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Impact of folate and homocysteine metabolism on human reproductive health

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Folates belong to the vitamin B group and are involved in a large number of biochemical processes, particularly in the metabolism of homocysteine. Dietary or genetically determined folate deficiency leads to mild hyperhomocysteinemia, which has been associated with various pathologies. Molecular mechanisms of homocysteine-induced cellular dysfunction include increased inflammatory cytokine expression, altered nitric oxide bioavailability, induction of oxidative stress, activation of apoptosis and defective methylation. Whereas the involvement of folate metabolism and homocysteine in ageing-related diseases, in several developmental abnormalities and in pregnancy complications has given rise to a large amount of scientific work, the role of these biochemical factors in the earlier stages of mammalian reproduction and the possible preventive effects of folate supplementation on fertility have, until recently, been much less investigated. In the present article, the possible roles of folates and homocysteine in male and female subfertility and related diseases are systematically reviewed, with regard to the epidemiological, pathological, pharmacological and experimental data of the literature from the last 25 years.

Key words: fertility/folates/homocysteine/MTHFR polymorphism/human reproduction

Introduction

Folates are a group of inter-convertible co-enzymes, differing by their oxidation state, number of glutamic acid moieties and one-carbon substitutions. They are involved in amino acid metabolism, purine and pyrimidine synthesis and methylation of a large number of nucleic acids, proteins and lipids. Of particular interest is the interface between folate metabolism and the homocysteine/methionine cycle. Homocysteine, a sulfhydryl-containing amino acid that is not used in protein synthesis, originates exclusively from the one-carbon-donating metabolism of methionine, and it is remethylated into methionine with folates acting as methyl donors (Lucock, 2000).

Over the past decade, there has been a growing body of evidence that even a moderately elevated serum homocysteine concentration is associated with an increased risk of ageing-related diseases, such as atherosclerotic, thromboembolic and neurodegenerative disorders, and also with early pathological events of life (Herrmann, 2001; Gueant *et al.*, 2003). The latter category includes a number of developmental abnormalities, particularly neural tube defects, as well as late pregnancy complications, such as pre-eclampsia, *abruptio placentae*, intrauterine growth retardation, preterm birth and intrauterine fetal death (Eskes, 2000; Nelen, 2001; Hague, 2003; Steegers-Theunissen *et al.*, 2004; Tamura and Picciano, 2006).

Whereas a large amount of scientific work has investigated the roles of folate metabolism and hyperhomocysteinemia in malformations and pathologies of the ongoing pregnancy, there is only little information on a possible involvement of these biochemical phenomena in the earlier stages of reproductive physiology and in related diseases. In the present article, current data on the influence of folate metabolism on male and female reproductive tracts and fertility are reviewed by means of a systematic review of the Medline database-indexed literature since 1980.

Biochemical background

In most mammalian cells, accumulating homocysteine is removed either by remethylation into methionine or by trans-sulfuration into cysteine (Scott and Weir, 1998; Fowler, 2005). In the trans-sulfuration pathway (Figure 1), homocysteine is condensed with serine in an irreversible reaction catalyzed by cystathionine-beta-synthase (CBS) to form cystathionine, which in turn is reduced to

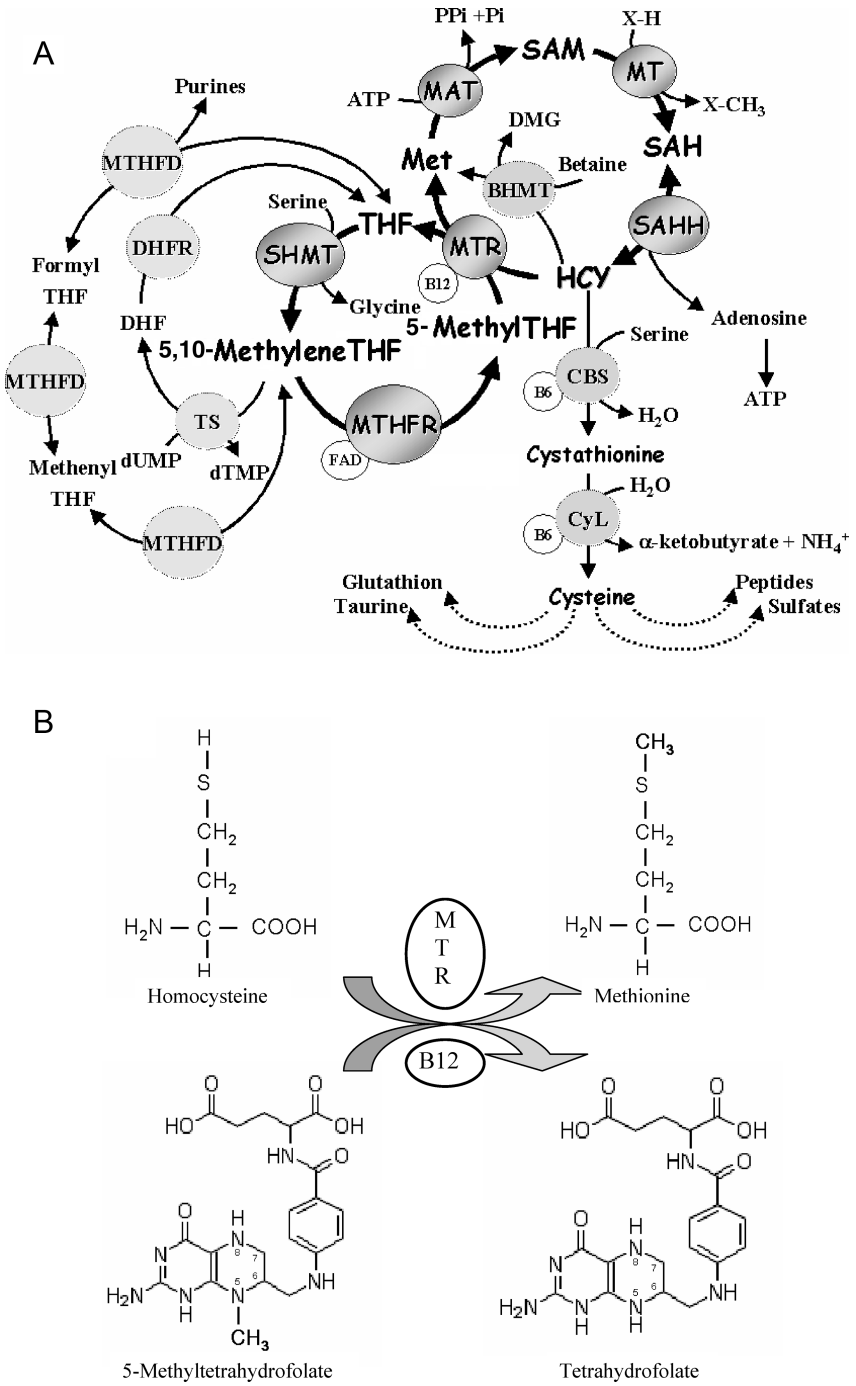

Figure 1. Biochemistry of folate and homocysteine

(**A**) Pathways of folate and homocysteine metabolism (the folate and methionine cycles are highlighted). Hcy is remethylated into methionine (Met) by MTR with 5-methylTHF as a methyl donor and cobalamin (B12) as a co-enzyme. 5-MethylTHF is produced by the FAD-dependent enzyme 5,10-MTHFR. 5,10-MethyleneTHF is also a one-carbon donor in the synthesis of thymidylate and after conversion into 5,10-methenyltetrahydrofolate (MethenylTHF) and further into 10-formyltetrahydrofolate (FormylTHF), in the synthesis of purines. After the release of their one-carbon unit, all of these substituted folates are converted to THF which is finally recycled into MethyleneTHF during the conversion of serine to glycine by the enzyme serine hydroxymethyltransferase (SHMT). Met is further transformed into SAM, the universal methyl donor for methylation of nucleic acids, proteins, polysaccharides, phospholipids, etc. After release of the methyl group, SAM is converted into SAH, which is reversibly hydrolyzed into Hcy. The alternative pathway by which Hcy is remethylated into Met takes place in the liver and uses betaine as a methyl donor; this reaction is catalyzed by BHMT. Hcy is also metabolized into cystathionine by the vitamin B6-dependent CBS which is further cleaved into cysteine and alpha-ketobutyrate by cystathionine lyase (CyL). Cysteine is further incorporated into peptides, glutathione and other molecules. Abbreviations: MAT, methionine adenosyltransferase; MT, methyltransferases; X, substrate to be methylated; SAHH, *S*-adenosylhomocysteine hydrolase; TS, thymidylate synthase; MTHFD, 5,10-methylenetetrahydrofolate dehydrogenase. (**B**) Chemical formulae of the key reaction, i.e. the remethylation of homocysteine, at the interface between the methionine and folate cycle. Methyltetrahydrofolate (MethylTHF) consists of a pteridine ring, para-aminobenzoic acid and glutamic acid (the three constituents of all biologically active folates), with the methyl group at position 5. In the present reaction, catalyzed by MTR, this methyl group is transferred to homocysteine, with cobalamin (B12) acting as an intermediate carrier.

cysteine and alpha-ketobutyrate by cystathionine lyase. Both of these enzymes depend on pyridoxal 5-phosphate, an active from of vitamin B6.

During the remethylation into methionine (Figure 1), a methyl group provided by 5-methyltetrahydrofolate (5-methylTHF) is transferred to homocysteine by methionine synthase (MTR). In this ubiquitous reaction, cobalamin (vitamin B12) is involved as an intermediate carrier of the methyl group. Alternatively, homocysteine can also be remethylated into methionine by betaine–homocysteine methyltransferase (BHMT), in which the methyl group is provided by betaine that is transformed into dimethylglycine. However, in contrast with the MTR reaction, this alternative pathway seems to be limited to the liver; in particular, the possibility of a BHMT-expression in human gonads has not yet been investigated (Chadwick *et al.*, 2000; Delgado-Reyes *et al.*, 2001). Whatever the remethylation pathway, the resulting methionine will be either incorporated into various peptides or transformed into *S*-adenosylmethionine (SAM) by methionine adenosyltransferase, which transfers an adenosyl group from ATP to methionine. SAM is the universal methyl donor in a large number of methylation reactions, and thus plays a key role in cellular function. During these reactions, which are catalyzed by specific methyltransferases, SAM has a methyl group removed to form *S*-adenosylhomocysteine (SAH). Finally, SAH is hydrolyzed in a reversible reaction into homocysteine. The total sequence of the preceding reactions is called the homocysteine/methionine cycle.

This cycle could not turn accurately without the normal functioning of a second cycle, the folate cycle. The methyl-donating 5-methylTHF originates from 5,10-methyleneTHF by the flavine adenine dinucleotide-dependent 5,10-methyleneTHF reductase (MTHFR). After the remethylation of homocysteine to methionine, demethylated THF will be converted again into 5,10-methyleneTHF during the conversion of serine to glycine. MTHFR has a pivotal regulatory function in the folate cycle, as it directs the folate pool toward the remethylation of homocysteine at the expense of DNA and RNA synthesis (Fowler, 2001); besides its conversion to 5-methylTHF by MTHFR, 5,10-methyleneTHF is further metabolized in several one-carbon transfer reactions during the synthesis of thymidylate (methylation of deoxyuridine 5′-monophosphate (dUMP) to deoxythymidine 5′-monophosphate dTMP), as well as during the synthesis of purines (Figure 1).

An impaired function of these metabolic pathways leads to accumulation of homocysteine, either by insufficient transsulfuration (through CBS mutations or vitamin B6 deficiency) or by a blockage of remethylation. In the latter case, folate or cobalamin deficiency may be involved; importantly, a single nucleotide polymorphism of the *MTHFR* gene, C677T, encodes a thermolabile variant of the enzyme, characterized by an alanine-to-valine substitution at position 222 and a 50% reduction in enzyme activity (Frosst *et al.*, 1995). As a consequence, the *MTHFR* C677T polymorphism has been associated with moderately elevated serum homocysteine concentration, particularly in patients with insufficient folate supply (Harmon *et al.*, 1996; Jacques *et al.*, 1996). The prevalence of the homozygous TT allele is ~10% in Caucasians, Australians and White Americans, but it seems to be influenced by ethnicity as well as by folate status (Botto and Yang, 2000). Consistently, our group found an association of T allele frequency and the rate of cases with folate deficiency among seven population samples: the prevalence of homozygotes was highest in Mexicans and Italians who also have the highest plasma folate concentrations, whereas it was lowest in West Africans who have the lowest folate status (Gueant-Rodriguez *et al.*, 2006). A second polymorphism of the same gene (A1298C) does not seem to be associated with hyperhomocysteinemia (van der Put *et al.*, 1998).

Clinical implications of the *MTHFR* C677T polymorphism include increased risk for several diseases, such as the pregnancy complications mentioned earlier, particularly in subjects with low folate status, but also for some other pathologies, such as colorectal neoplasias, in which the risk might be reduced in mutated subjects with sufficient folate supply (Ueland *et al.*, 2001; Peyrin-Biroulet *et al.*, 2004). The cellular and molecular mechanisms underlying these effects have been investigated principally in the field of atherosclerosis, using either animal models or *in vitro* culture of endothelial cells or other components of the vascular wall; these studies have been recently reviewed in detail elsewhere (Lawrence de Koning *et al.*, 2003; Austin *et al.*, 2004) and will be briefly recalled next (Figure 2).

First, homocysteine has been shown to induce vascular inflammation by enhancing the expression of pro-inflammatory cytokines, such as monocyte chemoattractant protein 1 (MCP-1), which regulates migration and activation of monocytes/macrophages, and interleukin 8 (IL-8), which is an important chemoattractant for neutrophils and T-lymphocytes (Poddar *et al.*, 2001). Second, homocysteine decreases the bioavailability of nitric oxide (NO), one of the major endothelium-dependent vasodilators that is produced by the endothelial isoform of nitric oxide synthase (eNOS). This effect is caused either by an accelerated oxidative inactivation of NO and/or eNOS (Zhang *et al.*, 2000; Romerio *et al.*, 2004) or by an increase in serum assymetric dimethylarginine, an endogenous inhibitor of eNOS (Stuhlinger *et al.*, 2003). Third, there is a large body of evidence that hyperhomocysteinemia is associated with the production of reactive oxygen species (ROS) in endothelial and smooth muscle cells. The mechanism of this oxidative stress relies either on auto-oxidation of the highly reactive thiol group of homocysteines (Starkebaum and Harlan, 1986) or on the formation of intracellular superoxide

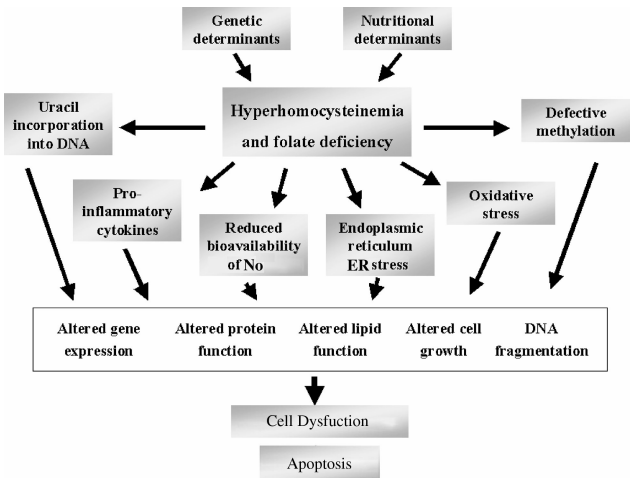

Figure 2. Cellular and molecular mechanisms of hyperhomocysteinemia-induced cell dysfunction.

and peroxyl radicals with concomitant inhibition of cellular antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase (Weiss, 2005). Fourth, a more recent concept concerns activation of the unfolded protein response (UPR) that is triggered when unfolded or misfolded proteins accumulate in the endoplasmic reticulum (ER) (Kaufman, 2002). This ER stress induces the expression of several molecular chaperones and other stress response proteins, which are aimed at restoring correct protein folding or retranslocating defective proteins back to the cytosol for degradation in the proteasomes. In case of a prolonged ER stress, the UPR extends the to activation of apoptosis by various signaling pathways (Xu *et al.*, 2005). This is precisely what happens in human endothelial cells after exposure to homocysteine *in vitro*: while inducing misfolding in the ER by altering the local redox potential and interfering with disulfide bond formation, homocysteine activates UPR and, subsequently, growth arrest (Outinen *et al.*, 1999) and apoptosis (Zhang *et al.*, 2001). Homocysteine-induced endothelial apoptosis probably also involves other mechanisms such as the classical p53 pathway (Lee *et al.*, 2005).

Furthermore, folate deficiency and genetically determined low MTHFR activity lead to an insufficient remethylation of homocysteine to methionine and a decreased SAM production and SAM/SAH ratio. Insufficient availability of SAM then results in impaired methylation reactions, with multiple consequences, especially as far as DNA methylation is concerned. In homozygous *MTHFR* 677 TT patients, the resulting deficit in 5-methylTHF has been associated with DNA hypomethylation in peripheral blood mononuclear cells (Stern *et al.*, 2000; Ueland *et al.*, 2001; Friso *et al.*, 2002). Thus, defective methylation may lead to aberrant gene expression resulting in abnormal fetal development and malignant diseases (Reik and Walter, 2001).

Finally, dietary folate deficiency and the resulting decreased cellular synthesis of 5,10-methyleneTHF, as well as reduced MTHFR activity lead to an accumulation of dUMP and thus to an excessive incorporation of uracil into DNA, with the subsequent repair mechanisms increasing the risk of chromosome breakage (Blount *et al.*, 1997; Fenech, 2001).

Whether all or some of these pathogenetic mechanisms of endothelial dysfunction are also involved in folate deficiency-induced alterations of male and female reproductive functions is currently unknown. However, several experimental data are in favor of such similarities, as will be discussed next.

Folates and male fertility

Epidemiological data

Several authors investigated the prevalence of the different *MTHFR* 677 genotypes among infertile male patients (Table I). In a German study, 255 patients seeking fertility counseling and 200 fertile controls were included, but precise inclusion criteria were not provided (Bezold *et al.*, 2001). The frequency of wild-type CC and heterozygous CT carriers was not significantly different in patients and controls. However, the prevalence of mutant TT homozygotes was significantly higher among patients when compared with controls (18.8% vs. 9.5%). The authors thus concluded a possible role of *MTHFR* polymorphism in the pathogenesis of male infertility. In contrast, this conclusion was not shared by a Dutch group who did not find any significant difference in the prevalence of *MTHFR* polymorphism between 113 healthy fertile men and 77 subfertile patients with moderate oligospermia and conception failure in female partners for at least 1 year: TT homozygosity was found in 9.1% of patients and in 13.3% of controls (Ebisch *et al.*, 2003). In Italian men, the prevalence of TT homozygosity was even higher, between 20.4% and 27.6%, and yet not significantly different in fertile and infertile patients (Stuppia *et al.*, 2003).

A further study included 151 Indian patients with severe male infertility (Singh *et al.*, 2005). These patients presented either with severe oligozoospermia or with azoospermia; those with known genetic causes (chromosomal abnormalities or Y chromosome microdeletions) had been excluded. In this population, there were significantly more homozygous TT carriers (4% vs. 0%), as well as heterozygous CT carriers (26.5% vs. 18.5%), compared with 200 fertile control subjects. The authors emphasized the importance of the general prevalence of *MTHFR* polymorphism and the nutritional status of the population, when comparing the results of different studies: in general, Western European people have rather high serum folate and low homocysteine concentrations that do not vary consistently among the different *MTHFR* 677 genotypes. Thus it is possible that despite a higher prevalence of TT homozygotes, the clinical or biological effects of this polymorphism might be masked because of a higher nutritional vitamin intake. In contrast, Indian people generally have low serum folate and high homocysteine concentrations because of a poorer nutritional status; thus, despite a very low frequency of the mutated T allele, an abnormal reproductive phenotype depending on the *MTHFR* polymorphism is easily detectable. Therefore the authors suggested that similar observations could probably be made in African and Asian subjects, and that *MTHFR* polymorphism-related infertility could be prevented by nutritional improvement in these populations. Actually, these conclusions are consistent with our study mentioned earlier, analysing folate metabolism and *MTHFR* polymorphism in Western Europeans, Mexicans and West Africans (Gueant-Rodriguez *et al.*, 2006). Furthermore, such an interaction between nutritional status and genotype has also been suggested in a Spanish population where the prevalence of *MTHFR* 677-mutated subjects doubled since the mid-1970s, which coincided with the development of folic acid supplementation programs for pregnant women (Munoz-Moran *et al.*, 1998). Thus, a systematic supplementation could have rescued mutated fetuses whose viability may otherwise be reduced: when both *MTHFR* polymorphisms, C677T and A1298C, were determined in another population sample from South Europe, the presence of three or four mutated alleles could only be detected in aborted fetuses, but not in living neonates (Isotalo *et al.*, 2000). In contrast, the genotype analysis of 1777 individuals, divided into four age groups, showed a progressive increase of genotypes with two mutated alleles in the younger subjects when compared with the older, reflecting an unblocking of the usual genetic selection by changes in the diet and folate intake (Reyes-Engel *et al.*, 2002).

In accordance with the Indian study (Singh *et al.*, 2005), Korean authors also found a higher prevalence of the *MTHFR* C677T mutant homozygotes only among men with unexplained infertility, normal karyotype and no chromosome Y microdeletions (Park *et al.*, 2005). These differences remained significant when

Table I. *MTHFR* polymorphism and male infertility

Reference	Geographic origin	Patients	Results
Bezold *et al.* (2001)	Germany	255 subfertile men	Higher prevalence of 677TT vs. fertile controls
Ebisch *et al.* (2003)	Netherlands	77 subfertile men	Prevalence of 677TT not different vs. fertile controls
Stuppia *et al.* (2003)	Italy	93 infertile men	Prevalence of 677TT not different vs. fertile controls
Singh *et al.* (2005)	India	151 infertile men (severe oligoastheonteratozoospermia)	Higher prevalence of 677TT vs. fertile controls
Park *et al.* (2005)	South Korea	373 infertile men (unexplained infertility)	Prevalence of 677TT higher; prevalence of 1298CC not different vs. fertile controls
Paracchini *et al.* (2006)	Italy	105 infertile men	Higher prevalence of 677TT vs. fertile controls

subgroups of infertile patients with either azoospermia or severe oligoasthenoteratozoospermia, but not with moderately altered sperm parameters, were investigated. The authors also determined the frequency of the A1298C mutation that was, however, equally distributed among infertile patients and fertile controls. Finally, in a prospective study based on the follow-up of 105 Italians, the authors confirmed the *MTHFR* C677T homozygous variant to be a significant risk factor for male infertility (Paracchini *et al.*, 2006). However, this was no longer the case in a subgroup of patients who, in addition to the *MTHFR* polymorphism, also presented with a common deletion of *GSTM1*, a gene encoding one of the glutathione transferases. This effect could be explained by the *GSTM1* deletion causing an increase in glutathione, which in turn stimulates SAM synthase activity, thus counteracting the deleterious effects of *MTHFR* polymorphism on the subsequent methylation reactions. The authors therefore emphasized that these types of gene interactions have to be taken into account when interpreting the results of case–control studies that focus only on one genetic parameter.

Pharmacological and experimental data

A few therapeutic trials investigating the possible effect of folate supplementation on male fertility have been reported. In the first of them, 40 either normozoospermic or oligozoospermic patients received 10 mg folic acid per day for a period of 30 days (Landau *et al.*, 1978). The authors did not notice any correlation between serum or seminal fluid folate concentrations and total sperm count, and there was no beneficial effect of folic acid on sperm parameters. However, the treatment period may have been too short, as spermatogenesis is generally thought to extend>3 months in the human. Therefore, another group administered a daily dose of 15 mg folinic acid for 3 months to 65 infertile men with an excessive round cell count in their ejaculate (Bentivoglio *et al.*, 1993). Semen analysis was performed twice: before as well as at the end of the treatment. All female partners had normal fertility evaluation. In contrast with the preceding study, the authors noted statistically significant modifications of all analysed sperm parameters at the end of the treatment period, particularly an increase in sperm density (from 15 to 22.6×10^6/ml) and motility (from 17.7% to 27.8%) as well as a decrease in round cell count (from 9.7 to 6.4×10^6/ml). Moreover, among these 65 couples presenting with infertility for 3.2 years on average, 24 conceived within 6 months following the folinic acid treatment and 17 women delivered. The concomitant improvement of sperm parameters and reduction in round cells led to the conclusion that folate supplementation had a beneficial effect on spermatogenesis, possibly by increasing cellular cohesion within the seminiferous epithelium, thus preventing abnormal release of immature germ cells into the lumen.

Further therapeutic intervention studies were performed using a randomized, placebo-controlled protocol to compare subfertile patients with fertile controls. Subfertility was defined as the absence of pregnancy within 1 year of unprotected intercourse and moderate oligozoospermia, i.e. a sperm concentration between 5 and 20×10^6/ml. In a first study, the authors included 94 patients and 99 controls who were divided into four groups, receiving either folic acid (5 mg/day) and a placebo, or zinc sulfate (66 mg/day) and a placebo, or both drugs (folic acid and zinc sulfate) or two placebos for 6 months (Wong *et al.*, 2002). Serum folate and zinc concentrations were not different in subfertile and fertile subjects before the onset of treatment. Only concomitant administration of folic acid and zinc led to a significant 74% increase in sperm density and total count in subfertile patients, whereas in fertile controls no significant improvement was detected. Folic acid alone increased the sperm density by 40% in subfertile patients, but this did not reach statistical significance. Thus in this study, folate supplementation alone was not sufficient to significantly improve sperm parameters in subfertile patients, but it showed a synergistic effect with zinc sulfate. The importance of this oligo-element in spermatogenesis has been investigated by several authors and recently reviewed (Wong *et al.*, 2000). Interactions between folate and zinc metabolism have also been emphasized: zinc deficiency impairs folate intestinal absorption, as zinc plays a role in the conversion of pteroylpolyglutamates to the monoglutamate form (Tamura and Kaiser, 1991; Favier *et al.*, 1993).

The same Dutch group subsequently investigated whether the effect of folate and zinc supplementation depended on the *MTHFR* 677 genotype (Ebisch *et al.*, 2003). Unexpectedly, it was shown that a significant increase in sperm count was limited to wild-type *MTHFR* 677 CC carriers, whereas no effect was observed in CT heterozygotes and in homozygous TT mutants. The authors hypothesized that residual MTHFR activity in heterozygous and homozygous mutant patients was insufficient to produce the expected effect following folic acid administration, compared with wild-type enzyme activity, although this is not consistent with the above-mentioned assumption that a high folate

status may overcome the biological effects of *MTHFR* mutants. Polymorphism of other enzymes of the folate metabolism might also interfere. However, it has to be mentioned also that in this study, results from subfertile and fertile men had been pooled. Recently, the same authors used an identical study design to confirm the beneficial effect of the combined folic acid and zinc sulfate supplementation in subfertile patients, although to a lesser extent, as the increase in sperm count only reached 18% (Ebisch *et al.*, 2006b). In this study they showed subfertile patients to have lower serum inhibin B and higher FSH baseline concentrations, as well as an identical testosterone concentration, compared with fertile controls, but none of these parameters was modified by the treatment, thus the observed beneficial effect was not involving endocrine function of the testis.

Unlike supplementation, folate deprivation is conceivable only in animal models. Rats fed a folic acid-deficient diet showed a major reduction in sperm count (Mayr *et al.*, 1999), whereas various dihydrofolate reductase (DHFR) inhibitors, such as etopride (Malik *et al.*, 1995) and pyrimethamine (Cosentino *et al.*, 1990; Kalla *et al.*, 1997) proved to be efficient male contraceptives in mice and rats, by significantly reducing sperm count as well as motility. Given the key role of MTHFR in folate metabolism, the most interesting animal model is the *MTHFR* knock out mouse, whose MTHFR activity has been abolished by gene targeting (Chen *et al.*, 2001b). These animals had a seriously compromised survival, and males presented with high homocysteine concentration, abnormal spermatogenesis and severe infertility (Kelly *et al.*, 2005). In the neonatal testis of MTHFR-deficient animals, the number of germ cells was extremely reduced, because of a lack of post-natal proliferation, as well as an abnormal activation of apoptosis. In the adult testis, seminiferous tubules appeared either devoid of germ cells or with a maturation blockage. Both the survival and the reproductive phenotype could be significantly improved by betaine supplementation. This further illustrates the need for an intact folate cycle to maintain normal spermatogenesis, and suggests that the alternative homocysteine remethylation pathway is also operating in the testis.

To summarize the data from the above-mentioned pharmacological interventions, folic acid supplementation seems to have a positive effect on spermatogenesis and sperm parameters, either alone or in combination with other nutritional factors, but the subgroup of patients who might benefit from such therapy, as well as the optimal doses, still have to be determined. Furthermore, it has to be emphasized that no controlled, randomized study investigating the impact of folate supplementation on the pregnancy and live birth rate in the female partners of the treated male patients is currently available.

Presence of folates in the male reproductive tract

Seminal plasma folate concentration and biochemical forms have been determined in 48 men with low habitual fruit and vegetable consumption, using a microbiological assay (Wallock *et al.*, 2001). Results showed that total folate concentration was 1.5 times higher in seminal than in blood plasma. No polyglutamates were detected, as the results were identical before and after treatment of the samples with folate conjugase. Furthermore, seminal plasma contained a significant proportion (26%) of non-methyltetrahydrofolates, whereas in the blood plasma, 5-methylTHF is largely predominant. Blood and seminal plasma folate concentrations were positively correlated, except for the non-methyltetrahydrofolates. Sperm density and total count in the ejaculate showed a significant positive correlation with non-methyltetrahydrofolate concentration, but not with total folate or 5-methylTHF, suggesting a role for non-methyltetrahydrofolates in spermatogenesis or sperm maturation.

Furthermore, seminal plasma has been shown to contain a high affinity folate-binding protein, which shares immunoreactivity with the human milk folate-binding protein (Holm *et al.*, 1991). Immunohistochemical and ultrastructural studies suggested this binding protein to be secreted by the epididymal and vas deferens epithelium, but not by the seminiferous epithelium, prostate or seminal vesicles. Moreover, part of this binding protein was associated with prostasome-like vesicles that adhere to the spermatozoa in the epididymal duct, whereas testicular spermatozoa are devoid of immunoreactive folate-binding protein (Malm *et al.*, 2005). This observation further underlines a possible physiological role of folate metabolism in male gamete maturation: the authors hypothesize either a bacteriostatic function on folate-requiring micro-organisms or a mechanism for folate uptake into spermatozoa, possibly via megalin-mediated endocytosis (Birn *et al.*, 2005). These observations clearly need further investigation, especially with regard to possible differences between fertile and infertile men. A recent Chinese study did not find any difference in seminal plasma folate and cobalamin concentrations in 44 infertile and 176 fertile men; instead, the concentration of seminal ROS was significantly higher in the infertile group and negatively correlated with seminal folate and cobalamin (Chen *et al.*, 2001a).

Finally, high affinity folate-binding sites have also been detected in human testicular tissue (Holm *et al.*, 1999), as well as MTHFR activity, which proved to be five times higher in the mouse testis than in other tissues (Chen *et al.*, 2001b), but the cellular origin of this activity has not been determined.

Pathophysiology of folate deficiency-induced male infertility

Besides a direct effect of folate deficiency on the nucleic acid synthesis and thus on the proliferation of rapidly dividing cells such as male germ cell precursors, most of the pathophysiological mechanisms that have been described in endothelial cells, in case of folate deficiency and subsequent accumulation of homocysteine, also operate in the male reproductive tract. However, in many aspects of male infertility, the causal relationship between these cellular and molecular events and altered folate metabolism has not yet been investigated.

Inflammatory cytokines, such as those induced by hyperhomocysteinemia, have been associated with impaired sperm parameters and male infertility: MCP-1 could play a role in testicular inflammation (Aubry *et al.*, 2000), whereas seminal IL-8 concentration was significantly higher in men with genital tract inflammation when compared with that in healthy controls (Sanocka *et al.*, 2003). The NO signaling pathways are involved in penile erection, spermatogenesis, dynamics of the blood–testis barrier, sperm motility, capacitation, acrosome reaction and fertilization (Rosselli *et al.*, 1998; Herrero *et al.*, 2003; Lee and Cheng, 2004). Thus, any alteration of NO bioavailability, e.g. by hyperhomocysteinemia, may have direct consequences

on male reproductive functions. A considerable amount of literature has demonstrated that although a small quantity of ROS is necessary for normal sperm function and sperm–oocyte fusion, excessive oxidative stress will induce sperm DNA damage and adversely influence sperm function, fertilization and early embryo development; these mechanisms have been recently reviewed (Lewis and Aitken, 2005; Weiss, 2005; Agarwal *et al.*, 2006). As mentioned above, another pathophysiological impact of homocysteine metabolism involves UPR-induced apoptosis. There is currently no information available on this phenomenon in male reproduction. However, it is well established that other cell death pathways such as Fas ligand-induced apoptosis play an important role in testicular physiology, by regulating the clonal expansion of germ cells, and abnormal apoptosis has been implicated in various andrological pathologies, such as impaired spermatogenesis, reduced sperm motility or increased sperm DNA fragmentation (Said *et al.*, 2004).

To our knowledge, sperm DNA methylation has not yet been investigated in patients with *MTHFR* polymorphism. It is clear, however, that methylation and the related gene imprinting play an important role during spermatogenesis. This is illustrated by the fact that in primordial germ cells, inherited imprinted genes have their methylation imprints erased (Mann, 2001). Subsequently, a paternal-specific remethylation occurs during spermatogonial and spermatocytic differentiation (Rousseaux *et al.*, 2005). The relationship between defective DNA methylation and impaired spermatogenesis has been demonstrated by bisufhite genomic sequencing of sperm DNA, with regard to H19, a paternally *de novo* methylated gene that is imprinted during the premeiotic stages of spermatogenesis (Marques *et al.*, 2004). In 15% of moderate oligozoospermia and in 30% of severe oligozoospermia, H19 was shown to be incompletely methylated, whereas all of the normozoospermic controls showed normal methylation. In another study, sperm DNA methylation has been proposed as a prognostic factor in *in vitro* fertilization (IVF): these authors used a 5-methylcytosine immunoassay with flow cytometry detection to quantify DNA methylation, and observed a pregnancy rate of 33% per cycle in the normal methylation group vs. 8.3% in the low methylation group (Benchaib *et al.*, 2005). These observations are in accordance with previous animal studies using 5-aza-2-deoxycytidine, a hypomethylating agent, in neonatal mice whose testes contain only premeiotic germ cells. This treatment completely blocked differentiation to the spermatocyte stage (Raman and Narayan, 1995). When administered to adult mice or rats, the resulting effect was a severe impairment of spermatogenesis with reduced pregnancy rates in females (Doerksen *et al.*, 2000; Kelly *et al.*, 2003).

Methylation defects also affect other molecules than DNA. Particularly, phospholipid methylation has been shown to be highly active during the synthesis of phosphatidylcholine in rat Leydig cells (Moger, 1985). The major methyl donor, SAM, stimulated hCG-mediated testosterone synthesis in purified rat Leydig cells *in vitro*, whereas SAH had opposite effects (Papadopoulos *et al.*, 1987). Thus folate deficiency-induced hypomethylation may impair not only the exocrine, but also the endocrine, functions of the male gonad.

Finally, it has also been suggested that the adverse reproductive outcome in patients with homozygous *MTHFR* polymorphism may be related to homocysteine-induced precocious atherosclerotic vascular alterations, impairing the blood flow in the testicular arteries (Rossato, 2004).

Folates and Female Fertility

Folates and homocysteine in the female reproductive tract

Folates, methionine, homocysteine and vitamins B6 and B12 have been measured in the follicular fluid of 14 patients undergoing oocyte retrieval for IVF (Steegers-Theunissen *et al.*, 1993). None of these women had a previous history of recurrent spontaneous absorption or malformations. When compared with serum concentrations, follicular fluid concentrations of folates and homocysteine were not different, but methionine as well as vitamin B6 and B12 concentrations were significantly lower. Follicular fluid results from passive blood plasma diffusion through the basement membrane between the theca interna and the granulosa layer, as well as from active secretion by granulosa cells. Actually, prior to ovulation, granulosa cells, and the oocyte they enclose, are avascular and therefore depend on the diffusion of blood plasma nutrients supplied by the thecal capillary network. Thus the question was raised whether disturbances of this microenvironment around the oocyte may have deleterious consequences on the reproductive competence of the female gamete. A Polish group measured homocysteine concentration in follicular fluid of 40 patients undergoing IVF, 20 of whom received folic acid supplementation (Szymanski and Kazdepka-Zieminska, 2003). These authors found follicular fluid homocysteine concentration to be significantly lower in folate-supplemented patients; moreover, there was a negative correlation between follicular fluid homocysteine concentration and the degree of maturity of the retrieved oocytes. The clinical significance of this observation remains to be determined, since in another study, follicular fluid homocysteine concentration was not predictive of the success of the IVF procedure; however, there were only 15 patients included in that study, with heterogeneous etiologies (Jerzak *et al.*, 2003). Recently, homocysteine and other thiol concentrations have been determined in the ejaculate and follicular fluid of 156 couples undergoing IVF (Ebisch *et al.*, 2006a). Follicular fluid homocysteine concentration was significantly higher in women with endometriosis when compared with patients having unexplained infertility. Moreover, follicular fluid as well as seminal plasma homocysteine concentrations showed a significant negative correlation with embryo quality on day 3 after IVF, but whether there was any further correlation with the chances of pregnancy has not been investigated in that study.

The question of a possible effect of folic acid on ovarian function was raised in the late 1960s, when it has been shown that in immature superovulated rats, either excess or deficiency of folates partially inhibited ovulation (Willmott *et al.*, 1968). In rhesus monkeys, a folate-restricted diet led to irregular menstrual cycles, whereas pre-ovulatory serum estradiol as well as mid-luteal progesterone concentrations decreased progressively when compared with animals under normal diet. Ovarian biopsies of folate-deprived monkeys demonstrated degeneration of graafian follicles, with an increase in atretic and cystic follicles, as well as a depletion of granulosa cells and a reduction or even an absence of corpora lutea (Mohanty and Das, 1982). Thus, a sufficient folate intake seems to be necessary to maintain normal

ovarian histology and endocrine function in this primate model. Unfortunately, fertility had not been assessed in these animals, but another group studied the probability of pregnancy in golden hamsters according to the diet (Mooij *et al.*, 1992). In animals fed with a folic acid-free diet for 2 weeks before mating, the number of pregnancies as well as the red blood cell folate concentration were not different when compared with control animals. However, when the folate-free diet was prolonged up to 16 weeks before mating, none of the females was fertile, while their folate concentrations had decreased significantly. Recently, we reported on a low vitamin B2 and B12, folate and choline diet fed to female rats 1 month before mating (Blaise *et al.*, 2005). These animals showed normal fertility, but pups exposed to this diet *in utero* and during the suckling period showed significant growth retardation and a 25% perinatal mortality. Gonadal histology has not yet been assessed in that model.

Folate metabolism and estrogens

The hypothesis of a relationship between folate metabolism and estrogens has been investigated in a large number of studies since the early 1970s, focussing on the possible involvement of oral contraceptives in a reduction of folate intestinal uptake and serum concentration (Lindenbaum *et al.*, 1975). Thirty years later, the interactions of sex steroids and folate metabolism still remain controversial, but there seems to be convergent, though not unanimous, evidence of an estrogen-induced decrease in serum homocysteine concentration.

Serum homocysteine concentration is actually lower in females than in males (Boers *et al.*, 1983), lower in premenopausal than in post-menopausal women (Wouters *et al.*, 1995; Hak *et al.*, 2000) as well as throughout the follicular phase of the menstrual cycle (Tallova *et al.*, 1999). Homocysteine concentration has also been shown to increase after bilateral oophorectomy in a series of 30 patients (Kapral *et al.*, 2002) and to decrease again after the onset of hormonal replacement therapy (HRT). The impact of HRT on homocysteine has been investigated by a number of authors, and most of them agree with the inhibitory effect of estrogens on homocysteine production. These studies have been recently reviewed (Mijatovic and van der Mooren, 2001). In several other studies, however, no significant effect of HRT on homocysteine concentration could be detected (Berger *et al.*, 2000; Farag *et al.*, 2003). Similarly, the impact of oral contraceptives on homocysteine metabolism needs to be clarified (Lindenbaum *et al.*, 1975; Brattstrom *et al.*, 1992; Steegers-Theunissen *et al.*, 1992b; Merki-Feld *et al.*, 2002; Lussana *et al.*, 2003), concerning the role of estrogens as well as that of the progestin component of these products. Finally, two groups have monitored serum homocysteine concentration throughout long protocol stimulation for IVF (Bettahar-Lebugle *et al.*, 2002; Roopnarinesingh *et al.*, 2006). These protocols include first a pituitary down-regulation by a GnRH agonist, followed by ovarian stimulation with hMG or FSH. Thus serum estrogen concentration varies between two extremes in these patients during the IVF protocol; nevertheless, in both studies, homocysteine concentration did not show any variations that could have been expected from the preceding results.

Folate metabolism and Polycystic ovary syndrome

According to the Rotterdam criteria, polycystic ovary syndrome (PCOS) is defined by at least two of the following abnormalities: (i) oligo-or anovulation, (ii) clinical or biological hyperandrogenism and (iii) polycystic ovaries on pelvic ultrasound (Azziz, 2004). PCOS is classically related to an increased cardiovascular risk, which may be accounted for by the associated insulin resistance, hyperandrogenism, or possibly, hyperhomocysteinemia. Thus a number of studies have investigated homocysteine and folate metabolism and confirmed the presence of increased serum homocysteine concentration in obese as well as in non-obese PCOS patients (Yilmaz *et al.*, 2005). *MTHFR* polymorphism does not seem to be more frequent in these patients than in healthy controls (Tsanadis *et al.*, 2002), and the possible determinants of elevated homocysteine concentration are still debated among authors who found significant correlations between homocysteine and insulin resistance or hyperandrogenism (Yarali *et al.*, 2001; Schachter *et al.*, 2003; Vrbikova *et al.*, 2003; Bayraktar *et al.*, 2004; Wijeyaratne *et al.*, 2004) and those who did not (Loverro *et al.*, 2002; Kilic-Okman *et al.*, 2004; Yilmaz *et al.*, 2005). Interestingly, administration of insulin sensitizers, such as metformin, as it is proposed in PCOS patients to improve ovulation induction and other parameters (Stadtmauer and Oehninger, 2005), has led to a further increase of serum homocysteine in these patients, despite the decrease in insulin resistance (Vrbikova *et al.*, 2002; Kilicdag *et al.*, 2005b). This effect may be explained by a metformin-induced folate depletion (Wulffele *et al.*, 2003), and may be prevented by concomitant folate supplementation (Kilicdag *et al.*, 2005a). An adequate folate supplementation will also prevent an increase in plasma homocysteine during weight loss, which is the first therapeutic measure to be taken in obese PCOS patients (Henning *et al.*, 1998; Volek *et al.*, 2002; Ortega *et al.*, 2006). Finally, only two studies did not report elevated homocysteine concentration among patients with polycystic ovaries: one study including patients with polycystic-appearing ovaries but possibly not all had PCOS (Sills *et al.*, 2001), and an Italian study including 70 PCOS patients with low folate intake and a very high prevalence of the mutated 677T allele, as is usually observed in that population (Orio *et al.*, 2003).

Folate metabolism and outcome of assisted reproduction techniques

To investigate the question whether folate metabolism had an impact on the ovarian response to ovulation induction treatments for IVF (Table II), 105 IVF patients were included in a prospective study and had their *MTHFR* 677 genotype determined (Thaler *et al.*, 2006). A total of 269 IVF cycles were started and 245 led to oocyte retrieval. The analysis of these cycles showed that patients with a *MTHFR* 677 CT or TT genotype required significantly higher FSH doses for ovulation induction than homozygous wild-type patients, whereas they nevertheless had a lower ovarian response. Similarly, the number of oocytes collected and the maximal serum estradiol concentration were significantly lower in patients carrying the mutated T allele, whether they were homozygous or heterozygous. Fertilization and implantation rates were not affected by the *MTHFR* polymorphism. The preceding differences remained significant when the subgroup of patients over

Table II. *MTHFR* metabolism and IVF outcome

Reference	Patients	Impact
Martinelli *et al.* (2003)	162 IVF patients	Prevalence of 677TT not different in case of post-IVF implantation failure (1st attempt) vs. fertile controls
Azem *et al.* (2004)	45 IVF patients	Higher prevalence of 677TT in case of at least four post-IVF implantation failures vs. fertile controls
Haggarty *et al.* (2006)	602 IVF patients	Higher chance of live birth in 1298AA vs. 1298CC and in 677CT vs. 677CC patients (all patients supplemented with folate)
Dobson *et al.*	197 IVF couples	No impact of MTHFR polymorphism on embryo quality, chance of pregnancy and risk of early pregnancy loss (all patients supplemented with folate)
Thaler *et al.* (2006)	105 IVF patients	Higher FSH doses, lower ovarian response in 677CT/TT vs. 677CC patients when >35 years; no differences <35 years

>years old was analysed; however in younger patients, no significant differences were observed. Thus there may be an accelerated depletion of the ovarian reserve in patients with *MTHFR* polymorphism, which becomes clinically evident beyond the age of 35. *MTHFR* polymorphism had no impact on fertilization and implantation rates, which was consistent with the findings of an Italian group who showed there was no difference in the prevalence of *MTHFR* polymorphism between 234 women who conceived spontaneously and 162 patients with implantation failure after IVF, most of whom were undergoing their first cycle (MartinelLi *et al.*, 2003). In contrast, an Israeli group reported a significantly higher prevalence of *MTHFR* C677T homozygotes as well as other inherited thrombophilia mutations in patients with post-IVF implantation failure (Azem *et al.*, 2004). These authors however selected only patients with at least four consecutive IVF implantation failures despite the transfer of at least three good quality embryos, thus *MTHFR* polymorphism could be involved in such repeated and otherwise unexplained failures. In a recent study including 602 IVF patients, women with a heterozygous *MTHFR* 677 CT rather than a homozygous CC genotype had a slightly, but significantly increased chance to have a viable pregnancy, whereas for the *MTHFR* 1298 polymorphism, the homozygous AA genotype was significantly linked to a better IVF outcome (Haggarty *et al.*, 2006). Finally, in another recent study on 197 IVF couples, neither A1298C nor C677T polymorphism, in any of the partners, was associated with embryo quality, the chance of pregnancy and the risk of early pregnancy loss (Dobson *et al.*, 2007). However, in the two latter studies all of the female patients had been taking folate supplementation prior to their IVF attempt, thus a possible effect of these polymorphisms could have been masked.

Nonetheless, the above mentioned results raise the question of whether folate supplementation could improve IVF outcome, particularly in patients beyond 35 years and presenting with *MTHFR* polymorphism, and, if so, at which doses and for which treatment duration.

Folate metabolism and the risk of multiple pregnancies

A negative impact of the *MTHFR* C677T polymorphism on follicular growth and maturation may also explain an earlier observation of a significantly lower prevalence of the *MTHFR* 677T allele in women who spontaneously conceived dichorionic twins when compared with those having spontaneous singleton pregnancies (Hasbargen *et al.*, 2000). The frequency of homozygous wild-type, heterozygous and homozygous mutation carriers was 48.7, 41.7 and 9.6%, respectively, in 156 mothers with singleton pregnancies, whereas in 40 women with dichorionic twin pregnancies, these frequencies were 72.5, 22.5 and 5.0%, respectively. Thus a reduced MTHFR activity and the ensuing reduced SAM availability or hyperhomocysteinemia could inhibit polyovulation, which is a condition for spontaneous dichorionic pregnancies (Hall, 2003). On the other hand, it may also be hypothesized that low folate availability and hypomethylation, either due to poor dietary folate intake or genetic polymorphism, could lead to undetected loss of a dichorionic co-twin, a relatively common phenomenon called the vanishing twin (Landy and Keith, 1998). Whatever the exact mechanism, the influence of folate metabolism on the probability of dizygotic twinning is further illustrated by the observation that the rate of twin births is low in populations with a high prevalence of mutated *MTHFR* alleles, whereas it is rather high in ethnic groups with a low mutation frequency (Hasbargen *et al.*, 2000). However, other authors did not find any association between the prevalence of dizygotic twinning and the *MTHFR* genotype in large samples of Australian and Dutch populations (Montgomery *et al.*, 2003).

Nevertheless, there has been considerable controversy about whether or not periconceptional folic acid supplementation to prevent neural tube defects, would expose women to a higher risk of spontaneous multiple pregnancy and birth. The question was first raised in a Hungarian placebo-controlled study including >5500 pregnant women and showing a 40% increase of the multiple birth rate in women who took vitamin supplements when compared with those who did not (Czeizel *et al.*, 1994). Other authors reported similar findings (Werler *et al.*, 1997; Ericson *et al.*, 2001; Vollset *et al.*, 2005). However, different confounding elements could have biased these results, such as the inclusion of pregnancies after infertility treatments that are known to induce up to 25% multiple pregnancies (Berry *et al.*, 2005), or the use of different kinds of supplements (folic acid alone at very low or very high doses, multivitamin preparations, and sometimes supplements of unknown composition), or the retrospective study design with self-reported supplementation or an extremely low proportion of women who used supple meats. In contrast, several studies investigating the prevalence of multiple births before and after the onset of systematic food fortification programs in countries where this was the case did not find any increase that could have been attributable to folic acid intake in the general population (Waller *et al.*, 2003; Kucik and Correa, 2004; Lawrence *et al.*, 2004; Signore *et al.*, 2005). Moreover, the data from a community intervention program of folic acid supplementation in a homogeneous Chinese rural population including

>242 000 women did not find any difference in multiple birth rate of women under periconceptional supplementation with 0.4 mg folic acid when compared with those who were not supplemented (Li *et al.*, 2003). The Hungarian authors extended their study and included over 38 000 pregnant women; when only folic acid supplementation (at a high dose of 6 mg/day) was considered, the frequency of twinning was still higher than in untreated women, but this did not reach statistical significance (Czeizel and Vargha, 2004). In a recent Swedish study, possible confounding factors, such as infertility treatments, immigration, maternal age, parity and smoking, have been taken into account; nevertheless, the authors conclude folic acid supplementation to be a relatively weak but significant risk factor for dizygotic twinning (Kallen, 2004). Finally, in a recent study including 602 patients undergoing IVF and 932 women who conceived naturally, the authors also demonstrated a small but significant increase in the risk of dizygotic twinning among the infertile population (Haggarty *et al.*, 2006). Thus, this question is still a matter of debate.

Folate metabolism and early pregnancy loss

The question of the involvement of folate metabolism in embryonic viability has also been raised with regard to the relationship among folates, homocysteine and early pregnancy loss. Such a relationship has been suggested first in CBS-deficient patients with homocystinuria who presented with severe hyperhomocysteinemia and a spontaneous abortion rate of almost 50% (Mudd, 1985), although currently, a careful monitoring of these patients has allowed a better pregnancy outcome to be achieved (Levy *et al.*, 2002). In a population-based case–control study, women with low plasma folate levels had a higher risk of early pregnancy loss than women with normal or high levels (George *et al.*, 2002). Moderate hyperhomocysteinemia has also been found to be a risk factor for recurrent early pregnancy loss (REPL) (Steegers-Theunissen *et al.*, 1992a; Wouters *et al.*, 1993; Coumans *et al.*, 1999; Del Bianco *et al.*, 2004) and even for first early pregnancy loss (Gris *et al.*, 2003). A recent meta-analysis confirmed an increased risk of hyperhomocysteinemia for REPL, defined as two or more spontaneous abortions before 16 weeks of menstrual age (Nelen *et al.*, 2000). As to the possible impact of *MTHFR* polymorphism, 677 TT homozygosity has been shown to increase the risk in some studies, whereas in others, no such effect could be detected; these studies have been reviewed elsewhere (Zetterberg, 2004). In a recent meta-analysis by Rey *et al.*(2003), this polymorphism was not found to increase the risk for REPL.

There are several reasons to explain the inconsistency of these results. First, there is no homogeneous definition of REPL (at least two or three consecutive spontaneous abortions), as well as of hyperhomocysteinemia (fasting or afterload concentrations). Second, the possible impact of the fetal *MTHFR* genotype on the risk of REPL has not been investigated in most of the studies. Recently, Zetterberg *et al.* (2002) emphasized the importance of this parameter, as they observed an OR of 14.2 (95% confidence interval: 1.78–113) in spontaneously aborted embryos presenting with one or more *MTHFR* 677T and 1298C alleles when compared with the wild-type (677CC and 1298AA) genotype. Thus the risk could even be higher when both the mother and her fetus are homozygous, as has been shown for the risk of neural tube defects (Christensen *et al.*, 1999). Third, possible gene–gene interactions have to be taken into account, as has already been mentioned in the context of male infertility. As an example, the interference of a transcobalamin (TC) polymorphism, *TC C776G*, which influences homocysteine metabolism has been investigated (Zetterberg *et al.*, 2003). TC is a vitamin B12-binding protein and plays a role in the transport and bioavailability of this vitamin. Patients with a heterozygous or homozygous *TC 776* mutation have lower serum TC concentration and a tendency toward a higher homocysteine concentration (Namour *et al.*, 2001). Genotype analysis in fetal tissues from 76 spontaneous abortions, most of them occurring earlier than week 12, showed that embryos presenting with a combined *MTHFR* 677TT and TC 776CG or TC 776GG genotype had an increased risk for REPL when compared with embryos that had only one of these mutated genotypes (Zetterberg *et al.*, 2003). Fourth, as suggested by the preceding observation, etiologies other than folate deficiency or *MTHFR* polymorphism may lead to hyperhomocysteinemia and subsequent pregnancy loss. The association between REPL and vitamin B12 has been illustrated by two case reports concerning a 38 year-old woman with four episodes of early spontaneous abortion vitamin B12 deficiency and bone marrow megaloblastosis (Candito *et al.*, 2003), and a 36 year-old patient with documented familial and personal history of Addison–Biermer disease, who had experienced 12 episodes of spontaneous abortion in the absence of any other known causes of REPL (Gueant *et al.*, 2004).

Despite several pathophysiological hypotheses including impaired cell proliferation, increased oxidative stress, apoptosis, reduced extra-embryonic vascular development and hypomethylation (Zetterberg, 2004; Latacha and Rosenquist, 2005), it is not clear whether hyperhomocysteinemia is causally related to REPL or whether it is only a marker of the increased risk of REPL. Actually, lowering homocysteine concentration by B-vitamin supplementation has been shown to have a positive effect in several case reports and in small series, with spontaneous pregnancies occurring after a few months of treatment in patients who had previously experienced between 4 and 12 early spontaneous abortions (Quere *et al.*, 1998, 2001; Candito *et al.*, 2003; Gueant *et al.*, 2004).

Pathophysiology of folate deficiency-induced female infertility

Possible mechanisms of the deleterious effects of folate deficiency and homocysteine accumulation on female fertility include, as in the male, reduced cell division (e.g. of oogonia during oogenesis or of granulosa cells during folliculogenesis), inflammatory cytokine production, altered NO metabolism, oxidative stress, apoptosis and defective methylation reactions.

Excessive MCP-1 and IL-8 production has been associated with endometriosis-related infertility (Calhaz-Jorge *et al.*, 2003; Gmyrek *et al.*, 2005), whereas NO is involved in nearly all steps of female reproduction, i.e. in ovulation, early embryonic cleavage, implantation, regulation of arterial pressure, uterine quiescence and, finally, labor contractions and cervical ripening. Importantly, physiological NO concentrations are within a narrow range and either excess or lack of NO will induce an adverse reproductive outcome (Maul *et al.*, 2003; Thaler and Epel, 2003). Similarly, oxidative stress and apoptosis play a role in physiological events, such as follicular development and

cyclic endometrial changes, as well as in various pathological situations, such as infertility, endometriosis, spontaneous abortions, pre-eclampsia, gestational diabetes, fetal embryopathies and preterm labor (Agarwal *et al.*, 2005; Hussein, 2005). Thus, *MTHFR* polymorphism-related hyperhomocysteinemia may activate apoptosis, leading to follicular atresia, as suggested by the above-mentioned rhesus model. Whereas homocysteine-induced apoptosis remains to be investigated in granulosa cells and other follicular constituents, it has already been demonstrated in other tissues, such as the endothelium and trophoblast (Steegers-Theunissen *et al.*, 2000; Di Simone *et al.*, 2003; Suhara *et al.*, 2004).

Finally, insufficient availability of methyl groups for DNA, protein and lipid methylation could impair proliferation and differentiation of the granulosa layer, thus inhibiting follicular maturation as well as steroidogenesis. In fact, it has recently been shown that DNA methylation is involved in the regulation of differential aromatase expression in bovine granulosa and corpus luteum cells (Vanselow *et al.*, 2005).

Conclusion

Folate metabolism is involved in a large number of physiological and pathophysiological processes in the field of andrology and gynecology. There is a growing body of evidence demonstrating a relationship between folate and other B vitamin deficiencies, hyperhomocysteinemia and gonadal abnormalities, such as altered spermatogenesis and impaired ovarian reserve, as well as male and female infertility. However, the exact mechanisms underlying these phenomena still need to be further investigated.

Whereas the benefit of a periconceptional folate supplementation in women has been generally accepted as far as the risk of neural tube defects is concerned, the effects of this preventive intervention on other developmental abnormalities of the growing fetus, as well as on the success rate of assisted reproduction techniques are still unclear. Similarly, in the male, there is a considerable lack of information on the exact role of folate metabolism and folate supplementation in normal reproductive functions as well as in the treatment of infertility.

Finally, it has to be emphasized that further studies need to take into account gene–gene as well as gene–environment interactions, which have often biased previous work and led to contradictory conclusions.

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