Neural Tube Defects and Folate Pathway Genes: Family-Based Association Tests of Gene–Gene–Environment Interactions

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Background: Folate metabolism pathway genes have been examined for association with neural tube defects (NTDs) because folic acid supplementation reduces the risk of this debilitating birth defect. Most studies addressed these genes individually, often with different populations providing conflicting results.

Objectives: Our study evaluates several folate pathway genes for association with human NTDs, incorporating an environmental cofactor: maternal folate supplementation.

Methods: In 304 Caucasian American NTD families with myelomeningocele or anencephaly, we examined 28 polymorphisms in 11 genes: folate receptor 1, folate receptor 2, solute carrier family 19 member 1, transcobalamin II, methylenetetrahydrofolate dehydrogenase 1, serine hydroxymethyltransferase 1, 5,10-methylenetetrahydrofolate reductase (MTHFR), 5-methyltetrahydrofolate-homocysteine methyltransferase, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase, betaine-homocysteine methyltransferase (BHMT), and cystathionine-beta-synthase.

Results: Only single nucleotide polymorphisms (SNPs) in BHMT were significantly associated in the overall data set; this significance was strongest when mothers took folate-containing nutritional supplements before conception. The BHMT SNP rs3733890 was more significant when the data were stratified by preferential transmission of the MTHFR rs1801133 thermolabile T allele from parent to offspring. Other SNPs in folate pathway genes were marginally significant in some analyses when stratified by maternal supplementation, MTHFR, or BHMT allele transmission.

Conclusions: BHMT rs3733890 is significantly associated in our data set, whereas MTHFR rs1801133 is not a major risk factor. Further investigation of folate and methionine cycle genes will require extensive SNP genotyping and/or resequencing to identify novel variants, inclusion of environmental factors, and investigation of gene–gene interactions in large data sets.

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Of 1,000 births worldwide, in one embryo the neural tube will fail to close properly 28 days after conception, resulting in some form of neural tube defect (NTD). Failed closure at the cranial end, known as anencephaly, is a lethal condition, whereas failed closure at the caudal end usually results in a myelomeningocele. NTDs are the most common debilitating birth defect. Familial studies indicate a significant genetic component to NTDs, with a 40-fold increase in risk in first-degree relatives (Elwood et al. 1992). Myriad environmental exposures have been implicated in the development of NTDs; most notably, a significant decrease in risk can be achieved by maternal folic acid supplementation before conception.

The mechanism by which dietary folate supplementation prevents NTDs is poorly understood (MRC Vitamin Study Research Group 1991). Folic acid derivatives are essential for the synthesis of DNA, cell division, tissue growth, and DNA methylation (Morrison et al. 1998). Methylation enables proper gene expression and chromosome structure maintenance, both of which are critical in the developing embryo (Razin and Kantor 2005). The folate and methionine cycles are linked by the conversion of homocysteine to methionine (Figure 1). In the absence of food frequency data, maternal vitamin supplementation can also serve as a proxy for overall health because of the positive correlation between supplement intake, diet, and a healthy lifestyle (Slesinski et al. 1996). Vitamin supplementation is an important cofactor to consider when studying nutritionally related genes.

Animal models demonstrate that periconceptional folate supplementation protects against congenital defects in the face, neural tube, and conotruncal region of the heart. Low folate could directly limit its availability to cells or indirectly disrupt methionine metabolism, thereby increasing homocysteine in the maternal serum (Rosenquist and Finnell 2001). Either mechanism implicates folate receptor and methionine–homocysteine regulatory genes.

Folate enters cells by folate receptor 1 (FOLR1; GenBank accession no. NM_016725 [http://www.ncbi.nih.gov.GenBank]) and folate receptor 2 (FOLR2; GenBank accession no. NM_000803) or carrier-mediated internalization by solute carrier family 19 member 1 (SLC19A1; GenBank accession no. U15939), also known as reduced folate carrier protein 1. Transcobalamin II (TCN2; GenBank accession no. NM_000355) imports vitamin B12, cobalamin, a cofactor for another folate enzyme, 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR; GenBank accession no. NM_000254). The reactions within the folate metabolism cycle can be very complex, with methylenetetrahydrofolate dehydrogenase 1 (MTHFD1; GenBank accession no. J04031), serine hydroxymethyltransferase 1 (SHMT1; GenBank accession no. NM_004169), and 5,10-methylenetetrahydrofolate reductase (MTHFR; GenBank accession no. NM_005957) being widely studied in the NTD literature.
methylation reactions (Essien and Wannberg 1993). Enzymes that degrade homocysteine regulate homocysteine levels; for example, MTR converts homocysteine to methionine and folate to tetrahydrofolate (Trembath et al. 1999). 5-Methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR; GenBank accession no. AF025794) maintains MTR in its active state. Betaine-homocysteine methyltransferase (BHMT; GenBank accession no. BC012616) remethylates homocysteine to methionine with a betaine cofactor (Morin et al. 2003). Cystathionine-beta-synthase (CBS; GenBank accession no. NM_000071) controls homocysteine levels by degrading homocysteine into cystathionine (Morrison et al. 1998).

Detecting moderate effects of multiple folate genes will be particularly difficult if they are interactive or additive with environmental impacts (Morrison et al. 1998). This complex pathway has several known metabolic interactions, such as MTRR maintaining MTR in an active state. Previous studies found an association of MTHFR and MTR (Gueant-Rodriguez et al. 2003; Wilson et al. 1999) plus CBS and the MTHFR thermolabile variant with NTDs (Afman et al. 2003; Ramsbottom et al. 1997; Speer et al. 1999).

Thus, genes involved in folate metabolism are compelling candidates for NTDs, from both a genetic and an environmental perspective.

Material and Methods

Sample population. All polymorphisms were genotyped in 304 families with at least one individual affected with an NTD and their first-degree relatives when available. These families represent 240 complete trios and 64 families with only one parent, whereas 16 of these families had two or more affected individuals. Cases with lumbosacral myelomeningocele were classified as affected in the narrow diagnostic criteria, and any level NTD was affected in the broad criteria. These Caucasian families were collected from 13 sites across the United States through myelodysplasia clinics, neurosurgical referrals, our study website, and word of mouth. The family-based study design is robust to potential population stratification and particularly useful when sampling over such a wide geographic area. Most affected individuals were ascertained as children (average age at sample, 14.3 years) with no sex differences. In 74% of NTD case mothers, extensive environmental exposure interviews were conducted, including pre- and postconceptional vitamin use. Table 1 outlines the sample sizes subdivided by diagnostic criteria and maternal folate supplementation. This study was approved by the Duke University Medical Center Institutional Review Board, and all data and samples were collected after informed consent of subjects.

**SNP genotyping.** Eleven genes of the folate pathway are included in our study and were selected from previously published NTD research (Table 2). Three genes that degrade homocysteine (MTR, BHMT, and CBS) were more thoroughly genotyped based on HapMap Release 19 (International HapMap Project 2005) tagging single nucleotide polymorphisms (SNPs) and location in the gene (Figure 2). All but two genetic variants were genotyped by commercially available TaqMan allelic discrimination assays (Assay-on-Demand and Assay-by-Design, Applied Biosystems, Foster City, CA). Previously published polymerase chain reaction (PCR) primers for a 68-bp insertion in CBS exon 8 (Morrison et al. 1998) produced results that did not pass the quality control measures outlined below. Sequencing of the insertion showed a tandem duplication such that the forward primer hybridized before and within the insertion. We used a forward primer 58 bp further upstream of the insertion producing 242 or 310 bases fragments (forward, 5'-CGGCGGATTG-GGCCACTC-3'; reverse, 5'-GGCCGGGCGTCTGGACTC-3'). The SLC19A1 SNP rs1051266 was genotyped by melting curve analysis in the MGB Eclipse Probe System (Belousov et al. 2004). All PCR amplification used the GeneAmp PCR system 9700 thermocyclers (Applied Biosystems) according to assay specifications. Fluorescence was detected with the ABI Prism 7900HT Sequence Detection System and analyzed with ABI Prism Sequence Detection System software (version 2.0; Applied Biosystems). Quality control measures consisted of two reference samples from the Centre d’Etude du Polymorphisme Humain in Paris, France, and 24 duplicated samples per 384-well plate plus blinded from laboratory technicians. These 26 samples had to match completely, and at least 90% of all samples had to be successfully genotyped for the polymorphism to pass quality control. Genotypes were also checked for Mendelian inconsistencies within families.

**Statistical analysis.** Family-based association analysis was performed using the pedigree disequilibrium test (PDT) (Martin et al. 2000) and association in the presence of linkage (APL) test (Martin et al. 2003). Because of the mixed family types and incomplete sampling in our data set, PDT will take advantage of multiplex families, whereas APL performs better with missing data. These tests were

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**Figure 1.** The folate and methionine cycles highlighting the 11 genes included in this study. Substrates are shown in rectangular boxes; enzymes are shown in ellipses. Adapted from Nijhout et al. (2004) and Reed et al. (2004).

**Table 1.** Sample set details for the narrow (lumbosacral myelomeningocele only) and broad (any level NTD) diagnostic groups divided by maternal vitamin supplementation that was available for approximately 75% of mothers of affecteds.

| Data set       | Narrow | Broad |
|----------------|--------|-------|
| Full data set  |        |       |
| Families       | 279    | 304   |
| Affecteds      | 297    | 332   |
| Samples        | 1,158  | 1,259 |

Folate before conception

| Genes          |        |       |
|----------------|--------|-------|
| Families       | 69     | 76    |
| Affecteds      | 75     | 85    |
| Samples        | 307    | 330   |

No folate before conception

| Genes          |        |       |
|----------------|--------|-------|
| Families       | 141    | 149   |
| Affecteds      | 151    | 165   |
| Samples        | 617    | 653   |
performed on all SNPs for the narrow and broad phenotypes in the overall data set as well as those subdivided by maternal folate supplementation, BHMT allele transmission, and MTHFR allele transmission. All SNPs were checked for Hardy-Weinberg equilibrium (HWE) separately in unrelated affected individuals and unaffected relatives in the complete data set using genetic data analysis (Weir 1996). The reported p-values have not been corrected for multiple testing, but a strict correction is not critical given the biological plausibility implicating these genes in NTDs. Linkage disequilibrium (LD) between the SNPs in the same gene was calculated using the Graphical Overview of Linkage Disequilibrium (GOLD) software package (Abecasis and Cookson 2000).

Table 2. SNPs genotyped in the data set.

| Gene symbol | Gene name                              | GenBank accession no. | rs no. | Type of SNP |
|-------------|----------------------------------------|-----------------------|--------|-------------|
| FOLR1       | folate receptor 1                      | NM_015725             | rs2071010 | Intrinsic   |
| FOLR2       | folate receptor 2                      | NM_000803             | rs2298444 | Intrinsic   |
| SLC1B4A1    | solute carrier family 19 member 1      | NM_00355              | rs1051266 | Nonsynonymous |
| TCN2        | transcobalamin II                      | NM_001469             | rs1970277 | Nonsynonymous |
| MTHFD1      | 5,10 methylene-tetrahydrofolate reductase | NM_005857             | rs1801131 | Nonsynonymous |
| MTHFR       | 5,10 methylene-tetrahydrofolate reductase | NM_000254             | rs1092535 | Intrinsic   |
| MTHFR       | 5,10 methylene-tetrahydrofolate reductase | NM_000254             | rs1092535 | Intrinsic   |
| CBS         | cystathionine-beta-synthase             | NM_000071             | rs234715  | Nonsynonymous |

Gene annotations are from GenBank (http://www.ncbi.nih.gov/GenBank). *844ins68 is a 68-bp insertion in exon 8 of CBS.

Results

Single gene associations with an environmental stratification. The initial analysis of the entire data set for 28 SNPs in 11 genes (Table 3) found associations: BHMT rs3733890 (narrow PDT p = 0.023, narrow APL p = 0.058, broad APL p = 0.025, broad APL p = 0.035) and BHMT rs558133 (broad PDT p = 0.025, broad APL p = 0.061). All SNPs were in HWE except the MTHFD1 SNP rs2236225 in affected individuals only (data not shown).

When subdivided by case mothers’ dietary supplementation with folate 3 months before conception, the BHMT associations were significant only in the supplemented group: rs3733890 (narrow PDT p = 0.027, narrow APL p = 0.055, broad PDT p = 0.016, broad APL p = 0.027) and rs558133 (narrow PDT p = 0.036, broad PDT p = 0.012).

When all SNPs were analyzed in the stratified data set, two other genes had significant associations (Table 3). MTHFR rs1801131 was associated by APL with the narrow phenotype in rs234715 (p = 0.046). Also in the nonsupplementing families, CBS was associated by PDT with the broad phenotype in rs234715 (p = 0.015) and rs4920037 (p = 0.037) and SNPs in MTR showed significance: rs1092535 (narrow PDT p = 0.066, narrow APL p = 0.031, broad PDT p = 0.040, broad APL p = 0.04) and rs4659743 (narrow APL p = 0.013, broad APL p = 0.010).

Table 3. Single-gene p-values from significant association tests with an environmental stratum.

| Gene symbol | SNP rs no. | Narrow | Broad |
|-------------|------------|--------|-------|
| BHMT        | rs3733890  | 0.023* | 0.025* |
|             |            | 0.058  | 0.035*|
|             |            | 0.367  | 0.390 |
|             |            | 0.901  | 0.865 |
| BHMT        | rs558133   | 0.114  | 0.026*|
|             |            | 0.124  | 0.061 |
|             |            | 0.765  | 0.867 |
|             |            | 0.038* | 0.012* |
| MTHFR       | rs1801131  | 0.203  | 0.363 |
|             |            | 0.112  | 0.263 |
|             |            | 0.153  | 0.102 |
|             |            | 0.529  | 0.657 |
| MTHFR       | rs1092535  | 0.877  | 0.715 |
|             |            | 0.794  | 0.865 |
|             |            | 0.066  | 0.040* |
|             |            | 0.456  | 0.886 |
| BHMT        | rs4659743  | 0.885  | 0.547 |
|             |            | 0.426  | 0.375 |
|             |            | 0.104  | 0.010*|
|             |            | 0.891  | 0.741 |
| CBS         | rs234715   | 0.287  | 0.160 |
|             |            | 0.617  | 0.328 |
|             |            | 0.056  | 0.015*|
|             |            | 0.527  | 0.654 |
| CBS         | rs4920037  | 0.514  | 0.277 |
|             |            | 0.787  | 0.525 |
|             |            | 0.122  | 0.037*|
|             |            | 0.423  | 0.086 |

*p < 0.05.
Despite being 96.6 kb apart, high LD (\(D' = 0.973, r^2 = 0.946\)) throughout MTR could account for both SNPs' associations (Table 4).

**Stratifying by other genes.** In complex conditions like NTDs, multiple genes are likely contributing to folate-related risks. To evaluate multigenic effects, families were grouped by preferential transmission of an allele to affected offspring and reevaluated for all other SNPs. For BHMT rs3733890, 79 families preferentially transmitted the A allele, 149 transmitted both equally or had homozygous transmission, whereas 17 could not be determined and were not included in the analysis (Table 5). When the G allele was preferentially transmitted, the CBS insertion was significant by PDT (\(p = 0.033\) for both diagnostic groups), whereas two SNPs were significant by APL: SHMT rs1979277 (\(p = 0.042\) narrow, \(p = 0.020\) broad) and MTR rs4659743 (\(p = 0.049\) narrow, \(p = 0.015\) broad). When segregating the A allele, MTHFD1 rs2236225 was significant by PDT in the broad phenotypic group (\(p = 0.016\)). Other SNPs in BHMT were significant in the stratified groups due to intermarker LD (Table 4).

We performed a similar analysis stratifying by the MTHFR rs1801133 thermolabile T allele (Table 6). Sixty-eight families were grouped for the T allele; 90 families were grouped for the C allele; 134 families did not preferentially transmit either allele; and 12 were excluded. With overtransmission of the T allele, BHMT rs3733890 is more significant than in any prior analysis (narrow PDT \(p = 0.007\), narrow APL \(p = 0.027\), broad PDT \(p = 0.010\), broad APL \(p = 0.047\), and TCN2 rs1801198 was associated by PDT with the broad phenotype \(p = 0.045\). For the C allele subset, rs1801394 in MTRR was significant by APL in the broad group \(p = 0.048\). When neither allele was preferred, the SHMT SNP is significant by PDT \(p = 0.050\) for narrow, 0.037 for broad).

**Discussion**

**BHMT contributes to the risk of NTDs.** BHMT is significantly associated with NTDs in our sample set, particularly when mothers were receiving preconceptional folate or parents preferentially transmitted the MTHFR rs1801133 T allele. It is not immediately apparent how BHMT would increase NTD risk in a folate-rich environment. In adults, BHMT functions predominantly in the liver, whereas MTR is active in all tissues (Zhu et al., 2005), but the expression patterns in the developing embryo are unknown and may be markedly different than that in the adult. BHMT is responsible for up to 50% of methylation in the adult liver (Finkelstein and Martin 1984).

The methyl cycle supplies 1-carbon units critical for a variety of methylation reactions essential for proper gene expression and maternal and paternal imprinting by methylated DNA (Razin and Kantor 2005). Growth factor genes are commonly imprinted in this manner, and nutrition can alter these methylation patterns (Waterland and Jirtle 2003). Faulty embryonic methylation of DNA due to abnormal folate levels or improper methyl cycle gene expression at a critical developmental juncture could inappropriately silence growth factors necessary for proper tube closure.

Homocysteine levels are also maintained by the methyl cycle and play a role in NTD risk. Large-dose oral betaine therapy, a BHMT cofactor, treats hyperhomocysteinemia by shunting homocysteine through a betaine-dependent remethylation pathway (Kang 1996). When folate dependent methionine synthesis is impaired, by either genetic or environmental factors, BHMT plays a critical role in homocysteine homeostasis (Weisberg et al. 2003). However, the BHMTR (G allele) and Q (A allele) proteins show no differences in thermostability or enzymatic Michaelis constant (\(Q = 2.7\) and \(R = 2.8\)) (Weisberg et al. 2003). The association of hyperhomocysteinemia with NTD risk implicates enzymes such as MTR, BHMT, and CBS that degrade homocysteine.

Our observed relationship between BHMT, folate supplementation, and NTD risk appears counterintuitive. It is possible that the stratification method inadvertently grouped families by an unidentified cofactor.
correlated with supplementation. The BHMT polymorphism could also create a highly efficient variant that causes the metabolic cycles to overfunction when combined with high folate levels. Human NTDs can only be studied at birth, not at the true point of incidence 28 days postconception, so we may fail to observe a high-risk group incompatible with life. Such individuals with insufficient BHMT and low folate may not be observable unless they also have an additional unknown protective factor. All these hypotheses are highly speculative, particularly in the absence of any biological support.

In the subset of families also transmitting the MTHFR T allele, affected children who have inherited at least one copy of the thermolabile allele from a heterozygous parent are even more likely to have also received the BHMT A allele. A gene–gene interaction between MTHFR and BHMT would require polymorphisms in both genes for the disorder, or additional correlated factors are involved and undetectable in this sample. These results implicate BHMT in NTD risk alone, in conjunction with maternal folate supplementation, and/or a polymorphism in MTHFR that properly folate metabolism.

Other folate pathway genes implicated.
The most widely studied gene in NTD research, MTHFR, is not a significant risk factor in our overall data set. In families that did not receive folate supplementation, the rs1801133 polymorphism was moderately significant. Significant prior research combined MTHFR with other genes, and our results found BHMT to be highly significant in the T allele subgroup.

MTHFR rs1801133 is not the only genetic NTD risk factor, particularly in Caucasian Americans. Some NTD cases are not folic acid preventable, and at most 25% of cases can be solely explained by rs1801133 (Posey et al. 1996; van der Put et al. 1996). Excluding TT genotype people, there is still a decrease in folate and increase in homocysteine levels in patients and their parents (van der Put et al. 1997).

Some previously investigated NTD-related genes included in this study are less likely to be involved because of their biochemical function. For example, FOLR2 is not the primary binder of folate, therefore the lack of significance does not affect the overall outcome of the study because MTHFD1 was not an implicated gene.

NTDs are a complex disorder involving many genetic and environmental factors. Future studies aimed at identifying these risk factors must approach the problem with a wide perspective including several genes and collecting as much environmental data as possible. Despite substantial efforts to associate NTDs with folate genes, there is no convincing evidence of an association for most of these genes. The role of folate in the etiology of NTDs could result from epigenetic effects or interactions with nonfolate genes. All previous research supports the multifactorial nature of NTDs underlining the necessity of multiple approaches in order to disentangle the contributors to this complex disorder.

REFERENCES

Abecasis GR, Cookson WD. 2000. GOLD—graphical overview of linkage disequilibrium. Bioinformatics 16:182–183.

Afman LA, Lievers KJA, Kluijtmans LAJ, Trijbels FJM, Blom HJ. 2003. Gene–gene interaction between the cystathionine beta-synthase 31 base pair variable number of tandem repeats and the methylenetetrahydrofolate reductase 677C→T polymorphism on homocysteine levels and risk for neural tube defects. Mol Genet Metab 79:211–215.

Altschuler D, Brooks LD, Chakravarti A, Collins FS, Daly MJ, Donnelly P. 2005. A haplotype map of the human genome. Nature 437:1299–1300.

Belousov YS, Welch RA, Sanders S, Mills JL, Kulchenko A, Dempcy R, et al. 2004. Single nucleotide polymorphism genotyping by two colour melting curve analysis using the MGB Eclipse Probe System in challenging sequence environment. Hum Genomics 1:209–217.

De Marco P, Calvo MI, Moroni A, Arata L, Merlole E, Finelli RH, et al. 2002. Study of MTHFR and MS polymorphisms as risk factors for NTD in the Italian population. J Hum Genet 47:319–324.

Elwood JM, Little J, Elwood JH. 1992. Epidemiology and Control of Neural Tube Defects. Oxford, UK: Oxford University Press.

Essien FB, Wannberg SL. 1993. Methionine but not folic acid or vitamin-B12 alters the frequency of neural-tube defects in inbred mutant mice. J Nutr 123:271–274.

Finkestein JD, Martin JJ. 1984. Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. J Biol Chem 259:8008–8013.

Forsyth P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. 1995. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 10:111–113.

Gonzalez-Herrera L, Garcia-Escalante G, Castillo-Zapata I, Canto-Herrera J, Ceballos-Quintal J, Pinto-Escalante D, et al. 2002. Frequency of the thermolabile variant C677T in the MTHFR gene and lack of association with neural tube defects in the Mexico City area. J Nutr 132:271–314.

International HapMap Project. 2005. A haplotype map of the human genome. Nature 437:1299–1302.

Kang SS. 1996. Treatment of hyperhomocysteinemia: physiological basis. J Nutr 126:1237–1242.

Martin ER, Bass MF, Hauser ER, Kaplan NL. 2003. According for linkage in family-based tests of association with missing parental genotypes. Am J Hum Genet 73:1016–1026.

Martin ER, Monks SA, Warren LL, Kaplan NL. 2000. A test for linkage and association in general pedigrees: the pedigree disequilibrium test. Am J Hum Genet 67:146–154.

Mills JL, McPartlin JM, Kirke PN, Lee YJ, Conley MR, Weir DG, et al. 1995. Homocysteine metabolism in pregnancies complicated by neural-tube defects. Lancet 345:149–151.

Morin I, Pratt RJ, Weisberg I, Sabaaghian N, Wu Q, Garrow TA, et al. 2003. Common variant in betaine-homocysteine methyltransferase (BHMT) and risk for spina bifida. Am J Med Genet A 119:172–176.

Morrison K, Papapetrou C, Ho FA, Mariman EC, Lynch SA, Burn J, et al. 1998. Susceptibility to spina bifida, an association study of five candidate genes. Am J Hum Genet 62:539–536.

MRC Vitamin Study Research Group. 1991. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. Lancet 338:131–137.

Nijhof HF, Reed MC, Budu P, Ulrich CM. 2004. A mathematical model of the folate cycle: new insights into folate homeostasis. J Biol Chem 279:50508–50516.

Posey DL, Khoury MJ, Mullineux SP, Stein JG, Wu MM Jr, Qu CY. 1996. Is mutated MTHFR a risk factor for neural tube defects? Lancet 347:686–687.

Rombout D, Scott JM, Molloy A, Weir DG, Kirke PN, Mills JL, et al. 1997. Are common mutations of cystathionine beta-synthase involved in the aetiology of neural tube defects? Clin Genet 51:39–42.

Ravits A, Kantom B. 2005. DNA methylation in epigenetic control of gene expression. Prog Mol Subcell Biol 38:151–167.

Reed MC, Nijhof HF, Sparks R, Ulrich CM. 2004. A mathematical model of the methionine cycle. J Theor Biol 226:33–43.

Revilla JLG, Hernandez FN, Marin-MG, Sabador MT, Romero JL. 2003. C677T and A1298C MTHFR polymorphisms in the etiology of neural tube defects in Spanish population. Med Clin 120:441–445.

Rosenquist TH, Finnell RH. 2001. Genes, folate and homocysteine in embryonic development. Proc Nutr Soc 60:53–61.

Rosenquist TH, Ratashak SA, Selhub J. 1996. Homocysteine induces congenital defects of the heart and neural tube: effect of folic acid. Proc Natl Acad Sci USA 93:15272–15273.

Shields DC, Kirke PN, Mills JL, Rombout D, Molloy AM, Burke H, et al. 1999. “The thermolabile” variant of methylenetetrahydrofolate reductase and neural tube defects: an evaluation of the genetic risk associated with the gene-enzyme relationships in the embryo and the mother. Am J Hum Genet 64:1045–1055.

Skirball MJ, Simic SF, Kahle LL. 1996. Dietary intake of fat, fiber and other nutrients is related to the use of vitamin and mineral supplements in the United States: the 1992 National Health Interview Survey. Am J Public Health 96:1301–2008.
Speer MC, Nye J, McLone D, Worley G, Molvin EC, Viles KD, et al. 1999. Possible interaction of genotypes at cystathionine beta-synthase and methylenetetrahydrofolate reductase (MTHFR) in neural tube defects. NTD Collaborative Group. Clin Genet 56:142–144.

Steegers-Theunissen RPM, Boers GHJ, Tijbels FJM, Finkelstein JD, Blom HJ, Thomas CMG, et al. 1994. Maternal hyperhomocysteinemia—a risk factor for neural-tube defects. Metab Clin Exp 43:1475–1480.

Stegmann K, Ziegler A, Ngo ETKM, Kohlschmidt N, Schröter B, Ermert A, et al. 1999. Linkage disequilibrium of MTHFR genotypes 677C/T-1298A/C in the German population and association studies in probands with neural tube defects (NTD). Am J Med Genet 87:23–29.

Trembath D, Sherbondy AL, Vandyke DC, Shaw GM, Todoroff K, Lammer EJ, et al. 1999. Analysis of select folate pathway genes, PAX3, and human T in a midwestern neural tube defect population. Teratology 59:331–341.

van der Put NM, Steegers-Theunissen RP, Frosst P, Tijbels FJ, Eskes TK, van den Heuvel LP, et al. 1995. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet 346:1070–1071.

van der Put NM, Thomas CMG, Eskes TK, Tijbels FJ, Steegers-Theunissen RP, Mariman EC, et al. 1997. Altered folate and vitamin B-12 metabolism in families with spina bifida offspring. J Mol Med 95:505–510.

van der Put NM, van den Heuvel LP, Steegers-Theunissen RP, Tijbels FJ, Eskes TK, Mariman EC, et al. 1996. Decreased methylene tetrahydrofolate reductase activity due to the 677C→T mutation in families with spina bifida offspring. J Mol Med 74:891–894.

Waterland RA, Jirtle RL. 2003. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol Cell Biol 23:5293–5300.

Weir BS. 1996. Genetic Data Analysis II: Methods for Discrete Population Genetic Data. Sunderland, MA:Sinaur Associates.

Weisberg IS, Park E, Ballman KV, Berger P, Numm M, Suh D, et al. 2003. Investigations of a common genetic methyltransferase (BHMT) variant in betaine-homocysteine in coronary artery disease. Atherosclerosis 167:205–214.

Wilson A, Platt R, Wu Q, Leclerc D, Christensen B, Yang H, et al. 1999. A common variant in methionine synthase reductase combined with low cobalamin (vitamin B-12) increases risk for spina bifida. Mol Genet Metab 67:317–323.

Zhu HP, Cury S, Wen S, Wicker NJ, Shaw GM, Lammer EJ, et al. 2005. Are the betaine-homocysteine methyltransferase (BHMT and BHMT2) genes risk factors for spina bifida and orofacial clefts? Am J Med Genet 135A:274–277.