

# The periconceptual period, reproduction and long-term health of offspring: the importance of one-carbon metabolism

Régine P.M. Steegers-Theunissen<sup>1,2,\*</sup>, John Twigt<sup>1</sup>, Valerie Pestinger<sup>3</sup>, and Kevin D. Sinclair<sup>3,\*</sup>

<sup>1</sup>Department of Obstetrics and Gynaecology, University Medical Centre, Dr Molewaterplein 50, Rotterdam 3000 DR, The Netherlands, <sup>2</sup>Clinical Genetics, Erasmus MC, University Medical Centre, Rotterdam 3000 DR, The Netherlands and <sup>3</sup>School of Biosciences, University of Nottingham, Sutton Bonington, Leicestershire LE12 6HD, UK

Correspondence address. Tel: +31-10-7036886 (R.P.M.S.-T.)/+44-115-951-6053 (K.D.S.); Fax: +31-10-7036815 (R.P.M.S.-T.)/+44-115-951-6060 (K.D.S.); E-mail: r.steegers@erasmusmc.nl (R.P.M.S.-T.)/kevin.sinclair@nottingham.ac.uk (K.D.S.)

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**BACKGROUND:** Most reproductive failures originate during the periconceptual period and are influenced by the age and the lifestyle of parents-to-be. We advance the hypothesis that these failures can arise as a partial consequence of derangements to one-carbon (I-C) metabolism (i.e. metabolic pathways that utilize substrates/cofactors such as methionine, vitamin B<sub>12</sub>, folate). I-C metabolic pathways drive the synthesis of proteins, biogenic amines and lipids required for early growth, together with the synthesis and methylation of DNA and histones essential for the regulation of gene expression. We review how deficiencies in periconceptual I-C metabolism affect fertility and development together with underlying mechanisms derived from animal studies.

**METHODS:** A literature search was performed using PubMed and bibliographies of all relevant original research articles and reviews.

**RESULTS:** We define 'periconception' as a 5–6-month period in women embracing oocyte growth, fertilization, conceptus formation and development to Week 10 of gestation (coinciding with the closure of the secondary palate in the embryo). During this period significant epigenetic modifications to chromatin occur that correspond with normal development. Subtle variations in I-C metabolism genes and deficiencies in I-C substrates/cofactors together with poor lifestyle, such as smoking and alcohol consumption, disturb I-C metabolism and contribute to subfertility and early miscarriage and compromise offspring health. Procedures used in assisted reproduction can also disturb these metabolic pathways and contribute to poor pregnancy outcomes.

**CONCLUSIONS:** Evidence presented indicates that parental nutrition and other lifestyle factors during the periconceptual period can affect reproductive performance via I-C metabolic pathways. This knowledge provides opportunities for treatment and prevention of reproductive failures and future non-communicable diseases.

**Key words:** preconception care / fertility / B vitamins / folate / epigenetics

## Introduction

Problems of reduced reproductive health and fitness among more affluent human populations is largely attributable to advanced reproductive age, but are also due in part to changes in nutrition and lifestyle, particularly in low-income groups (Wong *et al.*, 2000, 2003; Baird *et al.*, 2005; Homan *et al.*, 2007; Hammiche *et al.*, 2011). Indeed, around 15% of couples planning pregnancy experience subfertility during the first year of unprotected intercourse. Furthermore, many couples experience reproductive failures comprising implantation failure (30%), early pregnancy loss (30%), clinical miscarriage (10%), congenital malformations in newborns (5%) and placental-related complications, such as fetal growth restriction (10%); and all of these originate during the periconceptual period (Macklon *et al.*, 2002; Steegers-Theunissen, 2010). Malnutrition is prevalent in many developed as well as developing countries and is known to disturb several metabolic pathways. Prominent among these are one-carbon (I-C) metabolic pathways (Stabler and Allen, 2004; Thuesen *et al.*, 2010). These pathways deliver methyl groups via the linked folate–methionine cycles for use in critical processes such as DNA synthesis and phospholipid and protein biosynthesis.

Unfortunately, awareness of these problems among the target group of women, but also among healthcare professionals, is poor. The prevalence and severity of impairments to these pathways are largely determined by complex interactions between subtle genetic variations and parental nutrition and lifestyle (Vollset *et al.*, 2001; Steegers-Theunissen and Steegers, 2003; Refsum *et al.*, 2006). Indeed derangements to I-C metabolism at different stages during the life course are believed to contribute to a broad range of clinical sequelae that include non-alcoholic fatty liver disease (Corben and Zeisel, 2012), various forms of cancer (Lee, 2009; Xu and Chen, 2009), vascular disease (Wald *et al.*, 2006), cognitive decline and dementia (Almeida *et al.*, 2008) and age-onset frailty syndrome (Matteini *et al.*, 2010). Set against this general background is the need to develop a greater understanding of the mechanisms by which derangements in I-C metabolism prior to and immediately following conception impinge on developmental processes associated with pregnancy establishment and long-term health. Here, valuable insights can be gained from intervention studies using various animal models, and from experiments involving *in vitro* culture of mammalian somatic and embryonic cells. Such models have been used extensively to study the role of folate in the pathogenesis and prevention of neural tube defects (NTDs; Greene *et al.*, 2009; Harris and Juriloff, 2010; De-Regil *et al.*, 2010).

Plasma homocysteine (Hcy) concentration is a sensitive marker of deranged maternal I-C metabolism. Evidence is accumulating of associations between elevated maternal Hcy and offspring born small for gestational age (SGA; Hogeveen *et al.*, 2012) and with congenital heart disease (Verkleij-Hagoort *et al.*, 2006). Because Hcy is also related to cardiovascular disease risk, the recent finding of a higher prevalence of cardiovascular disease in (great-) grandparents of families with children displaying

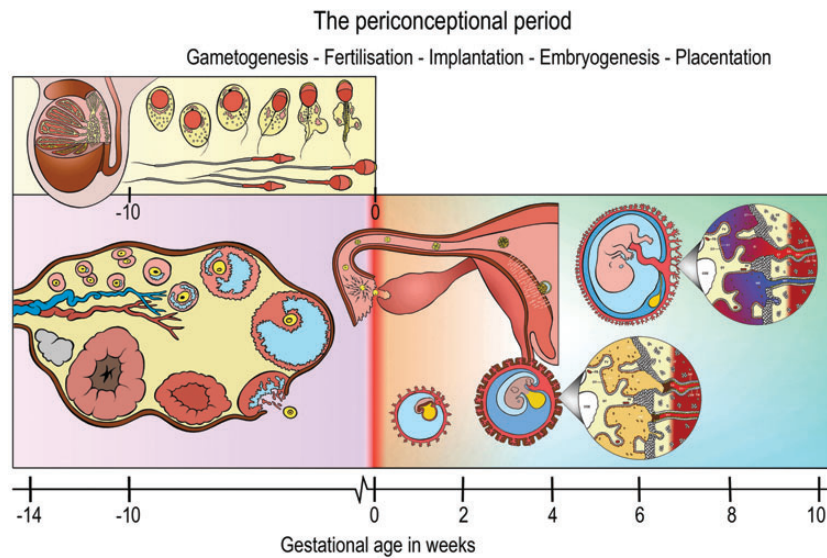
congenital heart disease is of concern (Wijnands *et al.*, 2012). Human studies associating maternal folate status with congenital malformations in offspring and placental-related complications, such as pre-eclampsia and fetal growth restriction, are summarized elsewhere (Twigt *et al.*, 2010). The current article, therefore, aims to provide a detailed and critical overview of the factors (e.g. nutrition, lifestyle, fertility treatment) that disturb I-C metabolism during the periconceptual period, and goes on to consider the implications that this can have for male and female fertility. It then goes on to consider longer-term effects, including developmental processes associated with pregnancy establishment and offspring health, and some of the underlying mechanisms associated with these effects drawing on evidence, where appropriate, from work conducted with animal models.

## Methods

This article provides a comprehensive review with reference to key studies in both the human and animal literature. Relevant studies were identified by searching PubMed using the following MeSH terms: 'Folic Acid'[Mesh], 'Pyridoxine'[Mesh], 'Vitamin B 12'[Mesh], 'Homocysteine'[Mesh], 'Methylenetetrahydrofolate Reductase (NADPH2)'[Mesh], 'Oocytes'[Mesh], 'Embryo, Mammalian'[Mesh], 'Placenta'[Mesh], 'DNA Methylation'[Mesh], 'Epigenomics'[Mesh], 'Epigenesis, Genetic'[Mesh], 'S-Adenosylmethionine'[Mesh], 'Oocyte'[Mesh], 'spermatozoa'[MeSH], 'spermatogenesis'[MeSH], 'assisted reproductive techniques'[MeSH], 'blastocyst implantation'[MeSH]. Articles were selected that, in the authors' view, constitute significant advances to our understanding of the topics covered by each of these MeSH terms.

## The periconceptual period

The periconceptual period has been a rather neglected and understudied stage of early human development, but has attracted greater attention in recent years (Steegers-Theunissen and Steegers, 2003; Steegers-Theunissen, 2010). This is partially due to the enhanced accessibility of sophisticated 3D-ultrasonography equipment (Rousian *et al.*, 2010, 2011). In the context of the current review, we base our definition of the periconceptual period on the physiology of reproduction and on the best implementation strategies for preconceptional care and research. We recognize that key but as yet poorly defined molecular events, including epigenetic modifications to DNA, occur in the oocyte during the extended period of ovarian follicular development. These can render the female gamete sensitive to external influences such as maternal nutrition and lifestyle. Biologically, the preconceptional phase may be considered to commence in women from around 26 weeks prior to conception when primordial follicles leave their resting state. However, because the most active phase of ovarian follicular development commences around 14 weeks preconception, we use this to define this as the preconceptional period (Griffin *et al.*, 2006; Fig. 1). This period is followed by the post-conceptual phase lasting through to 10 weeks after



**Figure 1** Definition of the periconceptual period in humans depicting the five stages of reproductive development considered in the current review set against their time course in weeks. The consecutive processes of spermatogenesis and spermiogenesis in men typically lasts around 10 weeks with final maturation of spermatozoa occurring in the epididymis. In women, primordial ovarian follicles leave their resting state around 26 weeks prior to terminal antral follicle maturation and ovulation, but extensive follicular growth (i.e. primary to late tertiary) commences from around 14 weeks preovulation (Griffin et al., 2006). Following fertilization, hatched blastocysts attach to the endometrial epithelium around Day 6 and the formation of the syncytiotrophoblast from the embryonic pole is observed from around Day 7 onwards. Implantation of the blastocyst is completed during the second week of embryonic development, although the process of placentation, involving the formation of the villous chorion and decidua basalis, continues for several more weeks. Gastrulation commences during the third week, giving rise to the three germ layers, and leads to the organogenetic period between Weeks 4 and 10. At the onset of this period the neural tube forms followed by development of all major organ systems, culminating in the closure of the secondary palate of the embryo by Week 10.

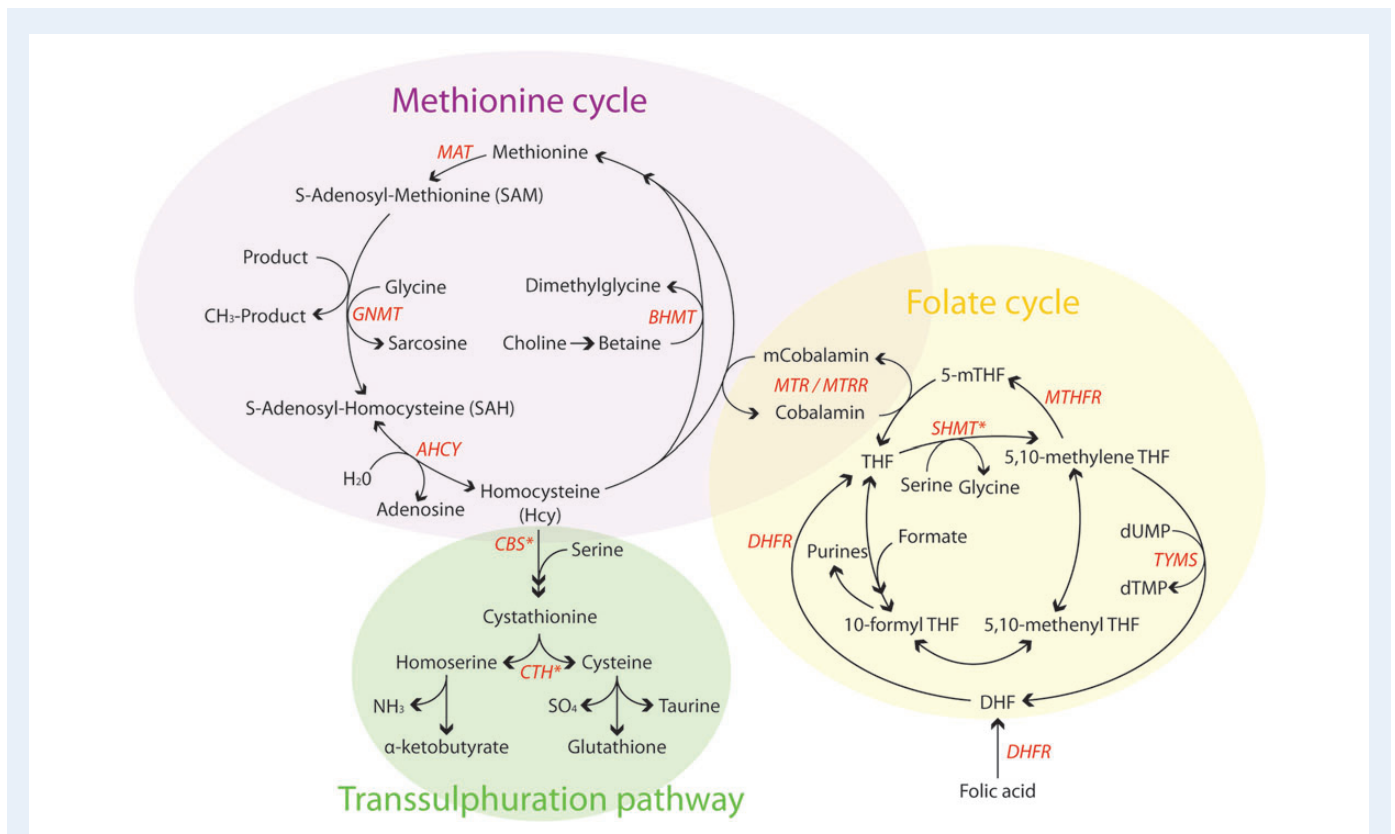
conception coinciding with the closure of the secondary palate of the embryo (Vermeij-Keers, 1990). In men, the analogous preconceptional phase would be around 10 weeks for the spermatogenic cycle. With regard to preconceptional counselling and interventions, it is important to communicate to clinicians and couples planning pregnancy that this period of human reproduction, so defined, is particularly sensitive to parental environment and diet. We therefore propose a period of 5–6 months as the periconceptual period in humans. In so doing we recognize that this represents a prolonged period of time and a broad range of biological processes that include gametogenesis, fertilization and early embryogenesis. Also, at present there is a lack of clinical data to identify the relative sensitivity of these separate stages of development to derangements in I-C metabolism, although later in this article we draw on emerging data from animal studies that provide important insights. Furthermore, as we discuss later in the article, dietary deficiencies in I-C substrates and cofactors are buffered to varying degrees by body reserves so that in clinical practice the sequence of pathophysiological changes that occur following micronutrient deprivation may take several weeks or indeed months to manifest.

Defining this period as we have, however, is important for the implementation of preconceptional care and research, and for communication with target groups of women and men, healthcare professionals, scientists and insurance companies. The importance of preconceptional nutrition and lifestyle came to prominence following the publication of data from randomized trials demonstrating the preventive effects of preconceptional folic acid use against NTDs in offspring (Locksmith and Duff,

1998). This led to the mandatory fortification of flour-based food products with folic acid in many countries, including the USA. In contrast, there has been resistance towards the introduction of such measures in many European states, where examples of potential detrimental effects of high folic acid are reported, such as a decreased natural killer cell cytotoxicity, a reduced response to antifolate drugs and an increased risk of insulin resistance in pregnant women and obesity in their offspring (Smith et al., 2008; Osterhues et al., 2009). Moreover, a combination of high folic acid and a low vitamin B<sub>12</sub> status is associated with an increased risk of cognitive impairment and anaemia, particularly in the elderly. However, in Europe, dietary inadequacies of folate and other B-vitamins prevail (Planells et al., 2003; Tabacchi et al., 2009; Gilsing et al., 2010), placing greater emphasis on the importance of preconceptional nutritional advice offered to couples planning pregnancy, and the recommendation of folic acid supplement use for mothers-to-be (Crozier et al., 2009; Pinto et al., 2009; Hammiche et al., 2011).

## Biochemistry and determinants of I-C metabolism

Here, we provide the briefest of overviews of the biochemistry that underlies I-C metabolism (Fig. 2) sufficient for the reader to follow the discussion that follows later in this article. More comprehensive reviews consider the biochemical aspects of these metabolic pathways in greater detail (Luccock, 2000; Stipanuk, 2004; Depeint et al., 2006;



**Figure 2** I-C metabolic pathways. Enzymes (in italic red): AHCY, S-adenosyl-L-homocysteine hydrolase; BHMT, betaine-homocysteine S-methyltransferase; CBS, cystathionine beta synthase; CTH, cystathionase (cystathionine gamma-lyase); DHFR, dihydrofolate reductase; GNMT, glycine N-methyltransferase; MAT, methionine adenosyltransferase; MTHFR, methylenetetrahydrofolate reductase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; SHMT, serine hydroxymethyltransferase; TYMS, thymidylate synthetase. Substrates: 5-mTHF, 5-methyl-tetrahydrofolate; DHF, dihydrofolate; dUMP, deoxyuridine monophosphate; dTMP, thymidine monophosphate; Hcy, homocysteine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate. \*CBS, CTH and SHMT are pyridoxine-dependent enzymes. Adapted from *Twigg et al. (2010)* in *Obeid et al. Vitamins in the prevention of human diseases*, with permission of Publishing House de Gruyter.

Loenen, 2006; Twigg et al., 2010). Critically, I-C metabolism delivers I-C moieties essential for the synthesis of DNA, polyamines, phospholipids, creatine and proteins, and methylation of DNA and histones via S-adenosylmethionine (SAM). Specific nutrients, such as choline (betaine), methionine, folate, vitamin B<sub>12</sub> (B12; cobalamin) and vitamin B<sub>6</sub> (B6; pyridoxine), act as substrates or cofactors in the synthesis and methylation of these substances.

The principal circulating form of folate in blood is 5-methyl-tetrahydrofolate (5mTHF), which serves together with choline as one of the most important I-C donors for the methionine cycle. The I-C moiety of 5mTHF is used by the cobalamin-dependent enzyme methionine synthase (MTR; EC 2.1.1.13) and methionine synthase reductase (MTRR; EC 2.1.1.135) in order to remethylate Hcy to methionine. These two enzymes essentially function as a unit where the latter enzyme (i.e. MTRR) regenerates a functional MTR from its inactive form (which arises following oxidation of its cob(II)alamin cofactor) via reductive methylation. Methionine is converted to SAM by methionine adenosyltransferase (MAT) encoded by three genes and comprises three principal isoforms (I, II and III; EC 2.5.1.6). Several methyltransferases use SAM as the I-C donor for methylation of lipids, proteins and chromatin. After transmethylation of SAM, S-adenosyl-homocysteine (SAH) is formed and reversibly hydrolysed

into Hcy and adenosine by SAH hydrolase (AHCY; EC 3.3.1.1). The optimum ratio of intracellular SAM:SAH is largely regulated by glycine N-methyltransferase (GNMT; EC 2.1.1.20). It facilitates the removal of excess SAM when SAM requirements for methylation reactions are met (Takata et al., 2003; Stipanuk, 2004). The folate-independent remethylation of Hcy is catalysed by betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5) utilizing I-Cs from the choline derivative betaine to remethylate Hcy. When methionine and folate levels are adequate, around 50% of Hcy is irreversibly transsulphurated into cystathionine, and cysteine and homoserine by the vitamin B<sub>6</sub> [pyridoxal-5'-phosphate (PLP)]-dependent enzyme cystathionine-β-synthase (CBS; EC 4.2.1.22) and cystathionine gamma-lyase (CTH; EC 4.4.1.1; Storch et al., 1990).

The conversion of 5mTHF into tetrahydrofolate (THF) provides I-Cs for the remethylation of Hcy to methionine after which THF is used in the folate cycle to provide I-C moieties for the synthesis of three of the four bases of DNA, i.e. guanine, adenine and thymine as well as for the synthesis of other compounds. Methylene-THF-reductase (MTHFR) is a key link between the methionine and folate cycles, because it determines whether 5,10-mTHF is utilized for 5-mTHF production or *de novo* synthesis of the DNA nucleotide precursor, thymidylate.



Finally, I-C metabolism is regulated by many of its substrates, cofactors and intermediates with the principal aim of maintaining optimal trans-methylation conditions so that, for example, when intracellular B12 levels are depleted, and thus MTR/MTRR activity is reduced, I-C moieties become trapped (the so-called methyl folate trap; Scott and Weir, 1981), leading to a decline in intracellular folates. Further examples of such regulatory processes are discussed elsewhere (e.g. Stipanuk, 2004).

## Nutrition and lifestyle

In Table I, the reference ranges for women not using vitamin supplements are depicted for the most important biomarkers of I-C metabolism in various fluids during the preconceptional, pregnancy and post-partum periods. Based on the available data on associations between plasma Hcy and reproductive outcomes, we classify homocysteinaemia as mild when the Hcy concentrations are between 9 and 15  $\mu\text{mol/l}$ , moderate when the concentrations are between 16 and 20  $\mu\text{mol/l}$  and severe when the concentrations are  $>20 \mu\text{mol/l}$ . Hcy concentrations of more than 100  $\mu\text{mol/l}$  are due to an extremely rare genetic defect also resulting in the excretion of very high Hcy concentrations in urine, i.e. classical homocystinuria ( $>400 \mu\text{mol/l}$ ; Kang et al., 1992; Welch and Loscalzo, 1998). At a very young age, these patients suffer from skeletal deformities, mental retardation, eye lens luxation, thrombosis, atherosclerotic complications and later reproductive failures.

Nutrition and lifestyle primarily, but also constitutional factors such as age, obesity, liver and renal function in combination with subtle variations in genes encoding I-C enzymes, can result in mild to moderate increases in plasma Hcy concentrations (Vollset et al., 2001). Dietary inadequacies in B vitamins are a growing problem in both developed and developing countries, leading to an increase in plasma Hcy of between 1 and 4  $\mu\text{mol/l}$  on average (McLean et al., 2008). Lifestyle factors, such as smoking, coffee and alcohol consumption, are also associated with

derangements in I-C metabolism (Refsum et al., 2006). Indeed, smoking and coffee consumption leads to average increases in plasma Hcy concentrations of between 1.5 and 2.0  $\mu\text{mol/l}$  independent of nutritional status. In chronic alcohol abusers, the elevation of plasma Hcy is much greater, but this is unknown in social alcohol users. It should be emphasized that although the effects of each of these separate 'lifestyle factors' on plasma Hcy are relatively small and without clinical symptoms, they often present together leading to mild to moderate increases (up to 30 and 50  $\mu\text{mol/l}$  Hcy) with clinical consequences in specific individuals. Observational studies reveal that multivitamin users have on average a 1.5–3.0  $\mu\text{mol/l}$  lower concentration of plasma Hcy. Genetic risk alleles, i.e. subtle genetic variations, in particular MTHFR 677C>T, together with ethnicity, gender, age, and liver- and renal dysfunction are considered constitutional factors that are also associated with moderate (16–20  $\mu\text{M}$ ) to severe ( $>20 \mu\text{M}$ ) hyperhomocysteinaemia (Nurk et al., 2004). Because of strong correlations among nutrition, lifestyle and constitutional factors, adjustments are important to avoid confounding in the assessment of environmental influences on I-C metabolism.

Independently of hyperhomocysteinaemia, global hypomethylation determined by a low SAM:SAH ratio has also been reported in subjects not taking B-vitamin supplements (Yi et al., 2000; Castro et al., 2003). Dietary folate is known to be a strong determinant of plasma Hcy. In contrast, body mass index (BMI) and body weight have been shown to be the strongest determinants of SAM and SAH (Van Driel et al., 2009). It remains to be shown, however, whether weight loss is a therapy by which I-C metabolism can be altered. These observations are consistent with the association of ageing and global DNA hypomethylation (Fraga and Esteller, 2007). It is clear that the aforementioned determinants can alter I-C metabolism and lead to mild to moderate hyperhomocysteinaemia by reducing the bioavailability of substrates, cofactors and intermediates. Hyperhomocysteinaemia in turn influences oxidative, vascular, apoptotic, inflammatory and methylation pathways, and protein, lipid and DNA

**Table I** Mean reference values (range or \*standard deviation) of biomarkers of I-C metabolism in several tissues without vitamin supplement use (Blood, Cikot et al, 2001; Follicular fluid, Steegers-Theunissen et al., 1993; Amniotic Fluid, Steegers-Theunissen et al., 1990, 1995, 1997; Imbard et al., 2013).

Tissue biomarker	Preconceptional	Pregnancy (Week)				Post-partum (Week)
Blood		6	10	20	32	6
Hcy, $\mu\text{M}$	12 (8–20)	12 (7–23)	10 (5–20)	9 (5–15)	10 (5–18)	15 (6–38)
Folate, nM	13 (7–24)	13 (7–23)	13 (7–22)	12 (7–20)	9 (5–17)	9 (5–18)
Vitamin B <sub>12</sub> , pM	296 (158–554)	260 (144–469)	240 (133–433)	199 (107–369)	164 (80–335)	310 (172–558)
Vitamin B <sub>6</sub> , nM		53 (30–76)	49 (28–70)	45 (23–67)	41 (15–66)	50 (17–82)
Follicular fluid						
Hcy, $\mu\text{M}$	7.4 (3.0–13.0)					
Folate, nM	16 (9.5–26.0)					
Vitamin B <sub>12</sub> , pM	200 (87–390)					
Vitamin B <sub>6</sub> , nM	36 (19–49)					
Amniotic fluid		8–12 weeks	14–18 weeks			
Hcy, $\mu\text{M}$		1.0 (0.5–1.7)	1.5 (1.1–2.4)			
Folate, nM			13 (6–27)			
Vitamin B <sub>12</sub> , pM			470 (70–1000)			
Vitamin B <sub>6</sub> , nM			14 (12*)			

synthesis (Van Mil *et al.*, 2010). Mild (9–15  $\mu\text{M}$ ) to moderate (16–20  $\mu\text{M}$ ) hyperhomocysteinaemia in later life is associated with cardiovascular disease, osteoporosis and Alzheimer's disease (Refsum *et al.*, 2006). In addition, a wide range of associations have been reported between folate, hyperhomocysteinaemia and reproductive failures, such as subfertility, polycystic ovary syndrome (PCOS), miscarriages, congenital malformations, fetal growth restriction, pre-eclampsia and cardiovascular disease later in life (Ebisch *et al.*, 2007; Twigt *et al.*, 2010; Toulis *et al.*, 2011; Wald *et al.*, 2011). From the available evidence, we propose that derangements in I-C metabolism during the periconceptual period can also lead to disturbances in gametogenesis, fertilization, implantation, embryogenesis and placentation with consequences for long-term health in current and future generations (Steegers-Theunissen and Steegers, 2003; Wijndans *et al.*, 2012).

In the clinical setting it is important to realize that, in most individuals, derangements in I-C metabolism, leading to hyperhomocysteinaemia, can be treated with B vitamins, especially synthetic folic acid, or a diet rich in vegetables and fruits (Brouwer *et al.*, 1999a, b). However, it should be noted that whilst such interventions can lower plasma Hcy concentrations, they may not always lead to improvements in clinical outcomes, which may instead be influenced by the residual non-responsive Hcy fraction (e.g. Ebbing *et al.*, 2010). Nevertheless, clinicians should address the (modifiable) determinants of I-C metabolism in the diagnosis and treatment of patients with reproductive failures, which in many cases will have consequences for health and disease risk in later life.

### Interim conclusions

Whilst separate effects of 'life-style' factors such as diet, BMI, smoking and alcohol consumption each lead to incremental (i.e. 1–4  $\mu\text{mol/l}$ ) increases in plasma Hcy concentrations, they often present together and therefore lead to moderate (>15  $\mu\text{mol/l}$ ) to severe Hcy levels (>20  $\mu\text{mol/l}$ ), particularly in individuals with genetic risk alleles. Such levels are associated with a range of reproductive problems including PCOS, recurrent miscarriages, malformations and pre-eclampsia. There is also emerging evidence that derangements to I-C metabolism, leading to hyperhomocysteinaemia, may disturb sperm and oocyte development, fertilization and preimplantation embryo development. Clinicians are therefore encouraged to address the modifiable determinants of I-C metabolism in the diagnosis of patients with reproductive failures.

## Assisted reproduction techniques

Biomarkers of I-C metabolism in human follicular fluid (FF) during *in vitro* fertilization (IVF) cycles have been detected with levels of folate and Hcy comparable with that found in blood, and lower levels measured for methionine, B12 and B6 (Table I, Steegers-Theunissen *et al.*, 1993). Studies have subsequently associated fertility and pregnancy outcome following assisted reproduction techniques (ART) with biomarkers of maternal I-C metabolism. Women harbouring specific subtle variants in key I-C enzymes, for example CC genotype of *MTHFR* 1298, are less likely to carry a pregnancy to term following ART (Haggarty *et al.*, 2006), although this improves when subjects take a daily supplement of 400  $\mu\text{g}$  folic acid (e.g. Dobson *et al.*, 2007). There is certainly scope to assess more fully the effects of polymorphic variants in other I-C genes on pregnancy outcomes following natural conception or ART. It

is also probable that procedures and culture media employed in ART disturb I-C metabolism in gametes and cleavage-stage embryos, and that this leads to epigenetic alterations to DNA and associated histone methylation in various developmentally important genes (Grace and Sinclair, 2009). Certainly, ovarian hyperstimulation in both the mouse and humans has been reported to alter the methylation status of several imprinted genes within the oocyte (Sato *et al.*, 2007; Market-Velker *et al.*, 2010a), although effects across studies are inconsistent. This may be due to the small and variable subsets of imprinted genes studied across experiments. Similarly, procedures such as IVF and intracytoplasmic sperm injection (ICSI) have been associated with loss of imprinting in animal models, although compelling evidence of similar effects in human embryos is lacking and frequently difficult to separate from confounding effects of maternal age and underlying causes of parental subfertility (Sinclair, 2008; Santos *et al.*, 2010). The variability of commercially available media is also of concern, particularly with respect to levels of I-C substrates and cofactors (Steele *et al.*, 2005). Recently, Market-Velker *et al.* (2010b) assessed the maintenance of DNA methylation for three imprinted genes in mouse zygotes cultured in five different commercially available media. Relative to *in vivo* controls, embryos cultured in all five media exhibited loss of imprinting to a greater or lesser degree. However, it was not possible to identify which media components contributed to these observations, and effects on the methylation status of non-imprinted loci were not investigated. However, effects of ovarian stimulation and culture were cumulative, resulting in increased disruption to genomic imprinting.

## Ovarian folliculogenesis, steroidogenesis and female fertility

Studies on associations between biomarkers of I-C metabolism and female fertility are scarce. We showed that FF Hcy levels were associated with endometriosis in subfertile women (Ebisch *et al.*, 2006). An intriguing observation was that Hcy (particularly that in FF) was dependent on folic acid supplement use. Hcy was negatively associated with the number of oocytes recovered, embryo quality and pregnancy outcome (Boxmeer *et al.*, 2008a, b, 2009). This has since been confirmed by others (Jerzak *et al.*, 2003; Pacchiarotti *et al.*, 2007). Consistent with these observations is the finding of an increased chance of pregnancy in couples undergoing IVF or ICSI and who adhere to a preconceptional 'Mediterranean-style' diet (i.e. a diet rich in I-C metabolites such as folate and B12 derived from vegetables, fruits and shellfish) or a highly adequate diet according to Dutch guidelines (Vujkovic *et al.*, 2010; Twigt *et al.*, 2012). This is important because maternal diet significantly affects I-C substrates in FF (Sinclair *et al.*, 2007; Boxmeer *et al.*, 2008a; Kanakkaparambil *et al.*, 2009). Women taking synthetic folic acid have increased FF folate and reduced Hcy (5.7–9.1 versus 7.8–19.1  $\mu\text{M}$ ; Twigt *et al.*, 2011). In contrast, in an animal model of deranged I-C metabolism, methionine, SAM, sarcosine, glutathione and taurine were reduced and Hcy increased (by 1.8-fold) in both FF and granulosa cells of ewes consuming a diet deficient in methionine, B12 and folate (Sinclair *et al.*, 2007; Kanakkaparambil *et al.*, 2009). Ovarian hyperstimulation in women is associated with reduced Hcy and other thiols in blood and FF (Boxmeer *et al.*, 2008b; Kralikova *et al.*, 2011). In these studies FF-Hcy was negatively correlated with follicular diameter, suggesting

that deranged I-C metabolism may impair ovarian follicle development. At present little is known about the putative effects of Hcy on ovarian folliculogenesis, and it is difficult to separate any specific effects of Hcy from other derangements of I-C metabolism. Recently, we have shown that physiological concentrations of Hcy (10–100  $\mu\text{M}$ ) added to culture media in the presence of FSH leads to a dose-dependent increase in ovine granulosa-cell proliferation associated with increased *FSHR* transcript expression (Kanakkaparambil et al., 2009). This may imply that Hcy enhances the sensitivity of granulosa cells to FSH. At present the mechanism by which Hcy increases *FSHR* expression is not known, although one current line of investigation concerns *FSHR* promoter-associated CpG island methylation. Altered methylation of cytosine residues in specific CpG dinucleotides within this region is known to regulate the transcription of *FSHR* in the rat (Griswold and Kim, 2001) and may do so also in other species by altering chromatin structure, thereby regulating the binding of transcription factors known to operate in this region (Xing et al., 2002; Xing and Sairam, 2002).

It follows that an increased sensitivity to FSH might also be expected to increase the number of antral follicles that respond to gonadotrophin treatment during controlled ovarian hyperstimulation, and this is what we have observed in both the sheep and humans (Kanakkaparambil et al., 2009; Twigt et al., 2011). Low dietary methionine (26  $\mu\text{M}$ ), B12 (230 pM) and folate (4.8 nM) in sheep and low dietary folate (17 nM) in women increased the number of estrogen-active antral follicles in response to 'conventional' ovarian hyperstimulation. In women, serum folate was negatively correlated with serum estradiol, whereas in sheep, granulosa-cell Hcy was positively associated with follicle number and FF-estradiol. Other than putative effects of I-C substrates and cofactors on *FSHR* expression, the mechanisms underlying associations between I-C metabolism and the selection of estrogen-active follicles remain obscure. Once again, however, an epigenetic effect cannot be discounted, as differential promoter-2-derived *CYP19* (aromatase cytochrome P450) expression in bovine granulosa and luteal cells is regulated by DNA methylation (Vanselow et al., 2005). In sheep increased antral follicle growth did not translate to an increased ovulation rate, which was similar to that of controls. Instead many large antral follicles failed to ovulate and became cystic.

To complicate matters further, transcriptional regulation of a number of genes encoding enzymes involved in the I-C pathway, e.g. *FOLR1* and *PEMT*, is sensitive to the prevailing steroid milieu (Resseguie et al., 2007; Sivakumaran et al., 2010). *FOLR1* encodes for folate receptor type alpha ( $\text{FR}\alpha$ ), which binds with high affinity ( $K_d < 10^{-9}\text{M}$ ) to 5-mTHF and facilitates cellular uptake. *FOLR1* is directly repressed by ligand binding of the hormone-bound estrogen receptor. In contrast, androgen, progesterone and glucocorticoid receptors activate *FOLR1* in response to ligand binding. Transcripts for *FOLR1* are expressed in germinal epithelium of the ovary (Elnakat and Ratnam, 2004), in human embryonic stem (ES) cells (Steele et al., 2005), and in oocytes and preimplantation embryos of all domestic animal species studied (Kwong et al., 2010; Pestinger and Sinclair, unpublished data). *PEMT* encodes for phosphatidylethanolamine *N*-methyltransferase (EC 2.1.1.17) and is intricately involved in choline metabolism, synthesizing phosphatidylcholine from phosphatidylethanolamine, where SAM acts as a methyl donor. Expressed predominantly in the liver (Vance and Ridgway, 1988), virtually nothing is known about its expression and activity within the testis and ovary, and in male and female germ cells. Nevertheless, *PEMT* in humans and mouse contains three estrogen response elements located in and

around the promoters and transcriptional start sites of this gene, the expression of which in primary hepatocytes is increased in response to 17- $\beta$  estradiol (Resseguie et al., 2007).

## Interim conclusions

In women and model animal species, follicular fluid levels of I-C metabolites (in particular Hcy) correlate with a range of reproductive disorders and pregnancy outcomes. In the context of assisted reproduction, ovarian responsiveness to gonadotrophin treatment is sensitive to levels of dietary folate, B12 and other I-C substrates and cofactors. Consequently, consideration should be given to the status of couples with respect to the provision of these essential micronutrients in the diet; although guidance concerning responses to specific stimulatory protocols and dietary supplements awaits further clinical investigation.

## Spermatogenesis, spermiogenesis and male fertility

Previous reviews of folic acid in human reproduction (Wong et al., 2000; Tamura and Picciano 2006; Ebisch et al., 2007; Twigt et al., 2010) noted that very little work had been undertaken to assess its effects on human male reproduction. An improvement in spermatozoa number and motility and a decrease in round cell number after a daily dose of 15 mg of folic acid in subfertile men were shown by Bentivoglio et al. (1993). In a randomized controlled trial, we showed a 74% increase in the total normal sperm count of subfertile men treated for 26 weeks with 5 mg of folic acid and 66 mg of zinc sulphate daily (Wong et al., 2002).

Dhillon et al. (2007) subsequently observed that plasma folate and B12 were decreased and Hcy was increased in subfertile men. Boxmeer et al. (2009) found that low seminal-plasma folate concentrations were associated with increased sperm DNA damage in fertile men. Most recently, however, Murphy et al. (2011) reported no association between serum folate, B12 and total Hcy concentrations with any of their semen quality parameters. Nevertheless, they did observe that polymorphic variants for two I-C-related genes were associated with male subfertility. The incidence of male subfertility was also increased in individuals homozygous for the *PEMT* variant M175C minor V allele and reduced in individuals heterozygous for the transcobalamin receptor gene variant rs172665. The literature related to associations between polymorphic variants of common I-C genes and semen quality or male fertility, however, is inconsistent. For example, whereas the common *MTHFR* C677T polymorphism was found to associate with idiopathic male subfertility in some study populations (Bezold et al., 2001; Safarinejad et al., 2011), the same mutation failed to associate with subfertility in other study populations (Ebisch et al., 2003; Dhillon et al., 2007), with perhaps an important difference in responsiveness between Asians and Caucasians being noted (Wu et al., 2012). These inconsistencies between studies probably reflect the limitation of investigating single or very few polymorphic variants in single or very few genes for complex traits in out-bred populations against often poorly defined nutritional backgrounds.

Whilst mice harbouring targeted deletions for specific I-C genes do not necessarily recapitulate the scenario where humans carry polymorphic variant(s) for those genes, they represent, nevertheless, a powerful model to investigate certain aspects of gene action and consequential metabolic and developmental outcomes. A case in point relates

to the aforementioned common polymorphic variant of *MTHFR* (i.e. C677T). The *MTHFR* enzyme has a dual function in regulating the flux of 1-C units into either methionine or dTMP and therefore DNA biosynthesis (Fig. 2; [Lucock and Yates, 2005](#)). In contrast to the *MTHFR* 677C variant, the *MTHFR* 677T variant favours the flux of 5,10-mTHF towards dTMP synthesis, thereby reducing uracil misincorporation into DNA in the presence of adequate levels of folate. The dual functionality associated with this polymorphism has important implications for cellular metabolism and development, which cannot be mimicked by targeted gene deletion. Nevertheless, studies in male BALB/c mice lacking *MTHFR* (*Mthfr*<sup>-/-</sup>) have demonstrated striking detrimental effects on spermatogenesis leading to subfertility ([Kelly et al., 2005](#)). The provision of betaine-supplemented water to dams throughout pregnancy and lactation, and to the pups following weaning, partially improved the sperm count and increased fertility significantly, confirming an important role of Hcy remethylation to methionine in male fertility. BALB/c *Mthfr*<sup>-/-</sup> mice were previously found to have altered SAM:SAH ratios and to exhibit global DNA hypomethylation in several tissues ([Chen et al., 2001](#)), and it is possible that similar epigenetic modifications to DNA methylation in developmentally important genes may have occurred in germ and somatic cells within the fetal and adult testis of this strain of mouse. A subsequent follow-up study by the same group revealed a milder phenotype in C57BL/6 *Mthfr*<sup>-/-</sup> mice, which had decreased sperm numbers but remained fertile ([Chan et al., 2010](#)). The acquisition of sex-specific DNA methylation within one maternally methylated and three paternally methylated imprinted genes in sperm was assessed but found to be unaltered. In contrast, genome-wide methylation of mature spermatozoa, determined by restriction landmark genome scanning (RLGS), revealed that, relative to *Mthfr*<sup>+/+</sup> mice, DNA methylation in a small subset of loci was altered in *Mthfr*<sup>-/-</sup> mice. Some of these loci were identified and found to be involved in cell cycle regulation. These studies, therefore, highlight a putative epigenetic-mediated mechanism to explain impaired male fertility following derangements to 1-C metabolism. Furthermore, differences in male fertility observed between different strains of *Mthfr*<sup>-/-</sup> mice recapitulate the random variation observed in out-bred human populations, highlighting the polygenic nature of this trait and the importance of polymorphic variants in 1-C genes other than *MTHFR*.

### Interim conclusions

Deficiencies in B-vitamin status in men lead to low sperm counts, particularly in subjects carrying risk allele variants. This can be partially treated by the diet and supplementation with appropriate B vitamins. Low sperm counts and poor sperm viability arises in part due to DNA damage, but emerging evidence in animal models also points to epigenetic alterations to DNA methylation, which may have long-term developmental consequences, although this awaits confirmation.

## Oocyte maturation and preimplantation embryo development

Elevated concentrations of Hcy in the FF of women undergoing IVF/ICSI treatment are associated with impaired oocyte and embryo quality ([Ebisch et al., 2006](#); [Sivakumaran et al., 2010](#)). Moreover, Hcy in the FF of subfertile women with endometriosis was higher than in women

with idiopathic subfertility (18.8 versus 9.2 nmol/mg protein; [Ebisch et al., 2006](#)). Hcy in blood is also frequently noted to be higher in women with polycystic ovarian syndrome (PCOS; 11–14 versus 7–10 μM for PCOS versus healthy women; [Schachter et al., 2003](#); [Kaya et al., 2009](#)) and, in such subjects, FF–Hcy concentrations are negatively associated with oocyte maturation and early embryo development ([Berker et al., 2009](#)). PCOS, however, is a complex metabolic and endocrine disease often presenting with anovulation and associated insulin resistance and hyperinsulinaemia ([Franks, 2006](#)). Negative effects on oocyte quality therefore cannot be attributed solely to Hcy as they could equally be attributed to insulin ([Adamiak et al., 2005](#)). Indeed, significantly positive correlations were found between all insulin-resistant indices and plasma Hcy in PCOS patients ([Schachter et al., 2003](#)). This arises because insulin inhibits the transcription of the gene encoding the rate-limiting transsulphuration enzyme, CBS (Fig. 2; [Ratnam et al., 2002](#)).

Transcripts for *Cbs* have been reported in cumulus cells of the mouse and increase following ovarian hyperstimulation ([Liang et al., 2007](#)). Given the function of *CBS*, expression of this enzyme in the ovary could account for the reduction in FF–Hcy in these women as discussed earlier. We have also detected low-abundance transcripts for *CBS* in all somatic cells within the ovary in the cow, in ovarian cells from other domesticated species, and in human ES and granulosa cells ([Steele et al., 2005](#); [Kwong et al., 2010](#); Pestinger and Sinclair, unpublished data). The functional suppression of *Cbs* in cumulus cells by RNA interference results in a significant increase in germinal vesicle-arrested oocytes in the mouse ([Liang et al., 2007](#)). Furthermore, *Cbs*<sup>-/-</sup> female mice are hyperhomocysteinaemic and infertile, although fertility is restored when *Cbs*-deficient ovaries are transplanted into normal ovariectomized females ([Guzman et al., 2006](#)). The latter observation highlights an important interplay between 1-C metabolism operating both systemically and locally within the ovary. Indeed, whilst considering consequences of maternal diet on the metabolic-regulation of developmental events occurring in germ cells within the ovary, and in the preimplantation embryo, one cannot lose sight of the fact that, in mammals, as much as 50% of methionine metabolism and up to 85% of all transmethylation reactions occur in the liver ([Mudd and Poole, 1975](#); [Finkelstein, 1990](#)). It follows that short-term nutritional studies timed to coincide with the periconceptual period in humans and animals are complicated by the fact that dietary deficiencies in 1-C substrates and cofactors are buffered to varying degrees by intra-cellular reserves stored in several tissue types ([Sinclair and Singh, 2007](#)). Consequently, the sequence of pathophysiological changes that occurs following micronutrient deprivation may take several weeks or months to manifest. In an attempt to partially overcome these limitations, [Anckaert et al. \(2010\)](#) cultured mouse pre-antral follicles for 12 days in standard control media (αMEM; Invitrogen), custom-made αMEM with methionine, folate, B12, B6 and choline removed, or custom-made αMEM with the aforementioned 1-C substrates and cofactors added back to match standard αMEM levels (i.e. methionine, 100 μM; B12, 1 μM; folic acid, 2.3 μM; B6, 5 μM; choline 7.2 μM). Antral follicular development and oocyte maturation were both impaired under 1-C-deficient conditions. Furthermore, the methylation status of a differentially methylated region (DMR) within one (i.e. *Mest*) out of four imprinted genes assessed was significantly reduced relative to that for oocytes derived under standard culture conditions. Whilst this study may be somewhat limited in scope (i.e. only four imprinted genes were assessed), and the artefacts



of *in vitro* culture, that includes serum and high levels of insulin, further limits one's ability to translate the findings into periconceptional nutritional recommendations, this study, nevertheless, sets an important precedent for the role of I-C substrates and cofactors in the development of the ovarian follicle-enclosed oocyte.

There is indirect evidence that the cytoplasm of the oocyte, which is by far the largest single cell in the mammalian body, may be an important source of I-C metabolites required for early preimplantation development. At present, however, no data are available on the content of I-C nutrients in oocytes. It is noteworthy, however, that during the preconceptional period, when the primordial follicle leaves its resting state and reaches the pre-ovulatory stage, oocyte volume increases 60–80-fold (depending on species). This constitutes a significant increase in mass and highlights the extent of cellular biosynthesis during this period which, in humans, can extend up to 26 weeks (Griffin et al., 2006). One can hypothesize, therefore, that maternal intake of folate and other I-C metabolites in the days and weeks leading up to conception may significantly affect the reserve of these nutrients in the oocyte and surrounding somatic cells of the peri-ovulatory follicle. This certainly merits further investigation.

The indirect evidence alluded to above, that the cytoplasm of the oocyte may be an important reserve of I-C metabolites, is supported by *in vitro* culture studies of mammalian zygotes in the presence of methotrexate (MTX), a structural folate antagonist known to reduce the intracellular content of reduced folates by competitively inhibiting DHFR. From a series of studies involving the culture of single cell mouse zygotes with varying doses of MTX (10-fold increases from 0.1 to 10 000  $\mu\text{M}$ ), either in the presence or absence of exogenous folic acid (up to 4  $\mu\text{g}/\text{ml}$ ) or thymidine (10-fold increases from 1 to 100  $\mu\text{M}$ ), O'Neill (1998) concluded that the cleavage-stage embryo has an absolute requirement for reduced folate, principally for thymidine synthesis, which can be met entirely by endogenous sources accumulated during oocyte growth. We recently extended these investigations to include the culture of bovine and ovine zygotes and observed development to occur unabated to the 8-cell stage in the presence of varying doses of MTX (0.5, 1 and 10  $\mu\text{M}$ ; Kwong et al., 2010). However, development beyond this stage was impeded, indicating that the reserve of reduced folates is exhausted by around the third cell cycle in embryos from these species. The inclusion of thymidine and the purine hypoxanthine rescued development of most embryos, although the blastocyst cell number was reduced. The addition of THF to media was without effect. We elected to add hypoxanthine because MTX, upon cellular uptake and polyglutamation, is also known to inhibit glycinamide ribonucleotide transformylase (EC 2.1.2.2) and aminoimidazole carboxamide ribonucleotide transformylase (EC 3.5.4.10) involved in purine synthesis (Chabner et al., 1985), and because earlier studies with human colon cancer cells indicated that MTX-induced differentiation was primarily due to intracellular deprivation of purines such as adenosine (Singh et al., 2006). Further evidence that MTX impeded embryo development by reducing intracellular pools of reduced folates came in the form of reduced uptake of [ $^{35}\text{S}$ ] methionine into intracellular SAM and SAH, and reduced uptake of both glutamate and tryptophan (the latter amino acid donates a formate group in the synthesis of 10-formyl-THF from THF; Kwong et al., 2010). It would seem, therefore, that the cleavage-stage embryo is heavily reliant on stores of reduced folates

acquired during the period of oocyte growth, although it also possesses carriers, e.g. FOLR1 and SLC19A1, required for folate uptake (Kwong et al., 2010). However, should it transpire that the preimplantation embryo is indeed largely reliant on oocyte reserves of I-C substrates and cofactors, this would emphasize the importance of preconceptional intake of foods, such as vegetables, fruits and nuts that are rich in I-C metabolites to intending mothers. It would also prioritize further animal and human studies to quantify directly the nature and level of reduced folates in oocytes from subjects receiving contrasting levels of dietary folate prior to oocyte collection. Data from such studies could then be related to measures of post-fertilization embryo development, pregnancy establishment and long-term well-being, and so provide direct evidence for the significance of preconceptional folate intake, and the relative importance of stores of reduced folates either of maternal or of oocyte origin.

The methionine requirements of cultured embryos were also recently investigated (Bonilla et al., 2010). A series of experiments working with a defined culture medium custom-formulated to exclude methionine revealed that the methionine requirement of the preimplantation bovine embryo is between 14 and 21  $\mu\text{M}$  in culture media. These requirements were based on assessments of zygote development to the blastocyst stage and on the blastocyst cell number, and are lower than the methionine levels in oviductal and uterine fluids (i.e. between 30 and 50  $\mu\text{M}$  (Hugentobler et al., 2007)). They are also much lower than levels found in many commercially available media (i.e. 112–200  $\mu\text{M}$ ) used to culture mammalian embryos. However, Bonilla et al. (2010) did not quantify methionine recycling via either BHMT or MTR nor did they assess compensatory fluxes through other components of the linked methionine–folate cycles (e.g. methylation of glycine to sarcosine by GNMT) (Fig. 2). The metabolic flux through these pathways can be adjusted in order to reduce cellular methionine requirements. Nevertheless, their values are consistent with our current thinking that methionine metabolism in germ and embryonic cells, together with the different somatic cell types within the ovarian follicle is relatively low, although quantitative requirements for methionine, especially in terms of the stoichiometry of associated reactions, have yet to be defined. Nonetheless, low methionine metabolism is evident from the complete lack of expression of BHMT, and no or very low expression of MAT1A in somatic cells of the bovine ovary, the oocyte and blastocyst (Kwong et al., 2010). These observations are being extended currently to include assessments in other model species and humans. As hepatic I-C metabolism is known to differ between ruminant and non-ruminant species (Snoswell and Xue, 1987), it follows that metabolic flux through these cycles in the ovary and in embryonic cells may also be species-specific.

## Interim conclusions

B-vitamin deficiencies in women, leading to elevated follicular fluid Hcy concentrations, impair oocyte quality and reduce success following assisted reproductive procedures such as IVF/ICSI. In animal studies, disturbances to I-C metabolism, arising either following genetic modification, or culturing oocyte/embryos in media containing non-physiological levels of I-C metabolites, are providing key insights into how disturbances to these metabolic pathways impair fertilization and preimplantation embryo development.

## Implantation

Methionine requirements during normal human pregnancy have been investigated, and the kinetics of its metabolism, including its rate of transmethylation and transsulphuration in women at different stages of gestation, have been quantified (Dasarathy *et al.*, 2010). Relative to the later stages of pregnancy, the first trimester was characterized as having a low rate of whole-body protein turnover and an elevated rate of transsulphuration of methionine. Relative hypoaminoacidaemia and hyperhomocysteinaemia (i.e. 5.1  $\mu\text{M}$ ; 1.2-fold increase) were also characteristic features of first relative to second and third trimester pregnancies. Collectively, these findings suggest a relatively high demand for methionine during the early stages of pregnancy driven perhaps by a high requirement for glutathione (GSH, discussed later).

In the context of periconceptional dietary requirements for I-C substrates and cofactors, however, it is worth noting that maternal adaptation to implantation begins during the proliferative phase of the menstrual cycle, commencing several days prior to ovulation and fertilization, and continues during the secretory phase, lasting around 13 days. The adaptive processes include decidualization of the endometrium where a significant degree of vascular remodelling and angiogenesis take place ahead of implantation (Plaisier, 2011). Human trophoblast invasion commences at around Day 8 following fertilization. These very early stages of blastocyst adhesion and subsequent invasion into the endometrial stroma are poorly characterized. Most studies are restricted to *in vivo* experiments in the mouse and *in vitro* experiments involving human endometrial explants, or co-culture models involving trophoblast and purified endometrial cell populations (Teklenburg and Macklon, 2009). The latter model systems have provided the most informative, albeit limited, data on the effects of derangements to I-C metabolism during the earliest stages of implantation.

*In vitro* studies of human cytotrophoblastic cells cultured in folate-free medium showed increased rates of apoptosis (Steegers-Theunissen *et al.*, 2000). Pathophysiological levels of  $\geq 20 \mu\text{M}$  Hcy, consistent with mild hyperhomocysteinaemia, induce a process of cell-detachment apoptosis in cultured human trophoblastic cells, which cease to secrete hCG; these effects can be alleviated by the addition of physiological levels of 20 nM folic acid (Di Simone *et al.*, 2003, 2004). Similarly, Hcy-thiolactone induces apoptosis in cultured primary human trophoblasts. This effect is attenuated following the addition of antioxidants such as ascorbic acid and *N*-acetyl-L-cysteine (Kamudhamas *et al.*, 2004). In contrast, culture of human primary extravillous trophoblast cells, derived from 7-week placental explants, with physiological concentrations of 10 nM folic acid increased trophoblastic cell proliferation and invasion of Matrigel-coated basement membranes (Williams *et al.*, 2011). The folic acid response was also associated with an increase in excretion of matrix metalloproteinases together with an increase in vascular density and a decrease in apoptosis. These direct interventional studies with cultured trophoblastic cells support previous observations that mild hyperhomocysteinaemia in women is associated with recurrent early pregnancy loss and defective chorionic villous vascularization (Steegers-Theunissen *et al.*, 1992; Nelen *et al.*, 2000).

The putative mechanisms by which these complex and varied biological effects arise involve excessive oxidative stress. It is known that interrelated reactive oxygen and nitrogen species, including nitric oxide, superoxide and peroxynitrite, participate in trophoblast invasion and determine subsequent vasculogenesis during normal placental

development. Only when oxidative and nitrate stress is heightened do these molecules lead to defective trophoblast invasion, characteristic of a number of pathological conditions including pre-eclampsia (Myatt, 2010; Burton and Jauniaux, 2011). Hcy is known to promote the production of both reactive oxygen and nitrogen species and to reduce activities of antioxidant enzymes such as superoxide dismutase, catalase and GSH peroxidase in cardiac and aortic endothelial cells (Lentz, 2005; Lubos *et al.*, 2007; Kolling *et al.*, 2011; Moreira *et al.*, 2011), and may do so also during endometrial stroma invasion by the syncytiotrophoblast. Hcy is known to bind to and antagonize peroxisome proliferator-activated receptor (PPAR)  $\alpha$  and  $\gamma$ . Induction of these two nuclear receptor proteins has been shown to attenuate oxidative nitrotyrosine production and oxidative stress in endocardial endothelial cells of *Cbs*<sup>-/+</sup> hyperhomocysteinaemic mice (Hunt and Tyagi, 2002; Tyagi *et al.*, 2011). Furthermore, whilst dietary cobalamin and folate can each serve to reduce circulating Hcy *in vivo*, these studies indicated that they may also serve as powerful antioxidants in their own right, sequestering superoxide and other reactive oxygen species.

There is interest among healthcare professionals in the concept that hypertensive disorders of pregnancy, including pre-eclampsia, may be linked to maternal I-C metabolism and other related micronutrients including long-chain omega-3 polyunsaturated fatty acids during early gestation (Garratt, 2009), although the experimental evidence is somewhat inconclusive (Tamura and Picciano, 2006; Oken *et al.*, 2007; Catov *et al.*, 2009; Thangaratinam *et al.*, 2011; Fowles *et al.*, 2012). For example, although there is some evidence that multivitamin use from conception is associated with a reduced incidence of pre-eclampsia, folic acid-only supplements appear to be less effective. Recently, Timmermans *et al.* (2011) reported lower uteroplacental vascular resistance following self-reported preconceptional folic acid use. However, the effects were small and not associated with the risk of hypertensive pregnancy disorders. Several recent case-control and prospective cohort studies, however, have associated elevated ( $\geq 6.9 \mu\text{M}$ ) maternal serum Hcy concentrations at different stages of pregnancy with pre-eclampsia and gestational hypertension (Braekke *et al.*, 2007; Dodds *et al.*, 2008; Acilmis *et al.*, 2011), where the absence of any relationship with maternal folate or vitamin B12 (Kaymaz *et al.*, 2011) could be due to interactions with the metabolism of long-chain polyunsaturated fatty acids (PUFAs) (Kulkarni *et al.*, 2011), although, once again, the protective effects of fish-oil-derived omega-3 PUFAs are equivocal (Oken *et al.*, 2007). There seems to be a general consensus, nevertheless, that whilst maternal Hcy is elevated in normotensive pregnancies that subsequently develop pre-eclampsia and Hcy increases further once pre-eclampsia is established, a direct causal relationship has yet to be established (Mignini *et al.*, 2005).

This conclusion is consistent with the limited data that have thus far emerged from animal studies. There are a number of hereditary and genetically modified mouse models of pre-eclampsia, e.g. endothelial nitric oxide synthase-deficient and catechol-*O*-methyltransferase-deficient mice, which have provided important insights into some of the mechanisms underlying hypertensive disorders of pregnancy, and confirm the utility of animal models (Ishida *et al.*, 2011). The aforementioned *Mthfr*<sup>-/+</sup> mouse on a BALB/c background becomes hyperhomocysteinaemic when fed a high-methionine/low-folate diet and presents with selective impairment of endothelium-dependent dilation of cerebral arterioles, but not aortic rings (Devlin *et al.*, 2004), and only modest impairment of acetylcholine-induced relaxation is seen in mesenteric

arteries recovered from late gestation pregnancies (Falcao et al., 2009). Maternal blood pressure was unaltered in that latter study and there was no increase in proteinuria which would be consistent with a disease phenotype. This does not rule out the possibility that either Hcy per se, or some related perturbation to I-C metabolism, e.g. SAH-induced inhibition of COMT activity (Shenoy et al., 2010), may contribute to the development of pre-eclampsia when other risk factors are present. Pre-eclampsia is a heterogeneous condition and, as discussed earlier, the consequences of targeted deletions in specific I-C genes vary between strains of mice.

### Interim conclusions

Hyperhomocysteinaemia ( $\geq 20 \mu\text{mol/l}$ ) in women is associated with recurrent early pregnancy loss and hypertensive pregnancy complications, including pre-eclampsia. *In vitro* studies with human primary cytotrophoblast and extravillous trophoblast cells implicate excessive oxidative stress and associated apoptosis as underlying contributory mechanisms that can be alleviated, in part, by addition of physiological levels of folic acid and antioxidants such as ascorbic acid. Results with folic acid and multivitamin use in several case-control and prospective cohort studies investigating hyperhomocysteinaemia during gestational hypertension, however, are less conclusive, highlighting the complex and heterogeneous nature of these conditions.

## Epigenetic programming and long-term development

The concept that maternal nutrition during pregnancy can impinge on the long-term well-being of offspring arose from retrospective cohort studies of Barker and Osmond (1986) in adult humans. However, it is the numerous animal studies conducted since then that have been most instructive in advancing our understanding of the mechanisms underlying this phenomenon. For example, the first demonstration that restricted dietary provision of I-C substrates and cofactors around the time of conception could lead to genome-wide epigenetic modifications to DNA methylation in offspring, with long-term implications for offspring health, was provided in sheep (Sinclair et al., 2007). In that study embryo-donor ewes were offered either a standard control or methyl-deficient diet, i.e. deficient in B12, methionine and folate, from 8 weeks prior and 6 days following conception by artificial insemination. Neither pregnancy rate nor birthweight was affected following single embryo transfer to normally fed surrogate ewes, but adult offspring were fatter, elicited altered immune responses to antigenic challenge, and were insulin resistant and hypertensive. In keeping with Low-Protein Diet studies in the rat (Kwong et al., 2000, 2006), these effects were most evident in male offspring. The altered methylation status of 4% of 1400 CpG islands examined by RLGS in the fetal liver revealed compelling evidence of a widespread epigenetic mechanism associated with this nutritionally programmed effect. Furthermore, more than half of the affected loci were specific to males. Strikingly, the same-sex-specific insulin-resistant phenotype was observed subsequently in a rat model of periconceptual methyl-metabolite restriction (Maloney et al., 2011). In that study, dams were offered either a nutritionally balanced semi-synthetic diet or one deficient in methionine, folate and choline from 3 weeks prior to mating and until Day 5 of gestation. Collectively, these intriguing observations point to conserved, sexually

dimorphic epigenetic programming during early preimplantation development and merit further investigation.

Our early investigations into the mechanistic basis of the 'large offspring syndrome' (LOS), which arises following the transfer of *in vitro* cultured embryos in farm animals, identified fundamental alterations in the allometric growth of key visceral organs (including the liver, heart and kidneys) in fetal sheep (Sinclair et al., 1999) associated with a loss of imprinting and expression of the gene encoding the type 2 insulin-like growth factor receptor (*IGF2R*; Young et al., 2001). This, in turn, arose as a consequence of a loss of methylation on the second intron DMR of that gene. Although we did not identify the specific constituents of culture media that contributed to the LOS, serum was an important component of media used at that time, and we subsequently demonstrated that its inclusion during zygote culture significantly altered the ratio of SAM:SAH and hence the transmethylation potential within blastocysts (Rooke et al., 2004). In this latter study, addition of  $\text{NH}_4\text{Cl}$  during culture also altered the ratio of SAM:SAH in granulosa cells, and this may be significant because elevated plasma ammonium and urea concentrations in zygote-donor ewes were negatively associated with *IGF2R* expression in the heart and kidney of LOS fetuses (Powell et al., 2006), indicating a putative involvement of nitrogen/urea metabolism in the aetiology of the LOS.

Continuing with the theme of imprinting, the feeding of a low-protein diet for just 4 days following natural mating in the rat led to post-natal hypertension in male offspring (Kwong et al., 2000), and a reduction in *H19* and *Igf2* transcript expression in fetal male, but not female, livers (Kwong et al., 2006), although *H19* transcript expression was not associated with altered methylation at its DMR in this latter study. Importantly, protein restriction in this rat model of developmental programming is associated with elevated plasma Hcy in the pregnant female during early gestation, which can be prevented when either glycine (Jackson et al., 2002) or folate (Lillicrop et al., 2005) is added to their diets. In contrast to the periconceptual rat studies cited above, Heijmans et al. (2008) reported reduced *IGF2* DMR methylation in genomic DNA from whole blood of human subjects who were prenatally exposed to famine during the Dutch Hunger Winter of 1944–1945 relative to their unexposed, same-sex siblings. Significantly, this only applied to those subjects who were exposed to famine as embryos during the first trimester of pregnancy and not as fetuses during the latter stages of gestation. More recently, periconceptual folic acid use of 400  $\mu\text{g/day}$  was shown to be associated with increased levels of *IGF2* DMR methylation in DNA extracted from whole blood of very young children (Steegers-Theunissen et al., 2009). Interestingly, *IGF2* DMR methylation in children from that study was correlated with the level of SAM in maternal blood.

Collectively, these and other studies have supported the long-held view that imprinted loci may be particularly susceptible to environmental influences and epigenetic programming, especially during the earliest stages of mammalian development. However, recent studies have challenged this tenet and it would seem that imprinted loci are neither more nor less susceptible to prenatal environmentally induced, epigenetic programming than non-imprinted loci (Tobi et al., 2009). Here, in human subjects participating in the aforementioned Hunger Winter Study in the Netherlands Tobi et al. assessed the methylation status of 7 imprinted genes (among which the *IGF2R* putative imprinted locus) and eight non-imprinted loci, that are involved in metabolic and cardiovascular disease. Following periconceptual famine exposure,

the methylation status of six loci, three imprinted and three non-imprinted, was altered in exposed individuals relative to that of their same-sex siblings, indicating that early environmental effects on the human epigenome may be more widespread than previously thought.

Indeed, the murine agouti gene represents an alternative non-imprinted locus susceptible to environmentally induced epigenetic regulation. The  $A^y$  allele contains an intracisternal A particle retrotransposon, the CpG methylation status of which is metastable and sensitive to the dietary provision of methyl groups. Working with the viable yellow agouti ( $A^y$ ) mouse, Waterland and Jirtle (2003) demonstrated that dietary supplementation with methionine, folate, B12, choline and betaine throughout gestation and lactation increased CpG methylation at the agouti locus, thereby recapitulating the mottled and healthy agouti phenotypes. At the time, these epigenetic effects were thought to have occurred, in part, during early embryo development because the  $A^y$  methylation status of tissues derived from the three germ layers was affected. Subsequent studies, however, indicated that both the embryonic and fetal environment can influence the epigenetic status of the  $A^y$  allele and thereby alter offspring phenotype (Cropley *et al.*, 2006; Morgan *et al.*, 2008). Other metastable epialleles sensitive to the provision of maternal methyl supplements during pregnancy exist in the mouse, e.g. *axin fused* ( $Axin^{Fu}$ ) (Waterland *et al.*, 2006), and recently several putative metastable epialleles have been identified in humans (Waterland *et al.*, 2010). DNA methylation at these loci was found to differ according to season of birth, i.e. rainy versus dry season in Gambia, and, because the inter-individual variation in methylation occurred in DNA from tissues representing all three germ layers, it provided evidence of a systemic effect of periconceptional diet/environment on the human epigenome. At present, however, the number of human metastable epialleles identified is few and the mechanisms that underlie epigenetic metastability are not understood. It is likely that many more such alleles will be identified in the future, and they certainly represent interesting putative candidates for diet-induced epigenetic programming of long-term development.

Finally, high-resolution genome-wide array and sequencing-based technologies are now increasingly being used to identify novel candidate loci associated with human metabolism and disease. By way of example, some recent studies investigating associations between specific I-C metabolites and pregnancy outcomes in humans have confirmed relationships between cord plasma Hcy concentrations, birthweight and DNA methylation in *LINE-1* sequences (Fryer *et al.*, 2009) and, more recently, with autosomal and X chromosome CpGs associated with consensus coding sequence genes (Fryer *et al.*, 2011). Functional characterization in this later study indicated that genes correlating with birthweight were associated with lipid metabolism, whereas those correlating with Hcy were more closely related to developmental processes. The relatively recent emergence of these high-resolution and high-throughput sequencing-based technologies promises major advances in our understanding of these complex relationships in the near future.

### Interim conclusions

There is emerging evidence from human studies to indicate that periconceptional derangements in I-C metabolism can lead to long-term epigenetic modifications to DNA methylation in children, which may extend beyond associated alterations in birthweight to affect long-term well-being, although this awaits further investigation. Animal studies,

however, indicate that periconceptional epigenetic programming of long-term health occurs, and that consideration should be given to both the effects of parental diet around the time of conception as well as to the metabolite composition of embryo culture media.

## General conclusions

The periconceptional period, encompassing a timeframe of 5–6 months around conception, is an important period in human life during which subfertility, miscarriage, congenital malformations, fetal growth restriction and placental-related disorders originate. This review presents accumulating evidence to indicate that derangements in I-C metabolism during this period are associated with reproductive failure. Specifically, poor nutrition relating to folate and B12, disturb I-C metabolism and contribute to these adverse outcomes. Of interest are findings that techniques used in ART can affect I-C metabolism in gametes and preimplantation embryos, indicating that further refinements to procedures and culture media are required. Emerging evidence also indicates that derangements in I-C metabolism can lead to epigenetic modifications to DNA methylation implicated in long-term programming of offspring health. This provides a mechanistic explanation for at least some of the associations between reproductive failure, age-related diseases and modifications induced by nutritional factors and lifestyle. For clinicians and scientists the periconceptional period, therefore, represents a timeframe in development worthy of further study, particularly with respect to the improvement of preventive care. This will lead to a reduction of perinatal morbidity and mortality, improvements in long-term health and well-being, and substantial reductions in healthcare costs.

## Authors' roles

R.P.M.S.-T. initiated and drafted the study. R.P.M.S.-T. and J.T. searched and summarized the literature of the human studies and mapped the biochemistry of the I-C pathway. K.D.S. and V.P. contributed by researching and summarizing the literature of animal and related human studies, and elaborated on the mechanistic aspects of relationships between derangements in I-C metabolism and clinical sequelae. K.D.S. co-wrote the final version with R.P.M.S.T. All authors contributed to the manuscript and approved the final version for publication.

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## References

- Acilims YG, Dikensoy E, Kutlar AI, Balat O, Cebesoy FB, Ozturk E, Cicek H, Pence S. Homocysteine, folic acid and vitamin B12 levels in maternal and umbilical cord plasma and homocysteine levels in placenta in pregnant women with pre-eclampsia. *J Obstet Gynaecol Res* 2011;1:45–50.
- Adamiak SJ, Mackie K, Watt RG, Webb R, Sinclair KD. Impact of nutrition on oocyte quality: cumulative effects of body composition and diet leading to hyperinsulinemia in cattle. *Biol Reprod* 2005;5:918–926.



- Almeida OP, McCaul K, Hankey GJ, Norman P, Jamrozik K, Flicker L. Homocysteine and depression in later life. *Arch Gen Psychiatry* 2008; **11**:1286–1294.
- Anckaert E, Romero S, Adriaenssens T, Smitz J. Effects of low methyl donor levels in culture medium during mouse follicle culture on oocyte imprinting establishment. *Biol Reprod* 2010; **3**:377–386.
- Baird DT, Collins J, Egozcue J, Evers LH, Gianaroli L, Leridon H, Sunde A, Templeton A, van Steirteghem A, Cohen J et al. ESHRE Capri Workshop Group. *Hum Reprod Update* 2005; **3**:261–276.
- Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* 1986; **8489**:1077–1081.
- Bentivoglio G, Melica F, Cristoforoni P. Folic acid in the treatment of human male infertility. *Fertil Steril* 1993; **60**:698–701.
- Berker B, Kaya C, Aytac R, Satioglu H. Homocysteine concentrations in follicular fluid are associated with poor oocyte and embryo qualities in polycystic ovary syndrome patients undergoing assisted reproduction. *Hum Reprod* 2009; **9**:2293–2302.
- Bezold G, Lange M, Peter RU. Homozygous methylenetetrahydrofolate reductase C677T mutation and male infertility. *N Engl J Med* 2001; **15**:1172–1173.
- Bonilla L, Luchini D, Devillard E, Hansen PJ. Methionine requirements for the preimplantation bovine embryo. *J Reprod Dev* 2010; **5**:527–532.
- Boxmeer JC, Brouns RM, Lindemans J, Steegers EA, Martini E, Macklon NS, Steegers-Theunissen RP. Preconception folic acid treatment affects the microenvironment of the maturing oocyte in humans. *Fertil Steril* 2008a; **6**:1766–1770.
- Boxmeer JC, Steegers-Theunissen RP, Lindemans J, Wildhagen MF, Martini E, Steegers EA, Macklon NS. Homocysteine metabolism in the pre-ovulatory follicle during ovarian stimulation. *Hum Reprod* 2008b; **11**:2570–2766.
- Boxmeer JC, Macklon NS, Lindemans J, Beckers NG, Eijkemans MJ, Laven JS, Steegers EA, Steegers-Theunissen RP. IVF outcomes are associated with biomarkers of the homocysteine pathway in monofollicular fluid. *Hum Reprod* 2009; **5**:1059–1066.
- Braekke K, Ueland PM, Harsem NK, Karlsen A, Blomhoff R, Staff AC. Homocysteine, cysteine, and related metabolites in maternal and fetal plasma in preeclampsia. *Pediatr Res* 2007; **62**:319–324.
- Brouwer IA, Dusseldorp van M, Thomas CMG, Duran M, Hautvast JGAJ, Eskes TKAB, Steegers-Theunissen RPM. Low-dose folic acid supplementation decreases plasma homocysteine: a randomized trial. *Am J Clin Nutr* 1999a; **69**:99–104.
- Brouwer IA, Dusseldorp van M, West CE, Meyboom S, Thomas CMG, Duran M, Van het Hof KH, Eskes TKAB, Hautvast JGAJ, Steegers-Theunissen RPM. Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. *J Nutr* 1999b; **129**:1135–1139.
- Burton GJ, Jauniaux E. Oxidative stress. *Best Pract Res Clin Obstet Gynaecol* 2011; **25**:287–299.
- Castro R, Rivera I, Struys EA, Jansen EE, Ravasco P, Camilo ME, Blom HJ, Jakobs C, Tavares de Almeida I. Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. *Clin Chem* 2003; **49**:1292–1296.
- Catov JM, Nohr EA, Bodnar LM, Knudson VK, Olsen SF, Olsen J. Association of periconceptional multivitamin use with reduced risk of preeclampsia among normal-weight women in the Danish National Birth Cohort. *Am J Epidemiol* 2009; **11**:1304–1311.
- Chabner BA, Allegra CJ, Curt GA, Clendeninn NJ, Baram J, Koizumi S, Drake JC, Jolivet J. Polyglutamation of methotrexate. Is methotrexate a prodrug? *J Clin Invest* 1985; **3**:907–912.
- Chan D, Cushnie DW, Neaga OR, Lawrance AK, Rozen R, Trasler JM. Strain-specific defects in testicular development and sperm epigenetic patterns in 5,10-methylenetetrahydrofolate reductase-deficient mice. *Endocrinology* 2010; **7**:3363–3373.
- Chen Z, Karaplis AC, Ackerman SL, Pogribny IP, Melnyk S, Lussier-Cacan S, Chen MF, Pai A, John SW, Smith RS et al. Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. *Hum Mol Genet* 2001; **5**:433–443.
- Cikot RJ, Steegers-Theunissen RP, Thomas CM, de Boo TM, Merkus HM, Steegers EA. Longitudinal vitamin and homocysteine levels in normal pregnancy. *Br J Nutr* 2001; **85**:49–58.
- Corben KD, Zeisel SH. Choline metabolism provides novel insights into non-alcoholic fatty liver disease and its progression. *Curr Opin Gastroenterol* 2012; **28**:159–165.
- Cropley JE, Suter CM, Beckman KB, Martin DI. Germ-line epigenetic modification of the murine A vy allele by nutritional supplementation. *Proc Natl Acad Sci USA* 2006; **46**:17308–17312.
- Crozier SR, Robinson SM, Borland SE, Godfrey KM, Cooper C, Inskip HM. SWS Study Group. Do women change their health behaviours in pregnancy? Findings from the Southampton Women's Survey. *Paediatr Perinat Epidemiol* 2009; **5**:446–453.
- Dasarathy J, Gruca LL, Bennett C, Parimi PS, Duenas C, Marczewski S, Fierro JL, Kalhan SC. Methionine metabolism in human pregnancy. *Am J Clin Nutr* 2010; **2**:357–365.
- Depeint F, Bruce WR, Shangari N, Mehta R, O'Brien PJ. Mitochondrial function and toxicity: role of B vitamins on the one-carbon transfer pathways. *Chem Biol Interact* 2006; **1–2**:113–132.
- De-Regil LM, Fernández-Gaxiola AC, Dowswell T, Peña-Rosas JP. Effects and safety of periconceptional folate supplementation for preventing birth defects. *Cochrane Database Syst Rev* 2010; **6**:CD007950.
- Devlin AM, Arning E, Bottiglieri T, Faraci FM, Rozen R, Lentz SR. Effect of MTHFR genotype on diet-induced hyperhomocysteinemia and vascular function in mice. *Blood* 2004; **7**:2624–2629.
- Dhillon VS, Shahid M, Husain SA. Associations of MTHFR DNMT3b 4977bp deletion in mtDNA and GSTM1 deletion, and aberrant CpG island hypermethylation of GSTM1 in non-obstructive infertility in Indian men. *Mol Hum Reprod* 2007; **4**:213–222.
- Di Simone N, Maggiano N, Caliendo D, Riccardi P, Evangelista A, Carducci B, Caruso A. Homocysteine induces trophoblast cell death with apoptotic features. *Biol Reprod* 2003; **4**:1129–1134.
- Di Simone N, Riccardi P, Maggiano N, Piacentani A, D'Asta M, Capelli A, Caruso A. Effect of folic acid on homocysteine-induced trophoblast apoptosis. *Mol Hum Reprod* 2004; **9**:665–669.
- Dobson AT, Davis RM, Rosen MP, Shen S, Rinaudo PF, Chan J, Cedars MI. Methylenetetrahydrofolate reductase C677T and A1298C variants do not affect ongoing pregnancy rates following IVF. *Hum Reprod* 2007; **2**:450–456.
- Dodds L, Fell DB, Dooley KC, Armon BA, Allen AC, Nassar BA, Perkins S, Joseph KS. Effect of homocysteine concentration in early pregnancy on gestational hypertensive disorders and other pregnancy outcomes. *Clin Chem* 2008; **2**:326–334.
- Ebbing M, Bønaa KH, Arnesen E, Ueland PM, Nordrehaug JE, Rasmussen K, Njølstad I, Nilsen DW, Refsum H, Tverdal A et al. Combined analyses and extended follow-up of two randomized controlled homocysteine-lowering B-vitamin trials. *J Intern Med* 2010; **268**:367–382.
- Ebisch IM, van Heerde WL, Thomas CM, van der Put N, Wong WY, Steegers-Theunissen RP. C677T methylenetetrahydrofolate reductase polymorphism interferes with the effects of folic acid and zinc sulfate on sperm concentration. *Fertil Steril* 2003; **5**:1190–1194.
- Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. *Hum Reprod* 2006; **7**:1725–1733.
- Ebisch IM, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. *Hum Reprod Update* 2007; **2**:163–174.
- Elnakat H, Ratnam M. Distribution, functionality and gene regulation of folate receptor isoforms: implications in targeted therapy. *Adv Drug Deliv Rev* 2004; **8**:1067–1084.
- Falcao S, Bisotto S, Gutkowska J, Lavoie JL. Hyperhomocysteinemia is not sufficient to cause preeclampsia in an animal model: the importance of folate intake. *Am J Obstet Gynecol* 2009; **2**:198.e1–198.e5.
- Finkelstein JD. Methionine metabolism in mammals. *J Nutr Biochem* 1990; **5**:228–237.
- Fowles ER, Walker LO, Marti CN, Ruiz RJ, Wommack J, Bryant M, Kim S, Timmerman GM. Relationships among maternal nutrient intake and placental biomarkers during the 1st trimester in low-income women. *Arch Gynecol Obstet* 2012; **4**:891–899.
- Fraga MF, Esteller M. Epigenetics and aging: the targets and the marks. *Trends Genet* 2007; **23**:413–418.
- Franks S. Genetic and environmental origins of obesity relevant to reproduction. *Reprod Biomed Online* 2006; **5**:526–531.
- Fryer AA, Nafee TM, Ismail KM, Carroll WD, Emes RD, Farrell WE. LINE-1 DNA methylation is inversely correlated with cord plasma homocysteine in man: a preliminary study. *Epigenetics* 2009; **6**:394–398.
- Fryer AA, Emes RD, Ismail KM, Haworth KE, Mein C, Carroll WD, Farwell WE. Quantitative, high-resolution epigenetic profiling of CpG loci identifies

- associations with cord blood plasma homocysteine and birth weight in humans. *Epigenetics* 2011; **1**:86–94.
- Garratt FN. Pre-eclampsia: a challenge to public health teams worldwide to ensure that maternal diets contain adequate levels of folic acid, n3 polyunsaturated fatty acids and vitamin D at conception. *Public Health* 2009; **1**:95–96.
- Gilsing AM, Crowe FL, Lloyd-Wright Z, Sanders TA, Appleby PN, Allen NE, Key TJ. Serum concentrations of vitamin B12 and folate in British male omnivores, vegetarians and vegans: results from a cross-sectional analysis of the EPIC-Oxford cohort study. *Eur J Clin Nutr* 2010; **9**:933–939.
- Grace KS, Sinclair KD. Assisted reproductive technology, epigenetics, and long-term health: a developmental time bomb still ticking. *Semin Reprod Med* 2009; **5**:409–416.
- Greene ND, Massa V, Copp AJ. Understanding the causes and prevention of neural tube defects: Insights from the splotch mouse model. *Birth Defects Res A Clin Mol Teratol* 2009; **85**:322–330.
- Griffin J, Emery BR, Huang I, Peterson CM, Carrell DT. Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig and human). *J Exp Clin Assist Reprod* 2006; **3**:2.
- Griswold MD, Kim JS. Site-specific methylation of the promoter alters deoxyribonucleic acid-protein interactions and prevents follicle-stimulating hormone receptor gene transcription. *Biol Reprod* 2001; **2**:602–610.
- Guzman MA, Navarro MA, Carnicer R, Sarria AJ, Acin S, Arnal C, Muniesa P, Surra JC, Arbones-Mainar JM, Maeda N et al. Cystathionine beta-synthase is essential for female reproductive function. *Hum Mol Genet* 2006; **21**:3168–3176.
- Haggarty P, McCallum H, McBain H, Andrews K, Duthie S, McNeill G, Templeton A, Haites N, Campbell D, Bhattacharya S. Effect of B vitamins and genetics on success of in-vitro fertilisation: prospective cohort study. *Lancet* 2006; **9521**:1513–1519.
- Hammiche F, Laven JS, van Mil N, de Cock M, de Vries JH, Lindemans J, Steegers EAP, Steegers-Theunissen RPM. Tailored preconceptional dietary and lifestyle counselling in a tertiary outpatient clinic in the Netherlands. *Hum Reprod* 2011; **9**:2432–2441.
- Harris MJ, Juriloff DM. An update to the list of mouse mutants with neural tube closure defects and advances toward a complete genetic perspective of neural tube closure. *Birth Defects Res A Clin Mol Teratol* 2010; **8**:653–669.
- Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, Slagboom PE, Lumey LH. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci USA* 2008; **44**:17046–17049.
- Hogeveen M, Blom HJ, den Heijer M. Maternal homocysteine and small-for-gestational-age offspring: systematic review and meta-analysis. *Am J Clin Nutr* 2012; **1**:130–136.
- Homan GF, Davies M, Norman R. The impact of lifestyle factors on reproductive performance in the general population and those undergoing infertility treatment: a review. *Hum Reprod Update* 2007; **13**:209–223.
- Hugentobler SA, Diskin MG, Leese HJ, Humpherson PG, Watson T, Sreenan JM, Morris DG. Amino acids in oviduct and uterine fluid and blood plasma during the estrous cycle in the bovine. *Mol Reprod Dev* 2007; **4**:445–454.
- Hunt MJ, Tyagi SC. Peroxisome proliferators compete and ameliorate Hcy-mediated endothelial cell activation. *Am J Physiol Cell Physiol* 2002; **4**:1073–1079.
- Imbard A, Blom HJ, Schlemmer D, Barto R, Czerkiewicz I, Rigal O, Muller F, Benoist JF. Methylation metabolites in amniotic fluid depend on gestational age. *Prenat Diagn* 2013; **23**:1–7.
- Ishida J, Matsuoka T, Saito-Fujita T, Inaba S, Kunita S, Sugiyama F, Yagami K, Fukamizu A. Pregnancy-associated homeostasis and dysregulation: lessons from genetically modified animal models. *J Biochem* 2011; **150**:5–14.
- Jackson AA, Dunn RL, Marchand MC, Langley-Evans SC. Increased systolic blood pressure in rats induced by a maternal low-protein diet is reversed by dietary supplementation with glycine. *Clin Sci (Lond)* 2002; **6**:633–639.
- Jerzak M, Putowski L, Baranowski W. Homocysteine level in ovarian follicular fluid or serum as a predictor of successful fertilization. *Ginekol Pol* 2003; **74**:949–952.
- Kamudhamas A, Pang L, Smith SD, Sadovsky Y, Nelson DM. Homocysteine thiolactone induces apoptosis in cultured human trophoblasts: a mechanism for homocysteine-mediated placental dysfunction? *Am J Obstet Gynecol* 2004; **2**:563–571.
- Kanakkaparambil R, Singh R, Li D, Webb R, Sinclair KD. B-vitamin and homocysteine status determines ovarian response to gonadotropin treatment in sheep. *Biol Reprod* 2009; **4**:743–752.
- Kang SS, Wong PW, Malinow PR. Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. *Ann Rev Nutr* 1992; **12**:279–298.
- Kaya C, Cengiz SD, Berker B, Demirtas S, Cesur M, Erdogan G. Comparative effects of atorvastatin and simvastatin on the plasma total homocysteine levels in women with polycystic ovary syndrome: a prospective randomized study. *Fertil Steril* 2009; **2**:635–642.
- Kaymaz C, Demir A, Bige O, Cagliyan E, Cimrin D, Demir N. Analysis of perinatal outcome by combination of first trimester maternal plasma homocysteine with uterine artery Doppler velocimetry. *Prenat Diagn* 2011; **31**:1246–1250.
- Kelly TL, Neaga OR, Schwahn BC, Rozen R, Trasler JM. Infertility in 5,10-methylenetetrahydrofolate reductase (MTHFR)-deficient male mice is partially alleviated by lifetime dietary betaine supplementation. *Biol Reprod* 2005; **3**:667–677.
- Kolling J, Scherer EB, da Cunha AA, da Cunha MJ, Wyse AT. Homocysteine induces oxidative–nitrosative stress in heart of rats: prevention by folic acid. *Cardiovasc Toxicol* 2011; **1**:67–73.
- Kralikova M, Melounova J, Crha I, Matejovicova M, Zakova J, Jarkovsky J, Tallova J. Intraindividual variability of homocysteine and related thiols concentrations in follicular fluid. *J Assist Reprod Genet* 2011; **9**:863–868.
- Kulkarni A, Mehendale S, Pisal H, Kilari A, Dangat K, Salunkhe S, Taralekar V, Joshi S. Association of omega-3 fatty acids and homocysteine concentrations in pre-eclampsia. *Clin Nutr* 2011; **1**:60–64.
- Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development* 2000; **19**:4195–4202.
- Kwong WY, Miller DJ, Ursell E, Wild AE, Wilkins AP, Osmond C, Anthony FW, Fleming TP. Imprinted gene expression in the rat embryo-fetal axis is altered in response to periconceptional maternal low protein diet. *Reproduction* 2006; **2**:265–277.
- Kwong WY, Adamiak SJ, Gwynn A, Singh R, Sinclair KD. Endogenous folates and single-carbon metabolism in the ovarian follicle, oocyte and pre-implantation embryo. *Reproduction* 2010; **4**:705–715.
- Lee SA. Gene-diet interaction on cancer risk in epidemiological studies. *J Prev Med Public Health* 2009; **6**:360–370.
- Lentz SR. Mechanisms of homocysteine-induced atherothrombosis. *J Thromb Haemost* 2005; **8**:1646–1654.
- Liang R, Yu WD, Du JB, Yang LJ, Yang JJ, Xu J, Shang M, Guo JZ. Cystathionine beta synthase participates in murine oocyte maturation mediated by homocysteine. *Reprod Toxicol* 2007; **1**:89–96.
- Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *J Nutr* 2005; **6**:1382–1386.
- Locksmith GJ, Duff P. Preventing neural tube defects: the importance of periconceptional folic acid supplements. *Obstet Gynecol* 1998; **91**:1027–1034.
- Loenen WA. S-adenosylmethionine: jack of all trades and master of everything? *Biochem Soc Trans* 2006; **34**(Pt 2):330–333.
- Lubos E, Loscalzo J, Handy DE. Homocysteine and glutathione peroxidase-1. *Antioxid Redox Signal* 2007; **11**:1923–1940.
- Lucock M. Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. *Mol Genet Metab* 2000; **1**:2:121–138.
- Lucock M, Yates Z. Folic acid - vitamin and panacea or genetic time bomb? *Nat Rev Genet* 2005; **3**:235–240.
- Macklon NS, Geraedts JP, Fauser BC. Conception to ongoing pregnancy: the black box of early pregnancy loss. *Hum Reprod Update* 2002; **8**:333–343.
- Maloney CA, Hay SM, Young LE, Sinclair KD, Rees WVD. A methyl-deficient diet fed to rat dams during the peri-conception period programs glucose homeostasis in adult male but not female offspring. *J Nutr* 2011; **1**:95–100.
- Market-Velker BA, Zhang L, Magri LS, Bonvissuto AC, Mann MR. Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner. *Hum Mol Genet* 2010a; **1**:36–51.
- Market-Velker BA, Fernandes AD, Mann MR. Side-by-side comparison of five commercial media systems in a mouse model: suboptimal in vitro culture interferes with imprint maintenance. *Biol Reprod* 2010b; **6**:938–950.
- Matteini AM, Walston JD, Bandeen-Roche K, Arking DE, Allen RH, Fried LP, Chakravarti A, Stabler SP, Fallin MD. Transcobalamin-II variants, decreased vitamin B12 availability and increased risk of frailty. *J Nutr Health Aging* 2010; **1**:73–77.

- McLean E, De Benoist B, Allen L. Review of the magnitude of folate and vitamin B12 deficiencies worldwide. *Food Nutr Bull* 2008;**29**:S38–S51.
- Mignini LE, Latthe PM, Villar J, Kilby MD, Carroli G, Khan KS. Mapping the theories of preeclampsia: the role of homocysteine. *Obstet Gynecol* 2005;**2**:411–425.
- Moreira ES, Brasch NE, Yun J. Vitamin B12 protects against superoxide-induced cell injury in human aortic endothelial cells. *Free Radic Biol Med* 2011;**4**:876–883.
- Morgan HD, Jin XL, Li A, Whitelaw E, O'Neill C. The culture of zygotes to the blastocyst stage changes the postnatal expression of an epigenetically labile allele, agouti viable yellow, in mice. *Biol Reprod* 2008;**4**:618–623.
- Mudd SH, Poole JR. Labile methyl balances for normal humans on various dietary regimens. *Metabolism* 1975;**6**:721–735.
- Murphy LE, Mills JL, Molloy AM, Qian C, Carter TC, Strevens H, Wide-Swennson D, Gwercman A, Levine RJ. Folate and vitamin B12 in idiopathic male infertility. *Asian J Androl* 2011;**6**:856–861.
- Myatt L. Review: reactive oxygen and nitrogen species and functional adaptation of the placenta. *Placenta* 2010;**31**(Suppl):S66–S69.
- Nelen WL, Bulten J, Steegers EA, Blom HJ, Hanselaar AG, Eskes TK. Maternal homocysteine and chorionic vascularization in recurrent early pregnancy loss. *Hum Reprod* 2000;**4**:954–960.
- Nurk E, Tell GS, Vollset SE, Nygård O, Refsum H, Nilsen RM, Ueland PM. Changes in lifestyle and plasma total homocysteine: the Hordaland Homocysteine Study. *Am J Clin Nutr* 2004;**5**:812–819.
- Oken E, Ning Y, Rifas-Shiman SL, Rich-Edwards JW, Olsen SF, Gillman MW. Diet during pregnancy and risk of preeclampsia or gestational hypertension. *Ann Epidemiol* 2007;**9**:663–668.
- O'Neill C. Endogenous folic acid is essential for normal development of preimplantation embryos. *Hum Reprod* 1998;**5**:1312–1316.
- Osterhues A, Holzgreve W, Michels KB. Shall we put the world on folate? *Lancet* 2009;**9694**:959–961.
- Pacchiarotti A, Mohamed MA, Micara G, Linari A, Tranquilli D, Espinola SB, Aragona C. The possible role of hyperhomocysteinemia on IVF outcome. *J Assist Reprod Genet* 2007;**10**:459–462.
- Pinto E, Barros H, dos Santos Silva I. Dietary intake and nutritional adequacy prior to conception and during pregnancy: a follow-up study in the north of Portugal. *Public Health Nutr* 2009;**7**:922–931.
- Plaisier M. Decidualisation and angiogenesis. *Best Pract Res Clin Obstet Gynaecol* 2011;**3**:259–271.
- Planells E, Sanchez C, Montellano MA, Mataix J, Llopis J. Vitamins B6 and B12 and folate status in an adult Mediterranean population. *Eur J Clin Nutr* 2003;**6**:777–785.
- Powell K, Rooke JA, McEvoy TG, Ashworth CJ, Robinson JJ, Wilmot I, Young LE, Sinclair KD. Zygote donor nitrogen metabolism and in vitro embryo culture perturbs in utero development and IGF2R expression in ovine fetal tissues. *Theriogenology* 2006;**8**:1901–1912.
- Ratnam S, Maclean KN, Jacobs RL, Brosnan ME, Kraus JP, Brosnan JT. Hormonal regulation of cystathionine beta-synthase expression in liver. *J Biol Chem* 2002;**45**:42912–42918.
- Refsum H, Nurk E, Smith AD, Ueland PM, Gjesdal CG, Bjelland I, Tverdal A, Tell GS, Nygård O, Vollset SE. The Hordaland Homocysteine Study: a community-based study of homocysteine, its determinants, and associations with disease. *J Nutr* 2006;**136**:1731S–1740S.
- Resseguie M, Song J, Niculescu MD, da Costa KA, Randall TA, Zeisel SH. Phosphatidylethanolamine N-methyltransferase (PEMT) gene expression is induced by estrogen in human and mouse primary hepatocytes. *FASEB J* 2007;**10**:2622–2632.
- Rooke JA, Ewen M, Mackie K, Staines ME, McEvoy TG, Sinclair KD. Effect of ammonium chloride on the growth and metabolism of bovine ovarian granulosa cells and the development of ovine oocytes matured in the presence of bovine granulosa cells previously exposed to ammonium chloride. *Anim Reprod Sci* 2004;**1**:2:53–71.
- Rousian M, Koning AH, van Oppenraaij RH, Hop WC, Verwoerd-Dikkeboom CM, van der Spek PJ, Exalto N, Steegers EA. An innovative virtual reality technique for automated human embryonic volume measurements. *Hum Reprod* 2010;**9**:2210–2216.
- Rousian M, Verwoerd-Dikkeboom CM, Koning AH, Hop WC, van der Spek PJ, Steegers EA, Exalto N. First trimester umbilical cord and vitelline duct measurements using virtual reality. *Early Hum Dev* 2011;**2**:77–82.
- Safarinejad MR, Shafiei N, Safarinejad S. Relationship between genetic polymorphisms of methylenetetrahydrofolate reductase (C677T, A1298C, and G1793A) as risk factors for idiopathic male infertility. *Reprod Sci* 2011;**3**:304–315.
- Santos F, Hyslop L, Stojkovic P, Leary C, Murdoch A, Reik W, Stojkovic M, Herbert M, Dean W. Evaluation of epigenetic marks in human embryos derived from IVF and ICSI. *Hum Reprod* 2010;**9**:2387–2395.
- Sato A, Otsu E, Negishi H, Utsunomiya T, Arima T. Aberrant DNA methylation of imprinted loci in superovulated oocytes. *Hum Reprod* 2007;**1**:26–35.
- Schachter M, Raziell A, Friedler S, Strassburger D, Bern O, Ron-El R. Insulin resistance in patients with polycystic ovary syndrome is associated with elevated plasma homocysteine. *Hum Reprod* 2003;**4**:721–727.
- Scott JM, Weir DG. The methyl folate trap. A physiological response in man to prevent methyl group deficiency in kwashiorkor (methionine deficiency) and an explanation for folic-acid induced exacerbation of subacute combined degeneration in pernicious anaemia. *Lancet* 1981;**8242**:337–340.
- Shenoy V, Kanasaki K, Kalluri R. Pre-eclampsia: connecting angiogenic and metabolic pathways. *Trends Endocrinol Metab* 2010;**9**:529–536.
- Sinclair KD. Assisted reproductive technologies and pregnancy outcomes: mechanistic insights from animal studies. *Semin Reprod Med* 2008;**26**:153–161.
- Sinclair KD, Singh R. Modelling the developmental origins of health and disease in the early embryo. *Theriogenology* 2007;**67**:43–53.
- Sinclair KD, McEvoy TG, Maxfield EK, Maltin CA, Young LE, Wilmot I, Broadbent PJ, Robinson JJ. Aberrant fetal growth and development after in vitro culture of sheep zygotes. *J Reprod Fertil* 1999;**1**:177–186.
- Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, Thurston A, Huntley JF, Rees WD, Maloney CA et al. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proc Natl Acad Sci USA* 2007;**49**:19351–19356.
- Singh R, Fouladi-Nashta AA, Li D, Halliday N, Barrett DA, Sinclair KD. Methotrexate induced differentiation in colon cancer cells is primarily due to purine deprivation. *J Cell Biochem* 2006;**1**:46–55.
- Sivakumaran S, Zhang J, Kelley KM, Gonit M, Hao H, Ratnam M. Androgen activation of the folate receptor alpha gene through partial tethering of the androgen receptor by C/EBPalpha. *J Steroid Biochem Mol Biol* 2010;**5**:333–340.
- Smith AD, Kim YI, Refsum H. Is folic acid good for everyone? *Am J Clin Nutr* 2008;**3**:517–533.
- Snoswell AM, Xue GP. Methyl group metabolism in sheep. *Comp Biochem Physiol B* 1987;**2**:383–394.
- Stabler SP, Allen RH. Vitamin B12 deficiency as a worldwide problem. *Annu Rev Nutr* 2004;**24**:299–326.
- Steeegers-Theunissen RPM. *Nieuw leven in een veranderende omgeving*. Oratiereeks Erasmus MC, 2010; ISBN 978-90-779-0672-9.
- Steeegers-Theunissen RPM, Steegers EAP. Nutrient–gene interactions in early pregnancy: a vascular hypothesis. *Eur J Obstet Gynaecol Reprod Biol* 2003;**2**:115–117.
- Steeegers-Theunissen RP, Steegers EA, de Boer R, Thomas CM, Kloosterman MD, Eskes TK. Elevated folate levels in amniotic fluid after oral supplementation. *Eur J Obstet Gynaecol Reprod Biol* 1990;**3**:283–291.
- Steeegers-Theunissen RPM, Boers GHJ, Trijbels JMF, Eskes TKAB. Hyperhomocysteinemia and recurrent spontaneous abortion or abruptio placentae. *Lancet* 1992;**339**:1122–1123.
- Steeegers-Theunissen RP, Steegers EA, Thomas CM, Hollanders HM, Peereboom-Stegeman JH, Trijbels FJ, Eskes TK. Study on the presence of homocysteine in ovarian follicular fluid. *Fertil Steril* 1993;**60**:1006–1010.
- Steeegers-Theunissen RP, Boers GH, Blom HJ, Nijhuis JG, Thomas CM, Borm GF, Eskes TK. Neural tube defects and elevated homocysteine levels in amniotic fluid. *Am J Obstet Gynecol* 1995;**172**:1436–1441.
- Steeegers-Theunissen RP, Wathen NC, Eskes TK, van Raaij-Selten B, Chard T. Maternal and fetal levels of methionine and homocysteine in early human pregnancy. *BJ Obstet Gynaecol* 1997;**104**:20–24.
- Steeegers-Theunissen RPM, Smith SC, Steegers EAP, Guilbert L, Baker PN. Folate affects apoptosis in human trophoblasts. *Br J Obstet Gynaecol* 2000;**12**:1513–1515.
- Steeegers-Theunissen RP, Obermann-Borst SA, Kremer D, Lindemans J, Siebel C, Steegers EA, Slagboom PE, Heijmans BT. Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child. *PLoS One* 2009;**11**:e7845.

- Steele W, Allegrucci C, Singh R, Lucas E, Priddle H, Denning C, Sinclair K, Young L. Human embryonic stem cell methyl cycle enzyme expression: modelling epigenetic programming in assisted reproduction? *Reprod Biomed Online* 2005;**6**:755–766.
- Stipanuk MH. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. *Annu Rev Nutr* 2004;**24**:539–577.
- Storch KJ, Wagner DA, Burke JF, Young VR. [1-13C; methyl-2H3]methionine kinetics in humans: methionine conservation and cystine sparing. *Am J Physiol* 1990; **5**(Pt 1):E790–E798.
- Tabacchi G, Wijnhoven TM, Branca F, Roman-Vinas B, Ribas-Barba L, Ngo J, Garcia-Alvarez A, Serra-Majem L. How is the adequacy of micronutrient intake assessed across Europe? A systematic literature review. *Br J Nutr* 2009; **101** (Suppl 2):S29–S36.
- Takata Y, Huang Y, Komoto J, Yamada T, Konishi K, Ogawa H, Gomi T, Fujioka M, Takusagawa F. Catalytic mechanism of glycine N-methyltransferase. *Biochemistry* 2003;**28**:8394–8402.
- Tamura T, Picciano MF. Folate and human reproduction. *Am J Clin Nutr* 2006; **5**:993–1016.
- Teklenburg G, Macklon NS. Review: in vitro models for the study of early human embryo-endometrium interactions. *Reprod Sci* 2009;**9**:811–818.
- Thangaratinam S, Langenveld J, Mol BW, Khan KS. Prediction and primary prevention of pre-eclampsia. *Best Pract Res Clin Obstet Gynaecol* 2011;**4**:419–433.
- Thuesen BH, Husemoen LL, Ovesen L, Jorgensen T, Fenger M, Linneberg A. Lifestyle and genetic determinants of folate and vitamin B12 levels in a general adult population. *Br J Nutr* 2010;**8**:1195–1204.
- Timmermans S, Jaddoe VW, Silva LM, Hofman A, Raat H, Steegers-Theunissen RP, Steegers EA. Folic acid is positively associated with uteroplacental vascular resistance: the Generation R study. *Nutr Metab Cardiovasc Dis* 2011;**1**:54–61.
- Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, Stein AD, Slagboom PE, Heijmans BT. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Hum Mol Genet* 2009;**21**:4046–4053.
- Toulis KA, Goulis DG, Mintzioris G, Kintiraki E, Eukarpidis E, Mouratoglou SA, Pavlaki A, Stergianos S, Poulasouchidou M, Tzellos TG et al. Meta-analysis of cardiovascular disease risk markers in women with polycystic ovary syndrome. *Hum Reprod Update* 2011;**6**:741–760.
- Twigt JM, Laven JSE, Steegers-Theunissen RPM. Chapter 6.1 folates in human health and disease. In: Hermann W, Obeid R (eds). *Vitamins in the Prevention of Human Diseases*. Berlin, DE: Walter de Gruyter Publishers, 2010, 101–114.
- Twigt JM, Hammiche F, Sinclair KD, Beckers NG, Visser JA, Lindemans J, de Jong FH, Laven JS, Steegers-Theunissen RP. Preconception folic acid use modulates estradiol and follicular responses to ovarian stimulation. *J Clin Endocrinol Metab* 2011;**2**:E322–E329.
- Twigt JM, Bolhuis ME, Steegers EA, Hammiche F, van Inzen WG, Laven JS, Steegers-Theunissen RP. The preconception diet is associated with the chance of ongoing pregnancy in women undergoing IVF/ICSI treatment. *Hum Reprod* 2012; **27**:2526–2531.
- Tyagi N, Qipshidze N, Sen U, Rodriguez W, Ovechkin A, Tyagi SC. Cystathionine beta synthase gene dose dependent vascular remodeling in murine model of hyperhomocysteinemia. *Int J Physiol Pathophysiol Pharmacol* 2011;**3**:210–222.
- Van Driel LM, Eijkemans MJ, de Jonge R, de Vries JH, van Meurs JB, Steegers EA, Steegers-Theunissen RP. Body mass index is an important determinant of methylation biomarkers in women of reproductive ages. *J Nutr* 2009; **12**:2315–2321.
- Vance DE, Ridgway ND. The methylation of phosphatidylethanolamine. *Prog Lipid Res* 1988; **1**:61–79.
- Van Mil NH, Oosterbaan AM, Steegers-Theunissen RP. Teratogenicity and underlying mechanisms of homocysteine in animal models: a review. *Reprod Toxicol* 2010; **4**:520–531.
- Vanselow J, Pohland R, Furbass R. Promoter-2-derived Cyp19 expression in bovine granulosa cells coincides with gene-specific DNA hypo-methylation. *Mol Cell Endocrinol* 2005; **1**–2:57–64.
- Verkleij-Hagoort AC, Verlinde M, Ursem NT, Lindemans J, Helbing WA, Ottenkamp J, Siebel FM, Gittenberger-de Groot AC, de Jonge R, Bartelings MM et al. Maternal hyperhomocysteinemia is a risk factor for congenital heart disease. *Br J Obstet Gynaecol* 2006; **12**:1412–1418.
- Vermeij-Keers C. Craniofacial embryology and morphogenesis: normal and abnormal. In: Stricker M, van der Meulen JC, Raphael B, Mazzola R, Tolhurst DE (eds). *Craniofacial malformations*. Edinburgh, UK: Churchill Livingstone, 1990, 27–60.
- Vollset SE, Refsum H, Nygard O, Ueland PM. Chapter 28 Lifestyle factors associated with hyperhomocysteinemia. In: Carmel R, Jacobsen DW (eds). *Homocysteine in health and disease*. Cambridge, UK: Cambridge University press, 2001, 341–355.
- Vujkovic M, de Vries JH, Lindemans J, Macklon NS, van der Spek PJ, Steegers EA, Steegers-Theunissen RPM. The preconception Mediterranean dietary pattern in couples undergoing in vitro fertilization/intracytoplasmic sperm injection treatment increases the chance of pregnancy. *Fertil Steril* 2010;**6**:2096–2101.
- Wald DS, Wald NJ, Morris JK, Law M. Folic acid, homocysteine, and cardiovascular disease: judging causality in the face of inconclusive trial evidence. *Br Med J* 2006; **338**:1114–1117.
- Wald DS, Morris JK, Wald NJ. Reconciling the evidence on serum homocysteine and ischaemic heart disease: a meta-analysis. *PLoS One* 2011;**2**:e16473.
- Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol* 2003; **15**:5293–5300.
- Waterland RA, Dolinoy DC, Lin JR, Smith CA, Shi X, Tahiliani KG. Maternal methyl supplements increase offspring DNA methylation at axin fused. *Genesis* 2006; **9**:401–406.
- Waterland RA, Kellermayer R, Laritsky E, Rayco-Solon P, Harris RA, Travisano M, Zhang W, Torskaya MS, Zhang J, Shen L et al. Season of conception in rural gambia affects DNA methylation at putative human metastable epialleles. *PLoS Genet* 2010; **12**:e1001252.
- Welch GN, Loscalzo J. Homocysteine and atherothrombosis. *N Engl J Med* 1998; **338**:1042–1050.
- Wijnands KPJ, Obermann-Borst SA, Sijbrands EJG, Wildhagen MF, Helbing WA, Steegers-Theunissen RPM. Maternal inheritance of cardiovascular disease in families and congenital heart disease in offspring. *Reprod Sci* 2012; **10**:345A.
- Williams PJ, Bulmer JN, Innes BA, Broughton Pipkin F. Possible roles for folic acid in the regulation of trophoblast invasion and placental development in normal early human pregnancy. *Biol Reprod* 2011;**6**:1148–1153.
- Wong WY, Thomas CMG, Merkus JMW, Zielhuis GA, Steegers-Theunissen RPM. Male factor subfertility: possible causes and the impact of nutritional factors. *Fertil Steril* 2000;**3**:435–442.
- Wong WY, Merkus HM, Thomas CM, Menkveld R, Zielhuis GA, Steegers-Theunissen RP. Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. *Fertil Steril* 2002;**77**:491–498.
- Wong WY, Zielhuis GA, Thomas CMG, Merkus JMW, Steegers-Theunissen RPM. New evidence of the influence of exogenous and endogenous factors on sperm count in man. *Eur J Obstet Gynaecol Reprod Biol* 2003; **1**:49–54.
- Wu W, Shen O, Qin Y, Lu J, Niu X, Zhou Z, Lu C, Xia Y, Wang S, Wang X. Methylene tetrahydrofolate reductase C677T polymorphism and the risk of male infertility: a meta-analysis. *Int J Androl* 2012; **1**:18–24.
- Xing W, Sairam MR. Retinoic acid mediates transcriptional repression of ovine follicle-stimulating hormone receptor gene via a pleiotropic nuclear receptor response element. *Biol Reprod* 2002; **1**:204–211.
- Xing W, Danilovich N, Sairam MR. Orphan receptor chicken ovalbumin upstream promoter transcription factors inhibit steroid factor-1, upstream stimulatory factor, and activator protein-1 activation of ovine follicle-stimulating hormone receptor expression via composite cis-elements. *Biol Reprod* 2002;**6**:1656–1666.
- Xu X, Chen J. One-carbon metabolism and breast cancer: an epidemiological perspective. *J Genet Genomics* 2009;**4**:203–214.
- Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ. Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. *J Biol Chem* 2000;**38**:29318–29323.
- Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet* 2001; **2**:153–154.