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Maternal nutritional status, C_1 metabolism and offspring DNA methylation: a review of current evidence in human subjects

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Evidence is growing for the long-term effects of environmental factors during early-life on later disease susceptibility. It is believed that epigenetic mechanisms (changes in gene function not mediated by DNA sequence alteration), particularly DNA methylation, play a role in these processes. This paper reviews the current state of knowledge of the involvement of C_1 metabolism and methyl donors and cofactors in maternal diet-induced DNA methylation changes *in utero* as an epigenetic mechanism. Methyl groups for DNA methylation are mostly derived from the diet and supplied through C_1 metabolism by way of choline, betaine, methionine or folate, with involvement of riboflavin and vitamins B_6 and B_{12} as cofactors. Mouse models have shown that epigenetic features, for example DNA methylation, can be altered by periconceptional nutritional interventions such as folate supplementation, thereby changing offspring phenotype. Evidence of early nutrient-induced epigenetic change in human subjects is scant, but it is known that during pregnancy C_1 metabolism has to cope with high fetal demands for folate and choline needed for neural tube closure and normal development. Retrospective studies investigating the effect of famine or season during pregnancy indicate that variation in early environmental exposure *in utero* leads to differences in DNA methylation of offspring. This may affect gene expression in the offspring. Further research is needed to examine the real impact of maternal nutrient availability on DNA methylation in the developing fetus.

DNA methylation: C_1 metabolism: DOHaD: Methyl donors

The 'Developmental Origins of Health and Disease' (DOHaD) hypothesis proposes not only that we are what we eat but also that we could be what our parents ate, and is a biologically and evolutionarily fascinating concept. The hypothesis postulates that early-life development is critically sensitive to inadequate nutrition and other environmental factors leading to permanent changes in metabolism that can alter susceptibility to complex diseases[(1)]. These early-life exposures can thereby be recorded and archived in the 'cellular memory', by inducing persistent adaptations in cellular function(s) with long-term effects[(2)]. Not only is this process of scientific importance, it also has relevance for public health, particularly in the framework of the twenty-first century pandemics of chronic diseases: the implication being that improvements in nutrition of one generation could prevent common complex diseases in future generations[(3)].

The foundations of the DOHaD theory were first formulated after Professor David Barker and colleagues from the MRC Environmental Epidemiology Unit in

Abbreviations: CpG, cytosine–guanidine; DOHaD, Developmental Origins of Health and Diseases; ME, metastable epialleles; SAH, S-adenosylhomocysteine; SAM, S-adenosyl-methionine.

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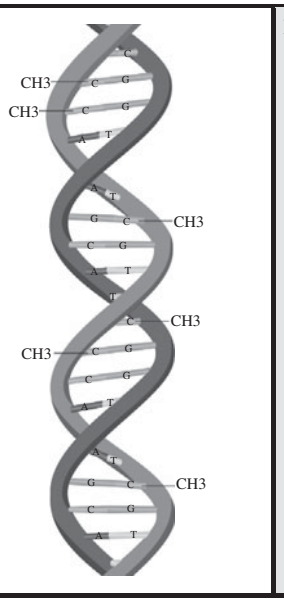

Box 1: Glossary

- Genome: The complete set of hereditary information possessed by an organism.
- Epigenetics: Changes in gene function not mediated by DNA sequence alteration
- Epigenome: The complete set of epigenetic information possessed by an organism.
- DNA methylation: The addition of methyl groups to the cytosine (C) in the DNA chain, thereby regulating gene expression.
- Imprinting: Parent-of-origin specific gene expression, whereby either the paternal or maternal allele is silenced.
- Metastable epiallele: DNA loci whose epigenetic state is independent of cell differentiation and which exhibit dynamic stochasticity.
- Epigenetic transgenerational inheritance: Epigenetic modifications that pass to the next generation through the gametes.

Fig. 1. DNA methylation.

Southampton (UK) studied retrospective data of geographical disease distribution and noticed that neonatal mortality was strongly associated with IHD rates in the UK[4]. Almost 30 years on, evidence has been extensively documented for a wide number of complex diseases including hypertension[5], type 2 diabetes[6] and cancers[7]. Yet, the mechanisms involved have not been fully elucidated.

This review discusses the current state of evidence that DNA methylation may be an important mediator of fetal programming in response to alterations in maternal diet and nutritional status during pregnancy, thus affecting later phenotypic outcomes.

The case for DNA methylation

The capacity for developmental plasticity likely requires many mechanisms to interact and current evidence suggests epigenetic mechanisms play an important role in this process[8]. Epigenetics refers to a number of mechanisms (namely DNA methylation, histone modification and RNA-related epigenetic marks) that establish layers of information in addition to DNA sequence information. They result in the 'epigenome' and lead to mitotically (and sometimes meiotically) replicable changes in gene expression potential that are not mediated by DNA sequence alteration[8]. These epigenetic mechanisms 'crosstalk' in an orchestrated manner to regulate gene expression throughout life[9].

Of all the epigenetic mechanisms so far described, DNA methylation is possibly the best characterised (Fig. 1). This process involves the covalent addition of a methyl group (CH_3) to the 5′ position of the pyrimidine ring of the cytosine base within cytosine–guanidine (CpG) dinucleotides, converting cytosine to 5-methylcytosine. This chemical modification alters the physical structure of the DNA, preventing DNA-binding proteins access during transcription processes, ultimately silencing the affected gene[10]. CpG are typically methylated. However, gene promoters contain CpG-rich DNA areas called 'CpG islands', which are usually unmethylated, highlighting the importance of DNA methylation in the regulation of gene expression[10,11]. Gene promoters are regulatory regions that contain transcription-factor binding sites to facilitate the transcription of the gene. Methylation in these regions acts as a gene expression-regulator switch, and thus can ultimately lead to phenotypic differences[12].

DNA methylation is catalysed by a number of DNA methyltransferases. The specific details of how these enzymes operate are unclear, but it is generally accepted that DNA methyltransferases 3a and 3b are mainly involved in *de novo* establishment of methylation patterns, whereas subsequent maintenance is guaranteed by different DNA methyltransferase 1 variants[13]. The role of DNA methyltransferase 2 remains undetermined[14].

For any mechanism to be a realistic candidate underlying the DOHaD hypothesis some premises should be met. It should be a molecular mechanism taking an active part in both prenatal development and the onset of a given disease. It should also be sensitive to environmental factors but have the ability to be stable over time. DNA methylation, as discussed in this review, meets these requirements.

Establishment of DNA methylation

Embryogenesis has been identified as a critical window in the establishment of the epigenome[8]. Gamete genomes are highly methylated when compared with somatic cells[11]. However, upon fertilisation, the newly formed zygote undergoes global demethylation, followed by *de novo* genome-wide methylation after implantation. New methylation patterns are established from pluripotent cells

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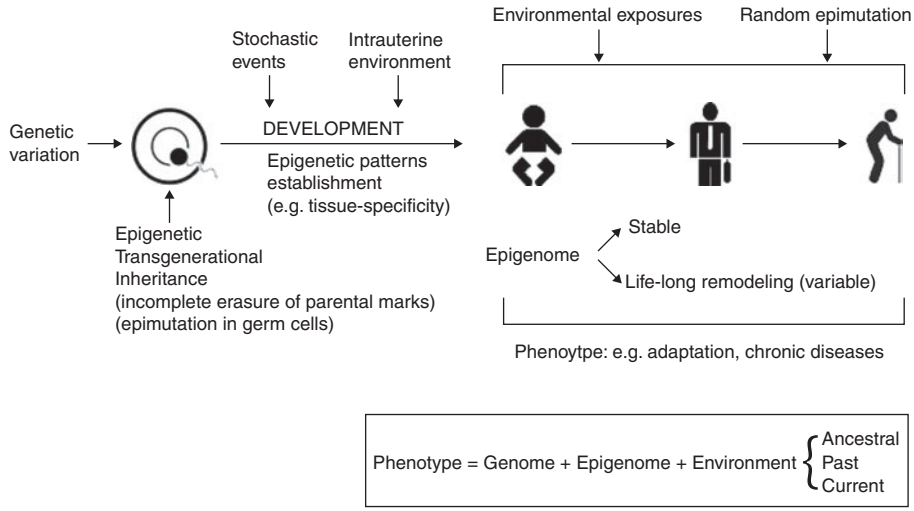

Fig. 2. Sources of individual epigenetic variation.

in a lineage-specific manner, through changes in gene expression leading to differentiation of organs and tissues[8]. The methylation patterns are perpetuated and propagated with high fidelity during rapid mitotic multiplication in fetal development[15]. However, the rules for the establishment of these patterns and the sources of individual epigenetic variation are not fully understood. It is believed that there are some genetically led patterns[16], while others are essentially stochastic[17] (Fig. 2). Timing is clearly critical but in human subjects the details remain largely undefined.

Classical examples of developmental DNA methylation are the X chromosome inactivation in women[18] and imprinting of genes (see box 1 in Fig. 1), such as the *IGF2* gene, where the maternal allele is suppressed (imprinted), while the paternal allele is activated (expressed)[19,20]. Imprinted genes play a critical role in regulation of placental and embryonic development, as well as in intrauterine growth and thus, early influences can have a substantial impact on human health later in life[10,21]. Imprinting marks are not erased during early embryonic development[22]. Misprogramming of the appropriate methylation patterns is associated with abnormal physical and mental development, including a number of 'epigenetic' developmental syndromes such as Beckwith–Wiedemann syndrome[23,24].

Large-scale erasure of methylation marks during early development limits the possibility of transgenerational inheritance, but this may occur through epigenetic marks that have failed to be erased before implantation or via epigenetic marks in germ line cells[25]. The vast majority of exposures alter only somatic cells but when epigenetic modifications occur in the germ line during embryonic gonadal sex determination, they become permanently programmed, and then the altered epigenome can appear in subsequent generations, in a sex-specific manner and in the absence of further environmental exposures[26,27].

Certain loci, known as metastable epialleles (ME), exist; their epigenetic state is independent of cell differentiation and they exhibit dynamic stochasticity[28]. At ME, epigenotype is established in a probabilistic manner in the early embryo and maintained thereafter in the differentiated cell lineages. This can lead to permanent phenotypic consequences, even in genetically identical individuals[29], and ME are thought to be particularly vulnerable to transient environmental influences[30].

The epigenome, unlike the genome, can be modified in response to interactions with environmental conditions within a lifetime. The epigenotype affects an individual response to a particular environment, and likewise the environment has the potential to change the epigenetic landscape, contributing to plasticity of phenotypes[8]. DNA methylation might explain how individuals can respond relatively quickly to changing external cues, such as deleterious compounds (e.g. tobacco or cooking smoke, arsenic or aflatoxins), infectious agents (e.g. *Helicobacter pylori*), or stress, either during development or throughout life[31,32]. Early DNA methylation alterations during cell-lineage differentiation tend to be irreversible while some later changes can be reversed[33,34].

DNA methylation depends specifically upon supply of dietary methyl groups, which are necessary throughout life to establish methylation patterns and to maintain these during repeated cycles of cell proliferation[35]. Once the epigenome is established, it is less responsive to external stimuli, but during early development, when tissue-specific patterns are undergoing establishment and maturation, the epigenome is sensitive to subtle changes[26]. Maternal nutritional deficiency or excess may thus result in permanent DNA methylation abnormalities (hypo- or hypermethylation) with the potential to affect gene expression[12].

In contrast to other epigenetic mechanisms, such as transient histone modifications, DNA methylation marks are chemically very stable and can be retained over time, thus potentially explaining long-term consequences for health. However, the global level of DNA methylation is thought to change over time and deteriorate due to oxidative stress and aging[36].

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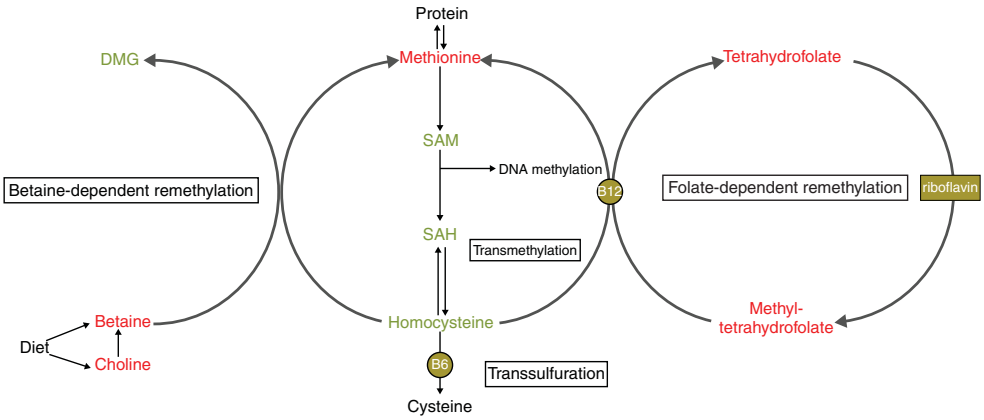

Fig. 3. (Color online) Diagram of C_1 metabolism. Methyl donors are shown in orange, functional biomarkers in green and cofactors are encircled. SAM, S-adenosyl-methionine; SAH, S-adenosylhomocysteine; DMG, dimethylglycine.

The role of epigenetic dysregulation and particularly of DNA methylation in the development of human disease has been increasingly recognised. Aging in human subjects is known to be associated with global hypomethylation, resulting in aberrant gene activation and age-related diseases[37,38]. Promoter-specific methylation has been associated with different diseases[39]. The most convincing evidence has been observed in relation to carcinogenesis[40,41], but there is also growing evidence that epigenetic dysregulation affects other life-course diseases such as CVD[42], type 2 diabetes[43] and obesity[44].

Taken together, such evidence supports DNA methylation as a plausible interface between the prenatal and early postnatal environment and adult disease risk.

Confirmatory evidence in animal models

Much of what is known to date is based on findings from animal studies. Murine models have provided direct evidence linking early-life programming through nutritional exposures with DNA methylation-mediated changes in gene expression, and hence phenotype[29]. Diets supplemented in methyl donors given to dams pre-conceptually and in pregnancy, can lead to permanent changes in gene regulation and to strong phenotypical modulation in the context of genetically identical inbred mice[45].

A classic example is the agouti viable yellow mouse, which has a yellow coat colour and is obese and hyperinsulinaemic[29]. This is caused by a dominant mutation of the agouti locus, caused by the insertion of a so-called IAP (intracisternal A particle) repeat element, which acts as an alternative promoter[46]. Such mobile elements render this genomic region epigenetically labile[29]. When this promoter is hypomethylated, it leads to expression of the agouti gene that regulates the production of yellow pigment and other pleiotropic effects including obesity. When it is silenced by methylation, the synthesis of yellow pigment is down-regulated and a pseudoagouti coat colour (darker) is produced[29]. Maternal methyl-donor supplementation (e.g. with folic acid, vitamin B_{12}, or betaine[45]) was confirmed to cause hypermethylation of the agouti viable yellow gene in the offspring, affecting offspring phenotype in a dose-dependent fashion. This genetic locus is considered an ME where methylation patterns are established stochastically within litter mates. Therefore, offspring present with a range of methylation at this locus and consequently variation in phenotypes, ranging from obese hyperinsulinaemic yellow, heavily to slightly yellow mottled and leaner non-hyperinsulinaemic pseudoagouti phenotypes. Other examples are known, such as the *Axin fused* gene that has a similar epigenetic behaviour, either leading to the expression or not of a kinked tail[47,48]. Furthermore, epigenetic transgenerational inheritance has been observed at ME in mice[49,50].

Research has also been conducted in sheep[51]. Ewes with restricted dietary methyl donors at physiological ranges, starting 8 weeks prior to conception until 6 d after conception, showed no effects in pregnancy establishment. However, offspring, and especially male descendants, had low weight at birth and were heavier and fatter in adulthood. They also had altered immune responses, were insulin resistant and had elevated blood pressure. Liver DNA methylation assessment showed altered widespread methylation status, in 4% of 1400 CpG islands examined. More than half of the affected loci were specific to males.

These animal models confirm the biological plausibility discussed earlier, and make it reasonable to think that similar processes could operate in human subjects.

Supply of methyl groups for DNA methylation

Methyl groups for fetal DNA methylation and development are provided through C_1 metabolism[52]. Pregnancy and lactation are times when methyl-donor supply is critical and demand for nutrients is higher[35]. The transfer of methyl groups depends ultimately on the availability of S-adenosyl-methionine (SAM). This is derived from methionine, which is converted through the action of methyltransferases to S-adenosylhomocysteine (SAH), while splicing off a methyl group and subsequently to homocysteine (Fig. 3).

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C_1 metabolism is characterised by a redundancy of pathways to conserve methionine that guarantees methyl-group availability, while removing unwanted homocysteine excess out of the system[53]. Two complementary remethylation pathways, one folate dependent, the other folate independent, intersect at the methionine cycle for the transformation of homocysteine into methionine. In the folate-dependent pathway, tetrahydrofolate is reduced to methyltetrahydrofolate by methyltetrahydrofolate reductase, with the intervention of riboflavin. Vitamin B_{12} catalyses the C_1 unit transfer from methyltetrahydrofolate to methionine by methionine synthase. The alternative folate-independent pathway is catalysed by the enzyme betaine homocysteine methyltransferase using betaine direct from the diet or from choline. In addition, homocysteine can be transformed into cysteine in certain tissues (liver, kidneys, pancreas, intestine and brain) by irreversible transsulphuration, in a process that requires vitamin B_6.

Clear interdependence exists between these two pathways, and perturbing the metabolism of any of the individual elements results in compensatory mechanisms via the alternative pathway[54] or in elevated plasma homocysteine concentrations[55,56]. The specific functioning of fetal C_1 metabolism is difficult to study, although it has been reported that the activities of methyltetrahydrofolate reductase and methionine synthase in preterm infant tissues were higher than those full-term or young children[57]. Therefore, prospective studies to investigate the role of maternal nutrition on the offspring DNA methylation need to be based on robust biomarkers for maternal C_1 metabolism.

Functional biomarkers of methylation capacity

Homocysteine, SAM, SAH and dimethylglycine are of metabolic origin and usually a good reflection of the overall status, i.e. excess or deficiency of substrates and cofactors within the C_1 metabolism[12,58]. DNA methylation is dependent on the balance between the substrate supply (SAM) and removal of the product (SAH), which is a potent inhibitor of SAM-dependent methyltransferase activity[59] and thereby methylation by way of a negative feedback loop. Hence, the clearing of SAH is the key for adequate methylation. The SAM:SAH ratio, sometimes called the 'methylation index', can be used as an indicator of the methylation potential of an individual[12].

Methylation regulation enzymes are differentially expressed in human tissues, leading to tissue-specific C_1 metabolism and thus tissue-specific homocysteine, SAM and SAH level regulation and methylation capacity[60]. For this reason, plasma SAM:SAH must be interpreted with caution and systemic SAM:SAH is not necessarily a meaningful indicator of tissue-specific methylation potential. Furthermore, efficiency of SAM transmembrane transport into the cells appears to be low[61]. Cells synthesise SAM from circulating methionine or homocysteine, as these cross the cell membrane easily. Thus, circulating homocysteine could be a better indicator of methylation potential[62]. Only the kidney appears capable of taking up SAH directly from plasma[63]. The transsulphuration of homocysteine to cysteine can alleviate SAM inhibition of methyltransferases[62].

Homocysteine is a non-protein-forming sulphur-containing amino acid that may exist free or bound to cysteine or albumin[64]. Elevated homocysteine is a good and well-studied indicator of C_1 metabolism disturbance and has consistently been associated with low concentrations of folate, vitamins B_{12} and B_6, choline and betaine[65–69]. Conversely, a meta-analysis of placebo-controlled trials of folic acid supplementation showed an average 25% reduction in homocysteine levels[70].

The Hordaland homocysteine study showed that high homocysteine concentrations were associated with risks of pre-eclampsia, premature delivery and low birth weight[71]. High homocysteine denoting aberrant methyl metabolism *in utero* is also linked with neural tube defects[72]. Usually, values of 5–15 μmol/l are considered normal for plasma homocysteine concentration, although different cut-off values have been used to define elevated concentrations[58]. There is a 30–60% decline in plasma homocysteine concentrations during pregnancy compared with non-pregnant women, due to various factors such as increased methionine requirements for fetal growth or changes in endocrine function[73,74].

Dimethylglycine is a by-product of choline–betaine metabolism, and also a derivative of glycine[75]. Plasma dimethylglycine levels appear to be lower in pregnant than in non-pregnant women (by 28%), and higher in fetal (2·44 μmol/l, SE 0·12) compared with maternal plasma (1·81 μmol/l, SE 0·12) as shown in a Canadian study[75].

Methyl donors

Methyl-group donors (also known as lipotropes) are all diet derived[76]. There is growing evidence that methyl donors are critical during pregnancy and that dietary excess or deficiency may have an impact on epigenetic programming in human subjects as in animals. Intake of folate and choline can be marginal during gestation and mismatch the biological requirements, leading to maternal depletion of stores and potentially to clinical deficiency[35]. The interactions between methyl donors for biological methylation and homocysteine removal make it difficult to separate their individual impact when studying reproductive outcomes. Furthermore, each of these methyl donors has specific roles in fetal development.

Folate is a B-vitamin (vitamin B_9), indispensable for the biosynthesis and repair of DNA and is a cofactor of numerous biochemical reactions[69]. Folate functions as a coenzyme and is key in the transfer of methyl groups[58]. Its function in normal neural tube closure in early gestation (21–28 d after conception in human subjects) has long been recognised[77]. Maternal supplementation with folic acid is implemented almost universally for prevention[78] and in some countries diet fortification has also been successful in reducing the incidence of neural tube defects[79]. In human subjects, maternal plasma folate is the main determinant of transplacental folate delivery to the fetus[80]. Blood folate in the fetus is several-fold higher than in the mother, and active transport occurs through a

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placental folate receptor. Plasma concentrations of folate fluctuate according to recent intakes and thus may reflect the effect of temporary changes in diet; low levels maintained over time indicate low folate intake and chronic depletion[58]. A cut-off point for deficiency of <7 nmol/l has been advised to prevent negative balance of folate[81], but higher levels above 16 nmol/l are required to reduce neural tube defects[82]. Pregnancy is associated with an increase in the demands of folate for fetal and uteroplacental organ growth[80]. Therefore, circulating folate concentrations decline during gestation in women who are not supplemented with folic acid[83], sometimes leading to overt folate deficiency. Furthermore, low folate in pregnant women does not appear to result in high plasma homocysteine suggesting effective homocysteine lowering mechanism(s) during pregnancy[84].

Choline is classified as part of the B-complex vitamins group, although it can be synthesised in the body from phosphatidylethanolamine. This *de novo* synthesis capacity, however, is limited[85]. Most men and postmenopausal women deprived of dietary choline develop symptoms of deficiency, including fatty liver or muscle damage[56,86]. Conversely, only about 44% of premenopausal women develop such problems in the absence of dietary choline because endogenous synthesis is upregulated by oestrogen[87]. Choline is also involved in the closure of the neural tube[88,89]. Most choline is oxidised by choline dehydrogenase to betaine to participate in C_1 metabolism[90]. Choline also has an important function later in pregnancy during neurogenesis of the fetal hippocampus, and deficiency of choline can have on visuospatial and auditory memory that persist in adulthood[91]. Folate appears to contribute to later brain development like choline and deficiency of either diminishes neurogenesis and increases neural cell death in the fetal brain[92,93]. Additionally, dietary choline can transform into acetylcholine for cholinergic neurotransmission, transmembrane signalling and lipid transport and metabolism or into phosphatidylcholine and sphingomyelin for cell membrane constitution and integrity[94].

Adequate levels of choline in plasma have not been defined as yet. Plasma choline has been reported to be up to 45% higher in pregnant compared to non-pregnant women[75,95] probably due to increased endogenous synthesis when oestrogen levels rise from 1 nmol/l to up to 60 nmol/l to support the higher demands[35]. Large amounts of choline are delivered to the fetus across the placenta, through a specific transporter-like protein[96], which may deplete the maternal stores[97]. Choline concentration in amniotic fluid is 10-fold greater than in maternal blood[98]. It has been estimated that about 60% of methyl groups are derived from choline, 20% from methionine and 10–20% from folate[76], indicating a central role of choline as a methyl donor.

Betaine conversion from choline primarily takes place in the liver and kidney and is irreversible[94]. Therefore, dietary betaine can potentially have a choline-sparing effect although not for all the functions of choline. Betaine is also an osmolyte that protects cells from environmental stress such as drought, high salinity or high temperatures[90]. It permits water retention in cells, thus protecting them from dehydratation. Plasma betaine is highly variable, in women typically 20–60 μmol/l in resting conditions[99]. Plasma betaine has been seen to decrease[75] in the first half of pregnancy, from 16·3 to 10·3 μmol/l and remains constant thereafter[100]. High concentrations of betaine are found in neonates, presumably linked to the high fetal-choline levels[101].

Methionine is a sulphur-containing amino acid, indispensable because human subjects cannot fix inorganic sulphur into organic molecules[64]. It is the precursor of cysteine. Cysteine cannot be used to synthesise methionine, but a derivative of cysteine, cystine, has a methionine-sparing effect and can replace approximately 70% of dietary requirements for methionine[102]. It has been observed that women with higher methionine intakes are at lower risk for neural tube defect affected pregnancies, irrespective of folate intake[103].

Enzyme cofactors

The main enzymatic cofactors in the C_1 metabolism cycle are the B-vitamins riboflavin, B_6 and B_{12}. These are essential and diet derived. They have all been shown to be associated with a reduction in neural tube-defect risk[104].

Riboflavin is an integral component of the coenzymes flavin mononucleotide and flavin-adenine dinucleotide; it catalyses numerous metabolic redox reactions, including energy production and production of pyridoxic acid from pyridoxal (vitamin B_6)[58]. During pregnancy the level of riboflavin carrier proteins in plasma increases, resulting in a higher rate of riboflavin uptake at the maternal surface of placenta and thus in transfer towards the fetus[105]. One of the most commonly used indicators for riboflavin status assessment is the erythrocyte glutathione reductase activity coefficient. Values of <1·2 have been traditionally considered to be adequate by representing complete tissue saturation; 1·2–1·4 is considered as low and >1·4 as deficient[69]; however, different cut-offs are often used by investigators.

Pyridoxine (vitamin B_6) and related compounds are involved, among others, in amino acid, lipid and glycogen metabolism, and neurotransmitter synthesis[58]. They are absorbed by passive diffusion. A cut-off point of 20 nmol/l has been proposed for plasma pyridoxal 5′-phosphate[69]. In pregnancy, pyridoxal 5′-phosphate is lower still in subjects with pre-eclampsia[106].

Cobalamin (vitamin B_{12}) is a cobalt-containing compound necessary for normal blood formation and neurological function[69]. Vitamin B_{12} is normally transported by transcobalamin II, produced in the liver and the placenta. The transfer from mother to fetus occurs via specific-receptor carriers[107]. Values between 148 and 220 pmol/l are considered subclinical deficiency of plasma vitamin B_{12} and values below this, clinical deficiency[82]. Levels are affected substantially by age[58].

Evidence for environmentally induced alterations in methylation patterns in human subjects

C_1 metabolism has been the subject of intense research, but concrete evidence of its involvement in DOHaD still needs

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to be built up systematically. The Pune Maternal Nutrition Study cohort in India has provided evidence of the importance of C_1 metabolism in fetal programming[108–110]. Low maternal levels of vitamin B_{12} (<150 pmol/l) correlated strongly with hyperhomocysteine levels (>15 μmol/l)[109] and predicted higher offspring adiposity and higher insulin resistance[108]. High levels of erythrocyte folate predicted increased body fat and insulin resistance too. Children from mothers with low vitamin B_{12} concentrations who were also folate replete were the most insulin resistant, possibly due to vitamin B_{12} disturbance of the methyl-group transfer for the folate-dependant pathway. The authors speculated about vitamin B_{12} trapping folate as 5-methyltetrahydrofolate, thereby preventing the generation of methionine from homocysteine and potentially DNA methylation, but this was not tested. Vitamin B_{12} and folate status at 18 weeks of pregnancy were more strongly associated than those at 28 weeks emphasising how critical these substances are in early-mid pregnancy[108]. Interestingly, lower folate and higher plasma homocysteine[110] were associated with a smaller newborn size suggesting caution with strategies based only on increasing fetal growth. Research is moving towards direct assessment of biomarker exposure(s) instead of employing birth weight as proxy, as the latter has a multifactorial origin and has proved to be a poor measure of exposure[111].

While, in recent years, different micronutrient trials have been conducted[112–114] that included folate and other cofactors (riboflavin and vitamins B_6 and B_{12}), these have so far only focused on short-term infant and adverse-pregnancy outcomes. Further investigations into DNA methylation patterns and longer-term follow up to explore disease risk will be important.

Comparable findings observed in animals of methyl-donor maternal supplementation inducing phenotypic changes mediated by DNA methylation are lacking in human subjects at present, but it is believed that similar mechanisms do take place. Some emerging evidence in human subjects has been provided by observational studies.

Monozygous twins are genetically identical; however, they can exhibit remarkable differences in susceptibility to diseases which may be affected by changes to DNA methylation. Thus, they offer a good opportunity for the study of epigenetics. Fraga *et al.*[36] observed that DNA methylation patterns in twins are indistinguishable at birth. This would be logical, as they share the same oviduct environment before implantation and although the nutrient supply might differ during placentation, it might be too late then to induce systemic epigenetic differences between twins[8]. Therefore, they are likely to establish very similar DNA methylation patterns. However, methylation patterns in twins diverge over their life-time[36], illustrating the plasticity of DNA methylation, probably under environmental pressure. Divergences can already be observed in early childhood[115].

Relevant to the DOHaD theory and illustrative of epigenetic programming was the first evidence of effects of the intra-uterine environment on DNA methylation, which comes from the 'natural experiment' of the Dutch Hunger Winter cohort[116]. This famine at the end of the Second World War provided a setting for the retrospective study of prenatal exposures: well-documented average daily rations dipping to 1673·6–3347·2 kJ/d (400–800 kcal/d), and detailed health care data on mothers. Long-term follow-up studies of individuals conceived during the famine have shown that prenatal undernutrition is associated with different adverse metabolic phenotypes, such as higher BMI, elevated serum cholesterol or impaired glucose tolerance and increased risk for insulin resistance, though this depends on the phase of development at exposure[116]. In an attempt to explain these observations, Heijmans *et al.*[117] tested for differences in DNA methylation of the *IGF2* gene locus comparing those exposed to periconceptional famine in early development with same-sex siblings conceived before or after the famine six decades later (Table 1). The authors found an average decrease of 5·2% in DNA methylation at this locus, thus suggesting that transient environmental conditions such as intra-uterine undernutrition can be recorded as persistent changes in the epigenome[117]. Interestingly, the association was only found when exposure was periconceptional but not later in gestation, highlighting the importance of timing of exposure. Steegers-Theunissen *et al.*[118] also looked at whether maternal folic acid periconceptional supplementation affected methylation of insulin-like growth factor 2 showing a 4·5% increase in methylation in individuals whose mothers had taken folate during early pregnancy.

The same group later tested a set of fifteen additional candidate genes and reported association of periconceptional undernutrition with DNA methylation for six of the genes[119]. Results for two genes indicated association with exposure during late gestation, thus suggesting that environmentally induced methylation changes are not limited to periconception (Table 1). Some of the associations were sex-dependent, in line with previous findings in human subjects[120] and sheep[51]. The differences in methylation were smaller than those seen for the insulin-like growth factor 2 locus and often showed an increase rather than an expected decrease in methylation with undernutrition. This is difficult to explain by deficiency in methyl donors, and thought to be part of an adaptative response[119]. These studies on the Dutch Hunger Winter cohort suggest that DNA methylation changes assumed to be established in early development can be persistent and may be frequent, but of relative small individual effect, implying that disease risk might entail a combination of multiple changes[2]. Interestingly, Tobi *et al.*[121] investigated (using the same cohort) whether the methylation level at defined loci was related to intrauterine growth restriction and children small for gestational age, both being phenotypes commonly used as measures of fetal environment, and found no association[121]. The authors concluded that these parameters may thus be associated with epigenetic changes in other loci not investigated or to non-epigenetic mechanisms.

To further our understanding in this area, Waterland *et al.*[122] set out to identify ME in human subjects. ME are more likely to be affected by environmental exposures and are not tissue-specific, which make it easier to analyse ME in human studies, through use of DNA from readily accessible peripheral blood samples as opposed to DNA from specific tissue samples. They designed a genome-wide methylation-specific analysis to screen for ME. Parallel

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Table 1. Effect of environmental (nutritional) exposure *in utero* on DNA methylation in human studies

Reference	Exposure	Timing of exposure	Sample size	Loci investigated	Effect observed	Timing of DNA-methylation measurement	Additional information
Heijmans *et al.*[117]	Famine (Hunger Winter in The Netherlands 1944–45) Average ration: 2790·72 kJ/d, 12% protein 19% fat 69% carbohydrates	Periconception	Sixty exposed Sixty control	IGF2	Hypomethylation at four CpG sites out of five Average difference = −5·2%, $P = 5{\cdot}9 \times 10^{-5}$	Six decades later	Results independent of sex Imprinted gene key in human growth and development
		Late gestation	Sixty-two exposed Sixty-two control	IGF2	No difference at five tested CpG site	Six decades later	
Tobi *et al.*[119]	Famine (Hunger Winter in The Netherlands 1944–45) Average ration: 2790·72 kJ/d 12% protein 19% fat 69% carbohydrates	Periconception	Sixty exposed Sixty control	GNASAS MEG3 IL-10 ABCA1 LEP INSIGF KCNQ1OT1 GRB10 GNASAB APOC1 IGF2R FTO CRH TNF NR3C1	1·1%, $P = 3{\cdot}1 \times 10^{-6}$ 0·5%, $P = 8 \times 10^{-3}$ 2·4%, $P = 1{\cdot}8 \times 10^{-6}$ 1·7%, $P = 8{\cdot}2 \times 10^{-4}$ 1·2%, $P = 2{\cdot}9 \times 10^{-3}$ −1·6%, $P = 2{\cdot}3 \times 10^{-5}$ NS NS NS NS NS NS NS NS NS	Six decades later	Sex differences in methylation Candidate genes for metabolic and CVD
		Late gestation	Sixty-two exposed Sixty-two control	GNASAS LEP IL-10 INSIGF KCNQ1OT1 APOC1 IGR2R CRH	−1·1%, $P = 1{\cdot}1 \times 10^{-7}$ Difference in men only ($P = 0{\cdot}017$) NS NS NS NS NS NS	Six decades later	Sex differences in methylation
Steeers-Theunisse *et al.*[118]	Folic acid supplementation intake (The Netherlands 2003–2007)	4 weeks after conception to 8 weeks after delivery	Eighty-six with FA Thirty-five without FA	IGF2	4·5%, $P = 0{\cdot}014$	Between 12 and 18 months after delivery	Methylation was associated with lower birth weight Imprinted gene key in human growth and development
Waterland *et al.*[122]	Seasonality (rainy season *v.* dry season, The Gambia, 1991, 1994, 1995, 1998)	Conception	Twenty-five per season	BOLA3 FLJ20433 PAX8 SLITRK1 ZFYVE28 LINE1 IGF2 GNASAS IL-10	>5%, $P = 0{\cdot}03$ <5%, $P = 0{\cdot}03$ >10%, $P = 0{\cdot}02$ <5%, $P = 0{\cdot}006$ >10%, $P = 0{\cdot}002$ Overall, $P = 0{\cdot}0001$ Non-significant control genes NS NS NS NS	Age: 8·9 years (0·5 SEM)	First description of human ME

aIGF2, insulin-like growth factor 2; CpG, cytosine–guanidine; ME, metastable epialleles.

screening of DNA from different tissues was used to exclude loci with tissue-specific methylation and discordance within monozygotic twin pairs provided support that inter-individual variation in methylation at the identified ME was stochastic. The establishment of ME in early development was then tested by studying differences in DNA methylation according to season of conception (dry/rainy) in rural Gambia (West Africa). Rural Gambia experiences dramatic seasonal fluctuations in food availability and maternal nutritional status, the rainy season being the most nutritionally challenged due to the high workload in the fields and the scarce stocks remaining from the previous harvest[123]. At the five loci investigated (Table 1), conception during the annual rainy season resulted in significantly higher DNA methylation, thus providing the first evidence of environmentally-associated changes in human ME. It is not clear whether these differences in DNA methylation in individuals conceived in the dry or the rainy season are mediated by seasonal differences in the availability or deficiency of methyl groups in the diet and maternal nutritional status. The differences in methylation by season were >10% for several of the loci and thus less subtle than those observed during the Dutch Hunger Winter studies[117, 119]. Since ME affect every cell type, they are more likely to be relevant to human disease. Notably, two of the five described genes, namely *PAX8* and *SLITRK1*, are known to be implicated in hypothyroidism and Tourette's syndrome, respectively[122].

Additionally, from the Swedish Overkalix cohort there are some hints of (male-line) transgenerational responses to nutrition in human subjects[124]. Food supply in adolescence of paternal grandparents correlated with the grandchild's longevity, including associations with risk of cardiovascular or diabetic death. There is currently no data on the mechanism(s) underlying these observations, however, epigenetic gametic inheritance may be a possible explanation[22].

From the studies outlined earlier several conclusions can be drawn. Human ME are likely to be susceptible to early environmental influences[122]. Differential methylation at other loci (e.g. *IGF2, GNASAS* or *IL-10*) (Table 1), not classified as ME, also appear to be affected by early nutritional exposures. Attention has to be given not only to the timing but also to the strength of the exposure. The Gambian study[122] investigated severe yet 'physiologically' mild differences in exposure as measured by season (which may or may not reflect maternal status), whereas in the Dutch studies[117] famine arguably is a more extreme exposure with regard to establishment of DNA methylation patterns in early development. It would be interesting to see whether the findings in Gambians can be reproduced in The Dutch Hunger Winter setting and whether there might be greater differences in methylation rate at the ME identified. Additionally, more data are needed in terms of accurate measurement of timing, dose and nutrient type(s) exposure periconceptionally or during pregnancy.

Conclusions

Epigenetics is still a recent and intricate science, where much is known but even more is yet to be determined. DNA methylation and other epigenetic mechanisms are becoming easier to study because of advances in technology, with the potential for providing a deeper understanding of complex diseases, even *in utero.*

There is an increasing body of evidence in animal models to suggest that many of the observed effects in fetal programming are mediated by epigenetic changes, but parallel evidence has to be further developed in human subjects. Furthermore, critical windows of exposure(s) that seem to exist during development have to be better defined, and also the balance between early acquired DNA methylation patterns as compared with ulterior modifications during life-course.

It is certainly a challenge to identify genomic regions that are likely to be more susceptible to methylation changes in response to prenatal environmental influences (human ME). Analogous to the development of the 'Human Genome Project', the 'Human Epigenome Project' aims to identify, catalogue and interpret DNA methylation patterns of all human genes in all major tissues (http://www.epigenome.org/). This and other research efforts will help understand epigenetic processes and their role in disease pathogenesis.

Redundancy in methyl-donor supply pathways as part of the C_1 metabolism means that changes in the level of one substance can potentially perturb the others through compensation mechanisms. Therefore, a comprehensive approach is needed when investigating these substances and how they affect DNA methylation.

All this needs to be taken into account in the design of new studies to better understand how maternal diet affects developmental epigenetics and the possible downstream consequences. Prospective studies, either observational or supplementation trials, should be designed, where accurate maternal nutritional status at specific times during pregnancy and the interactions between the different nutrients can be assessed in relation with DNA methylation. In addition, it will be important to link such basic research to measurable health effects in offspring phenotype, thus children need to be followed-up to ascertain the real impact of DNA methylation changes established during early development.

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