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Symposium on 'Micronutrients through the life cycle'

Genetic variation in genes of folate metabolism and neural-tube defect risk*

Ivon J. M. van der Linden, Lydia A. Afman, Sandra G. Heil and Henk J. Blom†
Laboratory of Pediatrics and Neurology, Radboud University Nijmegen Medical Center, PO Box 9101, 6500 HB Nijmegen, The Netherlands

Neural-tube defects (NTD) are common congenital malformations that can lead to severe disability or even death. Periconceptional supplementation with the B-vitamin folic acid has been demonstrated to prevent 50–70% of NTD cases. Since the identification of the first genetic risk factor of NTD, the C677T single-nucleotide polymorphism (SNP) in the methylenetetrahydrofolate reductase (MTHFR) gene, and the observation that elevated plasma homocysteine levels are associated with NTD, research has focused on genetic variation in genes encoding for enzymes of folate metabolism and the closely-related homocysteine metabolism. In the present review relevant SNP in genes that code for enzymes involved in folate transport and uptake, the folate cycles and homocysteine metabolism are summarised and the importance of these SNP discussed in relation to NTD risk.

Neural-tube defects: Folate: Genetic variation

Neural-tube defects (NTD) are common, costly and frequently fatal congenital anomalies with an aetiology that remains elusive. All infants with anencephaly are stillborn or die shortly after birth, whereas many infants with spina bifida survive, usually as a result of extensive medical and surgical care. Infants with spina bifida who survive are likely to have severe lifelong disabilities and are at risk for psycho-social maladjustment.

The causes of NTD are multifactorial. The evidence for genetic predisposition as a determinant for NTD is: a preponderance of NTD in females; prevalence differences related to racial and ethnic background (Buccimazza *et al*. 1994; Chatkupt *et al*. 1994); an increased prevalence in siblings (Hall *et al*. 1988). Environmental risk factors for NTD are the use of anti-epileptic drugs such as valproic acid (Lammer *et al*. 1987) and maternal conditions such as diabetes (Becerra *et al*. 1990), hyperthermia (Edwards *et al*. 1995; Graham *et al*. 1998), obesity (Shaw *et al*. 1996; Watkins *et al*. 1996; Werler *et al*. 1996) and certain professions e.g. agriculture or cleaning (Blatter *et al*. 1996).

One of the most promising clues to the causes of NTD is that women who use folic acid periconceptionally are at a 50–70% reduced risk for NTD-affected pregnancies (Smithells *et al*. 1980; Vitamin Study Research Group, 1991; Czeizel & Dudas, 1992). In an attempt to unravel the molecular mechanism underlying this protective effect of periconceptional folic acid supplementation, research on NTD has focused on folate uptake, the folate cycles and the closely-related homocysteine (Hcy) metabolism. The present review will focus on the genetics of NTD, in particular genetic variation in genes encoding for enzymes related to the folate pathway and Hcy metabolism.

Folate transport and uptake

Dietary folates mainly exist as polyglutamates (Tamura & Stokstad, 1973). As the uptake and transport of folates in the body occurs as monoglutamates, the dietary polyglutamated folates have to be deconjugated to

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Abbreviations: BHMT, betaine-homocysteine methyltransferase; cbl, vitamin B_{12}; CBS, cystathionine β-synthase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; FR, folate receptor; GCPII, glutamate carboxypeptidase II; Hcy, homocysteine; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, MTR reductase; NTD, neural-tube defects; RFC, reduced folate carrier; SHMT, serine hydroxymethyltransferase; SNP, single-nucleotide polymorphism; TC, transcobalamin; THF, tetrahydrofolate; TS, thymidylate synthase; VNTR, variable tandem repeat.

†**Corresponding author:** Dr Henk J. Blom, fax +31 24 3668754, email h.blom@cukz.umcn.nl

monoglutamates before absorption. The enzyme responsible for this deconjugation is folylpoly-γ-glutamate carboxypeptidase, which is associated with the intestinal apical brush border (Chandler *et al*. 1991) and is encoded by the glutamate carboxypeptidase II (GCPII) gene. After the deconjugation process the folate monoglutamates are absorbed in the proximal small intestine via a mechanism that involves reduced folate carrier (RFC). Once folate has entered the bloodstream it is mainly present as 5-methyltetrahydrofolate (THF) monoglutamate, which can enter the cell by means of folate receptor (FR) α. FR-α is a glycosylphosphatidylinositol-linked glycoprotein with a high affinity for the monoglutamate 5-methylTHF (Wang *et al*. 1992) and is expressed in a limited number of epithelial cells, predominantly in the proximal tubules of the kidney, the choroid plexus and the placenta (Kamen & Smith, 2004). The other FR, FR-β and FR-γ, possess a lower affinity for 5-methylTHF than FR-α. Besides receptor-mediated transport, 5-methylTHF can also enter the cell by carrier-mediated transport via RFC. In contrast to FR-α, RFC is ubiquitously expressed, although the affinity of RFC for 5-methylTHF is lower than that of FR-α. To retain the folate in the cell the enzyme folylpolyglutamate synthase adds glutamyl groups to the existing glutamyl group of folate, as polyglutamates are poorly transported across the cell membrane.

Genetic variation

Glutamate carboxypeptidase II

Genetic variation has been demonstrated in the GCPII gene. It has been reported (Devlin *et al*. 2000) that the C1561T single-nucleotide polymorphism (SNP) in the GCPII gene (His475Tyr) leads to a reduced activity of the folylpoly-γ-glutamate carboxypeptidase enzyme and subsequently decreased plasma folate levels and increased plasma Hcy levels. In contrast to these data Afman *et al*. (2003*b*) observed that the C1561T SNP results in increased plasma folate levels and they could not demonstrate an association between this SNP and NTD risk. Furthermore, other studies (Lievers *et al*. 2002; Morin *et al*. 2003*a*; Relton *et al*. 2003) have failed to find an effect of the GCPII C1561T SNP on NTD risk or metabolite levels. Parenthetically, the supplemented form of folate, folic acid, is a synthetic monoglutamate and does not require folylpoly-γ-glutamate carboxypeptidase to be absorbed. The use of periconceptional folic acid supplementation could thus attenuate possible effects of genetic variation in the GCPII gene.

Folate receptors α and β

Folate mainly enters the cell via FR-α, and the importance of FR-α is demonstrated by the observation that folate-binding protein 1 (the mouse orthologue of human FR-α) nullizygosity is embryonically lethal in knock-out mice (Piedrahita *et al*. 1999). Barber *et al*. (1998) have investigated the molecular genetic variation within the *FR*-α gene among a group of newborns with spina bifida. Using single-stranded conformation polymorphism analysis, dideoxy fingerprinting and sequence analysis they were unable to find any variation in exons 3–6 encoding for the mature protein. Furthermore, an analysis of the total coding region including the intron–exon boundaries and the signal sequences of human *FR-α* and *FR-β* in thirty-nine spina bifida patients, forty-seven mothers with a spina bifida-affected child and ten controls has also failed to identify any variation (Heil *et al*. 1999). Recently, two SNP in the 5′-untranslated region of the *FR-α* have been reported, both with a low prevalence (approximately 0·4% each; Nilsson & Borjel, 2004).

O'Leary *et al*. (2003) have focused on the *FR-β* gene instead of the *FR-α* gene and have examined five SNP in the *FR-β* gene that were present in the available database. Four of these SNP were not identified in their study population. However, they did confirm the presence of an A→T substitution in intron 1 with a high allele frequency, but no association with NTD risk was found.

The coding regions of the *FR-α* and *FR-β* genes do not show any variation. Some variants have been identified in the untranslated regions of these two genes, but none of the variation identified so far has been associated with NTD risk. It is possible that the FR genes do not tolerate any variation in their coding regions, and variants in the FR genes may not be compatible with life. This possible explanation is supported by data from the folate-binding protein 1-knock-out mouse model (Piedrahita *et al*. 1999).

Rothenberg *et al*. (2004) have recently identified the presence of autoantibodies directed against FR in the serum of women whose pregnancy is or was complicated by NTD. The autoantibodies were shown to block the binding of folic acid to the FR and to inhibit folate uptake by KB cells, a human epidermoid carcinoma cell line. An additional amount of folate could theoretically overcome this blockage, and thus the presence of FR autoantibodies may explain part of the preventive effect of periconceptional folic acid supplementation (Rothenberg *et al*. 2004). The findings of this study are promising; however, the study population was small and additional studies in larger populations are necessary to determine the exact role of FR autoantibodies in the aetiology of NTD.

Reduced folate carrier

An A80G substitution has been identified in the *RFC-1* gene that leads to the replacement of a histidine by an arginine (His27Arg; Chango *et al*. 2000). Shaw *et al*. (2002) have reported the absence of an association between the *RFC-1* A80G SNP and spina bifida risk in children, although they did observe a possible increase in spina bifida risk for children whose mothers did not use folic acid periconceptionally. De Marco *et al*. (2003) have identified the A80G SNP in the *RFC-1* gene as a risk factor for NTD in both patients and their mothers. A study in a Chinese population has reported an increased NTD risk in patients with the G80G genotype, especially when their mothers did not take folic acid supplements (Pei *et al*. 2005). Other studies (Relton *et al*. 2003, 2004*a*,*b*) have failed to find an association between the RFC-1 A80G SNP and maternal NTD risk, although one study (Morin *et al*. 2003*a*) has demonstrated an association between the

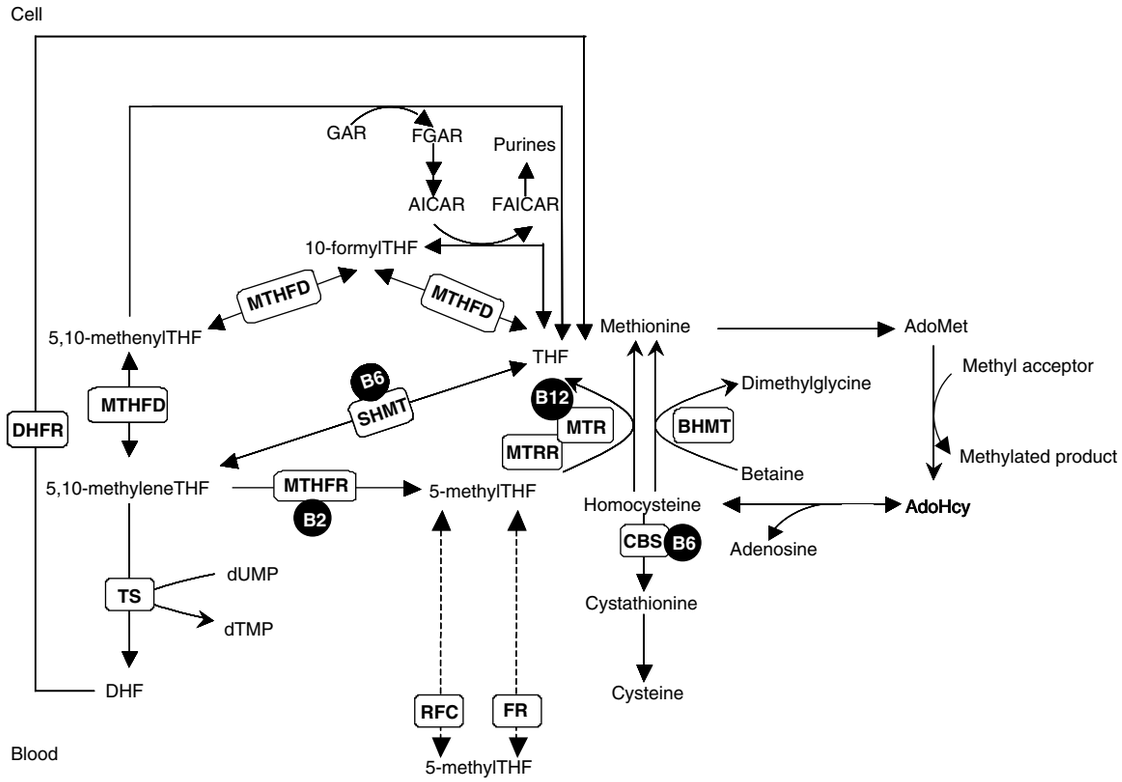

Fig. 1. Simplified overview of the folate pathway and homocysteine metabolism. GAR, glycinamide ribonucleotide; FGAR, formyl glycinamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; FAICAR, formyl 5-aminoimidazole-4-carboxamide ribonucleotide; THF, tetrahydrofolate; DHF, dihydrofolate; DHFR, dihydrofolate reductase; MTHFD, methylenetetrahydrofolate dehydrogenase; TS, thymidylate synthase; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; RFC, reduced folate carrier; FR, folate receptor; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; B6, pyridoxal 5′-phosphate; B12, cobalamin; B2, riboflavin.

RFC-1 G80G genotype and low erythrocyte folate levels. In summary, the RFC-1 A80G SNP may be a NTD risk factor, especially when maternal folate status is low, suggesting that sufficient folate can attenuate the effect of this polymorphism.

Folate cycle

On entering the cell 5-methylTHF functions as a methyl donor for the remethylation of Hcy to methionine, with subsequent formation of THF (Fig. 1). The THF formed after the demethylation of 5-methylTHF is used as a substrate in several reactions. THF can be converted to 10-formylTHF via a reversible reaction catalysed by formylTHF synthase, which is one of the three enzymic properties of the tri-functional enzyme methyleneTHF dehydrogenase (MTHFD). The other two enzymic properties of MTHFD are methenylTHF cyclohydrolase that reversibly converts 10-formylTHF to 5,10-methenylTHF and MTHFD that reversibly converts 5,10-methenylTHF to 5,10-methyleneTHF (Hum *et al*. 1988). Thus, the MTHFD enzyme plays a central role in the folate metabolism (Fig. 1). Folate metabolism acts as a C_1 unit donor in the synthesis of the purines adenine and guanine, which are building blocks for DNA. Another DNA building block is thymidine, the synthesis of which is catalysed by the enzyme thymidylate synthase (TS). In this reaction 5,10-methyleneTHF donates a methylene group to dUMP to form dTMP and dihydrofolate (DHF). The DHF is reduced back to THF by the enzyme DHF reductase (DHFR).

The enzyme serine hydroxymethyltransferase (SHMT) catalyses the reversible conversion of serine and THF to glycine and 5,10-methyleneTHF (Fig. 1) and is present in two isoforms, i.e. a cytosolic and a mitochondrial form. Both enzymes require pyridoxal phosphate, an active form of vitamin B_6 (Stover *et al*. 1997). The 5,10-methyleneTHF can be further reduced to 5-methylTHF by the enzyme methyleneTHF reductase (MTHFR). This enzyme is of great importance in the regulation of available folate for the remethylation of Hcy.

Genetic variation

Methylenetetrahydrofolate dehydrogenase

A study of the *MTHFD* gene in 117 patients with NTD by single-stranded conformation polymorphism analysis (Hol *et al*. 1998) has identified a G1958A SNP that results in the substitution of an arginine by a glutamine within the

10-formylTHF synthetase domain of the MTHFD enzyme (Arg653Gln). In this study the G1958A SNP had a similar frequency among patients with NTD and controls, and did not influence plasma Hcy concentration. More recently, in an Irish population it has been shown that the presence of this Arg653Gln SNP is associated with an increased risk for mothers to have an NTD-affected child, but not with an increased NTD risk for the patient (Brody *et al*. 2002). More studies on the *MTHFD* Arg653Gln variant are necessary to determine the influence of this SNP on NTD risk.

Thymidylate synthase

A 28 bp tandem repeat in the promoter enhancer region of the TS gene has been identified, typically containing two or three repeats (Kaneda *et al*. 1987). The triple repeat results in increased TS gene expression, whereas the double repeat is associated with decreased TS gene expression (Kaneda *et al*. 1987; Horie *et al*. 1995). The repeat is associated with decreased plasma folate and total plasma Hcy concentrations in a Chinese population (Trinh *et al*. 2002); however, no such effect was found in a north-western European population (Brown *et al*. 2004). Two studies (Volcik *et al*. 2003; Wilding *et al*. 2004) have examined the association between the repeat and NTD risk. Volcik *et al*. (2003) have shown that the double repeat is associated with NTD risk in infants, especially in non-Hispanic US whites, whereas Wilding *et al*. (2004) were unable to demonstrate an association between the repeat and NTD risk in subjects with NTD and their parents.

A 6 bp deletion in the 3′-untranslated region has been suggested to play a role in TS mRNA stability and translation (Ulrich *et al*. 2000) and the non-deleted genotype has been associated with increased NTD risk only in non-Hispanic US white subjects (Volcik *et al*. 2003). The only study that has examined the 6 bp deletion in relation to plasma Hcy and folate levels (Kealey *et al*. 2005) has reported an association between this TS variant and erythrocyte folate levels and plasma Hcy levels in non-smoking individuals. Based on these data, more studies on the relationship between the TS variants and folate status and NTD risk are warranted.

Dihydrofolate reductase

All folic acid in vitamin supplements and food fortification is present in the unreduced form and requires the action of DHFR before it can participate in cellular processes.

Recently, a 19 bp deletion has been described within intron-1 of the DHFR gene, which eliminates a potential SP1 transcription factor-binding site, possibly affecting DHFR gene expression (Johnson *et al*. 2004). In this study the 19 bp deletion was shown to increase the risk of having a child with spina bifida. These data warrant more studies on the association between the 19 bp deletion in the DHFR gene and NTD risk.

Serine hydroxymethyltransferase

A study has been conducted to identify genetic variation in both the cytosolic and mitochondrial isoforms of the *SHMT* gene in seventy patients with NTD (Heil *et al*. 2001). Several SNP were identified, of which the C1420T substitution in the cytosolic *SHMT* gene and the 4 bp deletion in the 3′-untranslated region of the mitochondrial isoform of the *SHMT* gene were common. The C1420T SNP, changing a leucine into a phenylalanine (Leu474Phe) in the cytosolic protein, was not found to be associated with NTD risk in mothers of patients with NTD, although the C1420C genotype resulted in elevated plasma Hcy concentrations and decreased erythrocyte and plasma folate levels in the mothers. The 4 bp deletion in the 3′-untranslated region of the mitochondrial isoform of the *SHMT* gene did not influence NTD risk, nor plasma Hcy and folate levels (Heil *et al*. 2001).

Other studies (Relton *et al*. 2004*a*,*b*) that have also looked at the C1420T SNP in the cytosolic isoform of *SHMT* have demonstrated a non-significant protective effect of the T allele in mothers. The studies of Geisel *et al*. (2003) and Relton *et al*. (2004*a*) have not shown a relationship between the C1420T SNP in the cytosolic isoform *SHMT* and plasma Hcy and erythrocyte folate. In conclusion, the C1420T SNP in the cytosolic isoform of *SHMT* is at most a minor risk factor for NTD risk.

Methylenetetrahydrofolate reductase

In collaboration with researchers at Montreal Children's Hospital, Montreal, Canada, Frosst *et al*. (1995) have identified the C677T (Ala222Val) SNP in the *MTHFR* gene that results in a mildly dysfunctional 'thermolabile' MTHFR enzyme and have demonstrated an association between the T677T genotype and elevated plasma Hcy levels. Furthermore, an increase in spina bifida risk for both mothers and children with the *MTHFR* T677T genotype has been reported (van der Put *et al*. 1995), thus identifying the first genetic risk factor for spina bifida.

Following the identification of the *MTHFR* C677T SNP many studies have investigated this SNP in relation to NTD risk. Some results (Mornet *et al*. 1997; Speer *et al*. 1997; Koch *et al*. 1998; Botto & Yang, 2000) have been contradictory and inconclusive, in part because of the considerable variation in *MTHFR* C677T allele frequency among different geographic regions and ethnicities.

A meta-analysis (van der Put *et al*. 1997*a*) on the association between the *MTHFR* C677T SNP and NTD risk has been carried out by combining all published data of control groups and families affected by NTD. Mothers with the *MTHFR* T677T genotype were found to have an overall 60% increase in risk of having NTD-affected children (odds ratio 1·6 (95% CI 1·1, 2·3)) and children with the *MTHFR* 677TT genotype were 80% more likely to have NTD (odds ratio 1·8 (95% CI 1·3, 2·5)). Botto & Yang (2000) have also conducted a meta-analysis on the *MTHFR* C677T SNP in relation to NTD risk. They have reported a 2-fold increase in risk for being a mother of a NTD-affected child (odds ratio 2·0 (95% CI 1·5, 2·8)) and a 1·8-fold increased NTD risk (odds ratio 1·8 (95% CI 1·4, 2·2)) for infants who have the *MTHFR* T677T genotype. In summary, the *MTHFR* T677T genotype is a genetic risk factor for NTD in both patients with NTD and their mothers.

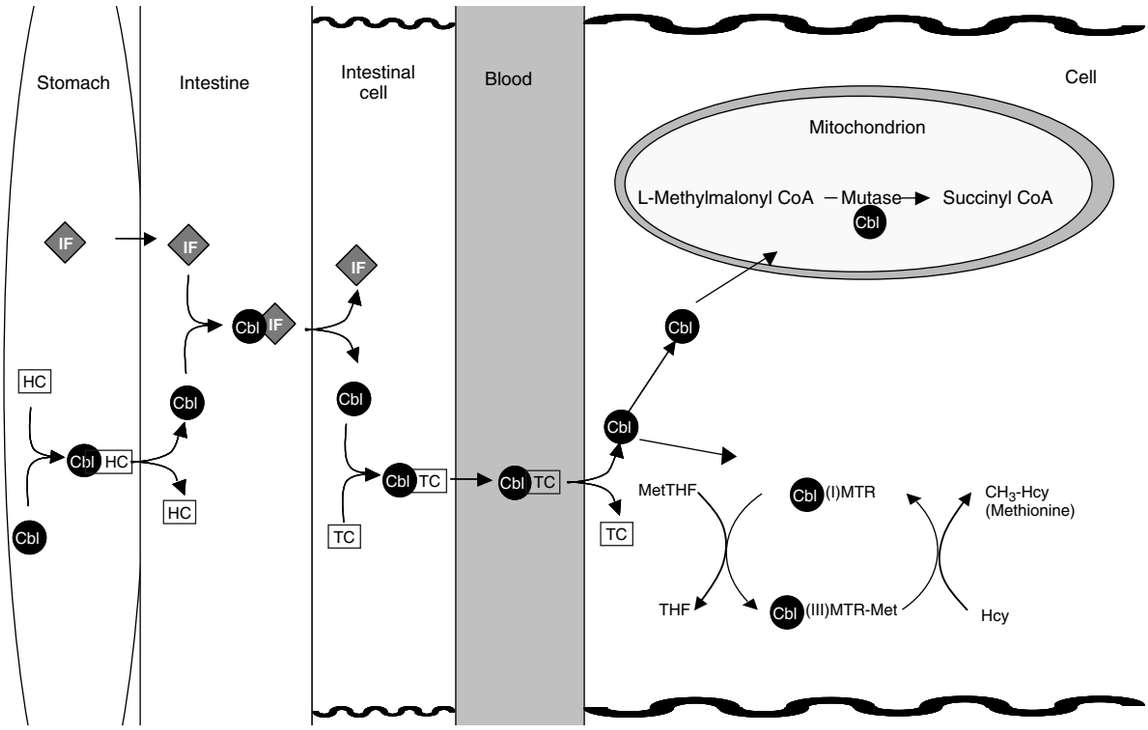

Fig. 2. Vitamin B_{12} pathway. cbl, Cobalamin; IF, intrinsic factor; HC, haptocorrin; TC, transcobalamin; mutase, L-methylmalonyl-CoA mutase; Met, methyl; THF, tetrahydrofolate; MTR, methionine synthase; Hcy, homocysteine.

A second SNP in the *MTHFR* gene involves the substitution of an adenine by a cytosine on position 1298 (A1298C), leading to an amino acid change of a glutamate into an alanine (Gln429Ala; van der Put *et al*. 1998). This SNP has also been associated with decreased MTHFR enzyme activity, although not as pronounced as that of the *MTHFR* C677T SNP (van der Put *et al*. 1998; Weisberg *et al*. 1998; Botto & Yang, 2000). To date, only one study (De Marco *et al*. 2002) has found an association between the *MTHFR* A1298C SNP and NTD risk, and the SNP does not seem to influence plasma Hcy and folate levels (van der Put *et al*. 1998; Weisberg *et al*. 1998; Stegmann *et al*. 1999; Volcik *et al*. 2000; Cunha *et al*. 2002; Parle-McDermott *et al*. 2003). The *MTHFR* A1298C SNP is not likely to be a risk factor for NTD.

Homocysteine metabolism

Remethylation

Remethylation of Hcy by the enzyme methionine synthase (MTR) takes place in all cells, except the erythrocytes, and involves the donation of a methyl group from 5-methylTHF to Hcy leading to the formation of methionine and THF (Fig. 1). The enzyme MTR requires vitamin B_{12} (cobalamin; cbl) as a cofactor, and the resulting complex, cbl(I)MTR, can bind the methyl group of 5-methylTHF to form methylcbl(III)MTR. The transfer of this methyl group to Hcy leaves the reformed cbl(I)MTR complex available for another methyl donation by 5-methylTHF. However, the cbl(I)MTR complex is sensitive to oxidation into the inactive cbl(II)MTR complex, which can be reactivated to the functional methylclb(III)MTR by the enzyme methionine synthase reductase (MTRR) and the donation of a methyl group from S-adenosylmethionine (Fig. 2).

While the MTR enzyme is expressed in almost every cell, another Hcy-remethylation system, the betaine-Hcy methyltransferase (BHMT) enzyme, is mainly expressed in the liver and kidneys. BHMT can remethylate Hcy by donating a methyl group from betaine and is responsible for 50% of the Hcy remethylation.

Vitamin B_{12} uptake, transport and metabolism

In plasma only 20% of the total vitamin B_{12} is bound to holo-transcobalamin (TC), the remaining 80% is bound to holo-haptocorrin and is not available for cellular uptake. Only vitamin B_{12} bound to TC is recognised by a specific carrier and taken up by the cell (Fig. 2), and the function of holo-haptocorrin is not clear. Thus, plasma holo-TC concentrations may be a better indicator of vitamin B_{12} status than total plasma vitamin B_{12} levels (holo-TC + holo-haptocorrin). In the cell vitamin B_{12} is converted into two metabolically-active forms, i.e. methylcbl required as a cofactor for MTR, which is present in the cytosol, and 5′-deoxyadenosylcbl necessary for the function of methylmalonyl-CoA mutase, which is present in the mitochondria (Fig. 2) and converts L-methylmalonyl-CoA to succinyl-CoA. As a metabolic consequence, vitamin B_{12} deficiency will result in elevated plasma Hcy levels and elevated plasma methylmalonic acid (Elin & Winter, 2001).

Transmethylation

Methionine adenosyltransferase catalyses the biosynthesis of S-adenosylmethionine from methionine and ATP. Methionine adenosyltransferase is present in two isoforms; one form is encoded by the methionine adenosyltransferase 2a gene, which is present in nearly all tissues, and the other form is encoded by the methionine adenosyltransferase 1a gene, which is expressed only in the liver. S-adenosylmethionine is the ultimate source of methyl groups for methylation reactions of, for example, DNA, RNA, proteins and lipids. The transfer of a methyl group from S-adenosylmethionine to a methyl acceptor results in the formation of S-adenosylHcy, which is hydrolysed to adenosine and Hcy by the enzyme S-adenosylHcy hydrolase. The equilibrium of this reversible reaction favours S-adenosylHcy formation, which is an allosteric inhibitor of methylation. Thus, Hcy and adenosine need to be metabolised rapidly in order to maintain low S-adenosylHcy levels.

Trans-sulfuration

In the transmethylation and remethylation pathway Hcy is retained. In the trans-sulfuration pathway Hcy is irreversibly degraded to cysteine by two pyridoxal phosphate-dependent enzymes, i.e. cystathionine β-synthase (CBS), which catalyses the condensation of serine and Hcy to cystathionine, and γ-cystathionase, which catalyses the hydrolysis of cystathionine to cysteine and α-ketobutyrate.

Genetic variation

Methionine synthase

Sequencing analysis of the coding region of the *MTR* gene (van der Put *et al*. 1997*b*) has revealed a A2756G SNP, changing an aspartic acid residue (believed to be part of a helix involved in cofactor binding) to a glycine (Asp919Gly). Several studies have examined the *MTR* A2756G SNP in relation to NTD. In some of these studies the presence of the G allele has been shown to be associated with an increased risk for the mother to have a child with NTD (Doolin *et al*. 2002), an increased NTD risk in the child (Gueant-Rodriguez *et al*. 2003; Zhu *et al*. 2003) or both (Gos *et al*. 2004). However, in other studies no association between the *MTR* SNP and NTD risk has been found (van der Put *et al*. 1997*b*; Morrison *et al*. 1998; Lucock *et al*. 2000, 2001; De Marco *et al*. 2002), while in one study the *MTR* 2756GG genotype has been found to be associated with a decreased NTD risk in the patients (Christensen *et al*. 1999).

Plasma Hcy levels have been reported in some studies to be increased for the *MTR* 2756AA genotype (Harmon *et al*. 1999; Tsai *et al*. 2000), although the relationship is not always significant (Chen *et al*. 2001; Kluijtmans *et al*. 2003), and in other studies it is present (van der Put *et al*. 1997*b*; Jacques *et al*. 2003; Klerk *et al*. 2003). The *MTR* A2756A genotype does not seem to influence plasma folate levels (Harmon *et al*. 1999; Jacques *et al*. 2003; Klerk *et al*. 2003; Kluijtmans *et al*. 2003), although one study has reported increased plasma folate levels for the *MTR* G2756G genotype (Chen *et al*. 2001). Data on the association between the *MTR* A2756G SNP and plasma Hcy and plasma folate concentrations as well as the relationship between this polymorphism and NTD risk are inconclusive. If there is a relationship between the *MTR* A2756G SNP and NTD risk, it is at most a rather moderate association.

Methionine synthase reductase

Wilson *et al*. (1999) have described a common variant in the FMN-binding domain of the gene encoding for the MTRR enzyme; the A66G SNP, which leads to an amino acid substitution of an isoleucine by a methionine (Ile22Met). They have reported an increased NTD risk for both mothers and patients with the *MTRR* G66G genotype, but only when plasma vitamin B_{12} concentration is low (Wilson *et al*. 1999). A later study (Zhu *et al*. 2003) has reported that the *MTRR* 66AG/GG genotype is associated with an increase in NTD risk for both mothers and patients. In a Polish study the association between the *MTRR* 66GG genotype and NTD risk was found to be confined to lumbosacral NTD (Pietrzyk *et al*. 2003). In other studies (Wilson *et al*. 1999; Lucock *et al*. 2001; Gos *et al*. 2004; O'Leary *et al*. 2005) the *MTRR* 66GG genotype was not found to be significantly associated with NTD risk for mothers and their children. In contrast to the previously discussed data, Relton *et al*. (2004*a*,*b*) have designated the A allele as a risk factor for NTD; however, the *MTRR* genotype distribution was not in Hardy-Weinberg equilibrium. Using a transmission disequilibrium test Doolin *et al*. (2002) have also shown that the A allele is a risk factor for NTD, but only in mothers. Recently, a study was carried out into the association between the *MTRR* A66G polymorphism and spina bifida risk (IJM van der Linden, M den Heijer, LA Afman, H Gellenkink, SHHM Vermeulen, LAJ Kluijtmans and HJ Blom, unpublished results). It was shown that the *MTRR* G66G genotype is a risk factor for spina bifida in mothers, and after performing a transmission disequilibrium test for eighty-two complete triads no preferential transmission of the *MTRR* risk allele from parents to their spina bifida-affected child was identified. A meta-analysis of eight relevant studies on the relationship between the *MTRR* A66G variant and maternal NTD risk has demonstrated the *MTRR* G66G genotype to be associated with an overall 48 % increase in NTD risk in mothers (odds ratio 1·48 (95 % CI 1·00, 2·19); IJM van der Linden, M den Heijer, LA Afman, H Gellenkink, SHHM Vermeulen, LAJ Kluijtmans and HJ Blom, unpublished results).

Olteanu *et al*. (2002, 2004) have shown that the substitution of an isoleucine by a methionine at position 22 in the MTRR enzyme results in a less-efficient repair of the MTR, possibly as a result of a reduced affinity for this enzyme. The *MTRR* A66G SNP has been associated with elevated plasma Hcy levels (Gaughan *et al*. 2001, 2002), although in most studies (Wilson *et al*. 1999; Jacques *et al*. 2003; Kluijtmans *et al*. 2003; Feix *et al*. 2004) an effect of the *MTRR* SNP on plasma Hcy has not been observed.

Table 1. Genetic variants in genes related to the folate cycles and homocysteine metabolism

Gene	SNP	Amino acid substitution	Allele frequency	NTD
GCPII	C1561T*	His475Tyr	approx 0·06 (T)	–
RFC-1	A80G	His27Arg	0·46–0·56 (G)	+/–
MTHFD	G1958A*	Arg653Gln	0·41–0·46 (A)	+/–
TS	28 bp rpt		0·17–0·48 (two rpt)	+/–
	6 bp del		0·29–0·42 (del)	+/–
DHFR	19 bp del		0·45 (del)	+
cSHMT	C1420T	Leu474Phe	0·32–0·36 (T)	–
mSHMT	4 bp del		0·02 (del)	–
MTHFR	C677T*	Ala222Val	0·10–0·50 (T)	+
	A1298C*	Glu429Ala	0·25–0·40 (C)	–
MTR	A2756G	Asp919Gly	0·15–0·20 (G)	+/–
MTRR	A66G	Ile22Met	0·39–0·59 (G)	+
TC	C776G	Pro259Arg	approx 0·45 (G)	–
BHMT	G716A	Arg239Gln	0·25–0·37 (A)	+/–
CBS	844ins68		approx 0·09 (ins)	–
	31 bp VNTR		approx 0·77 (eighteen rpt)	–

SNP, single-nucleotide polymorphism, NTD, neural-tube defect; GCPII, glutamate carboxypeptidase II; RFC-1, reduced folate carrier-1; MTHFD, methylenetetrahydrofolate dehydrogenase; TS, thymidilate synthase; DHFR, dihydrofolate reductase; cSHMT, mSHMT, cytosolic and mitochondrial serine hydroxymethyltransferase respectively; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; TC, transcobalamin; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; del, deletion; ins, insertion; rpt, repeat; VNTR, variable number of tandem repeats; approx, approximately; +, positive relationship with SNP; –, no relationship with SNP; +/–, possible relationship with SNP.
*Coding position is not according to nomenclature.

In summary, the *MTRR* G66G genotype seems to be a NTD risk factor in mothers, without altering plasma Hcy concentration.

Transcobalamin

TC transports vitamin B_{12} into the cell, where it is used as a cofactor in Hcy remethylation and L-methylmalonyl-CoA conversion. Decreased TC saturation with vitamin B_{12} has been reported in mothers with children with NTD (Afman *et al*. 2001). Furthermore, Afman *et al*. (2002) identified five sequence variants in the *TC* gene, of which the C776G SNP (Pro259Arg) has been described previously (Li *et al*. 1994). An analysis of the frequency of each SNP has been conducted in a population comprising mothers of NTD-affected children and controls (Afman *et al*. 2002). None of the SNP was found to be associated with NTD risk or with plasma Hcy concentration, although a trend was observed for the C776G SNP and elevated Hcy levels. The C776G SNP was shown to be associated with low total-TC, holo-TC and apo-TC levels and with a decreased holo-TC : total-TC that could be explained by a reduced binding of vitamin B_{12} to TC (Afman *et al*. 2002). The effect of this variant on TC level and saturation has been confirmed in a different study population (Miller *et al*. 2002).

Other studies have also investigated the relationship between genetic variants in the *TC* gene and NTD risk. Pietrzyk & Bik-Multanowski (2003) have shown that the G776G genotype is associated with an increase in maternal NTD risk, whereas studies by Gueant-Rodriguez *et al*. (2003) and Swanson *et al*. (2005) have found no such effect of the C776G SNP or the other SNP in the *TC* gene on NTD risk. Furthermore, no relationship between the *TC* C776G SNP and plasma Hcy concentrations has been found (Geisel *et al*. 2003; Winkelmayer *et al*. 2004; Gueant-Rodriguez *et al*. 2005; von Castel-Dunwoody *et al*. 2005). In summary, the *TC* C776G SNP does not seem to be a NTD risk factor.

Betaine-homocysteine methyltransferase

An analysis of the *BHMT* gene (Heil *et al*. 2000) has identified the G595A SNP (Gly199Ser), the G716A (Arg239Gln) SNP previously reported by Park & Garrow (1999) and the G1218T (Gln406His) SNP, but has not established an association between any of these SNP and plasma Hcy levels. A more recent study (Weisberg *et al*. 2003) has also found no relationship between the G716A SNP and plasma Hcy concentrations. However, the G716A SNP has been shown to be associated with a decrease in NTD risk in both children and mothers (Morin *et al*. 2003*b*), although a recent study (Zhu *et al*. 2005) did not find a protective effect of the A716A genotype in patients with NTD.

In mouse embryos *BHMT* is not expressed until neural-tube closure is almost completed (Fisher *et al*. 2002), which makes it less likely that the *BHMT* G716A SNP will be a risk factor of NTD in the fetus. Since the BHMT enzyme is responsible for 50% of the liver Hcy remethylation, variation in the maternal BHMT gene could affect maternal Hcy metabolism, thereby influencing the risk of having NTD-affected offspring.

Cystathionine β-synthase

Although CBS is only expressed in the liver and kidneys, it has been reported (Quere *et al*. 1999) that CBS is

expressed during early embryogenesis, and variation in the *CBS* gene may thus influence embryogenesis.

The 68 bp insertion (844ins68) and the 31 bp variable tandem repeat (VNTR) in the *CBS* gene are the most-frequently-studied variations in relation to NTD. In most studies the 844ins68 is not associated with NTD risk in children or mothers (Ramsbottom *et al*. 1997; Morrison *et al*. 1998; Speer *et al*. 1999; Richter *et al*. 2001; Gos *et al*. 2004), although there may be a trend towards a protective effect (Akar *et al*. 2000; Richter *et al*. 2001). A possible gene–gene interaction between the *MTHFR* C677T polymorphism and the *CBS* 844ins68 has also been examined in relation to NTD, but no such association has been reported (Ramsbottom *et al*. 1997; Morrison *et al*. 1998; Speer *et al*. 1999; Richter *et al*. 2001). In conclusion, the *CBS* 844ins68 does not seem to be a NTD risk factor.

The 31 bp VNTR has been described by Kraus *et al*. (1998) and further characterised by Lievers *et al*. (2001). It has also been shown (Lievers *et al*. 2001) that the 31 bp VNTR results in alternative splicing and a subsequent decrease in enzyme activity that is negatively correlated with the number of repeat units, while the number of repeat units is positively associated with plasma Hcy concentrations. A more recent study (Afman *et al*. 2003*a*) has also demonstrated that the 18/18 VNTR genotype is associated with elevated plasma Hcy levels when compared with the 17/18 and 18/19 VNTR genotypes. In addition, it has been reported that plasma Hcy levels decrease in individuals with the 16/17 and 17/18 VNTR genotypes compared with the 17/17 VNTR genotype (Yang *et al*. 2000). However, the 31 bp VNTR does not influence the risk of NTD (Afman *et al*. 2003*a*).

Conclusions

Since the observation that periconceptional folic acid supplementation reduces the risk of having a NTD-affected pregnancy by 50–70% and the identification of the first genetic risk factor of NTD (i.e. the C677T SNP in the *MTHFR* gene), research on these birth defects has focused on genetic variation in genes encoding for the enzymes involved in the folate cycles and the closely-related Hcy metabolism. Many genetic variants have been identified, but only a few of these variants have been associated with NTD risk (see Table 1). Of all variants discussed in the present review the *MTHFR* C677T SNP and the *MTRR* A66G SNP are the only two SNP that can be considered risk factors for NTD. Other genetic variants reported in the present review are less likely to be associated with NTD risk. More studies in sufficiently large populations are required to determine possible associations between most of these SNP and NTD risk.

In order to identify new genetic determinants of NTD, investigation of other genes that are not related to the folate pathway and Hcy metabolism may be required. New strategies, such as the SNP array that enables the identification of thousands of polymorphisms at the same time, can be used in future research to identify new genetic risk factors of NTD.

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