH, Hutchings BL *et al.* (1945) Isolation of an antianemia factor (vitamin Bc conjugate) in crystalline form from yeast. *Science* **102**, 2644–2645.
5. Hibbard BM (1964) The role of folic acid in pregnancy; with particular reference to anaemia, abruption and abortion. *J Obstet Gynaecol Br Commonw* **71**, 529–542.
6. MRC Vitamin Study Research Group (1991) Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet* **338**, 131–137.
7. Czeizel AE & Dudas I (1992) Prevention of the first occurrence of neural-tube defects by periconceptual vitamin supplementation. *New Engl J Med* **327**, 1832–1835.
8. Pfeiffer CM, Caudill SP, Gunter EW *et al.* (2005) Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Examination Survey 1999–2000. *Am J Clin Nutr* **82**, 442–450.
9. De Wal P, Tairou F, van Allen MI *et al.* (2007) Reduction in neural-tube defects after folic acid fortification in Canada. *New Eng J Med* **357**, 135–142.
10. Locasale JW (2013) Serine, glycine and one-carbon metabolism in full circle. *Nat Rev Cancer* **13**, 572–583.
11. Lamprecht SA & Lipkin M (2003) Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. *Nat Rev Cancer* **3**, 601–614.
12. Clarke M, Ward M, Strain JJ *et al.* (2014) B-vitamins and bone in health and disease: the current evidence. *Proc Nutr Soc* **73**, 330–339.
13. Yajnik CS (2004) Early life origins of insulin resistance and type 2 diabetes in India and other Asian countries. *J Nutr* **134**, 205–210.
14. Yajnik CS, Chandak GR, Jogle C *et al.* (2014) Maternal homocysteine in pregnancy and offspring birthweight: epidemiological association and Mendelian randomisation analysis. *Int J Epidemiol* **43**, 1487–1497.
15. Krishnaveni GV, Veena SR, Karat SC *et al.* (2014) Association between maternal folate concentrations during pregnancy and insulin resistance in Indian children. *Diabetologia* **57**, 110–121.
16. Gluckman PD, Hanson MA & Spencer HG (2005) Predictive adaptive responses and human evolution. *Trends Ecol Evol* **20**, 527–533.
17. Vaiserman A (2015) Epidemiological evidence for association between adverse environmental exposures in early life and epigenetic variation: a potential link to disease susceptibility? *Clin Epigen* **7**, 96.
18. Mathers JC, Strathdee G & Relton CL (2010) Induction of epigenetic alterations by dietary and other environmental factors. *Adv Genet* **71**, 3–39.
19. Waterland RA & Jirtle RL (2003) Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol* **23**, 5293–5300.
20. Rakyan VK, Blewitt ME, Druker R *et al.* (2002) Metastable epialleles in mammals. *Trends Genet* **18**, 348–351.
21. Waterland RA, Kellermeyer R, Laritsky E *et al.* (2010) Season of conception in rural Gambia affects DNA methylation at putative human metastable epialleles. *PLoS Genet* **6**, e1001252.
22. Dominguez-Salas P, Moore SE, Baker MS *et al.* (2014) Maternal nutrition at conception modulates DNA methylation of human metastable epialleles. *Nat Commun* **5**, 3746.
23. Silver MJ, Kessler NJ, Hennig BJ *et al.* (2015) Independent genomewide screens identify the tumor suppressor *VTRNA2-1* as a human epiallele responsive to periconceptual environment. *Genome Biol* **16**, 118.
24. Moore SE, Cole TJ, Poskitt EM *et al.* (1997) Season of birth predicts mortality in rural Gambia. *Nature* **388**, 434.
25. McKay JA, Waltham KJ, Williams EA *et al.* (2011) Folate depletion during pregnancy and lactation reduces genomic DNA methylation in murine adult offspring. *Genes Nutr* **6**, 189–196.
26. McKay JA, Xie L, Harris S *et al.* (2011) Blood as a surrogate marker for tissue-specific DNA methylation and changes due to folate depletion in post-partum female mice. *Mol Nutr Food Res* **55**, 1026–1035.
27. Leamon CP, Reddy JA, Dorton R *et al.* (2008) Impact of high and low folate diets on tissue folate receptor levels and antitumor responses toward folate-drug conjugates. *J Pharmacol Exp Ther* **327**, 918–925.
28. Langie SA, Achterfeld S, Gorniak JP *et al.* (2013) Maternal folate depletion and high-fat feeding from weaning affects DNA methylation and DNA repair in brain of adult offspring. *FAEB J* **27**, 3323–3334.
29. McKay JA, Xie L, Manus C *et al.* (2014) Metabolic effects of a high-fat diet post-weaning after low maternal dietary folate during pregnancy and lactation. *Mol Nutr Food Res* **58**, 1087–1097.
30. Burdge GC & Lillycrop KA (2010) Nutrition, epigenetics and developmental plasticity: implications for understanding human disease. *Annu Rev Nutr* **30**, 315–339.
31. Mathers JC (2008) Session 2: personalised nutrition. Epigenomics: a basis for understanding individual differences? *Proc Nutr Soc* **67**, 390–394.
32. Public Health England (2015) National diet and nutrition survey supplementary report: blood folate. https://www.gov.uk/government/statistics/national-diet-and-nutrition-survey-supplementary-report-blood-folate (accessed 26 June 2015).
33. Health Survey for England – 2012 (2013) Adult anthropometric measures, overweight and obesity. http://www.hscic.gov.uk/catalogue/PUB13218/HSE-Ch10-Adult-BMI.pdf (accessed 26 June 2015).
34. da Silva V, Hausman DB, Kauwell GPA *et al.* (2013) Obesity affects short-term folate pharmacokinetics in women of childbearing age. *Int J Obes* **37**, 1608–1610.
35. Stern SJ, Matok I, Kapur B *et al.* (2011) A comparison of folic acid pharmacokinetics in obese and nonobese women of childbearing age. *Ther Drug Monit* **33**, 336–340.
36. WHO (2008) Conclusions of a WHO technical consultation on folate and vitamin B_{12} deficiencies. *Food Nutr Bull* **29**, S238–S244.