

## A genetic defect in 5,10 methylenetetrahydrofolate reductase in neural tube defects

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### Summary

It is now well-established that folic acid, taken periconceptionally, can reduce the risk of neural tube defects (NTDs). Recent work has demonstrated that an abnormality of homocysteine metabolism is a critical factor. The gene for 5,10 methylenetetrahydrofolate reductase, an enzyme important in homocysteine metabolism, was studied in relation to NTDs. To determine the frequency of the allele for the thermolabile form of the reductase, DNA samples were collected from people with NTDs, parents of people with NTDs, and normal controls. Of 82 people with NTDs, 15 (18.3%) were homozygous for the abnormal, thermolabile allele. This was

significantly higher ( $p=0.01$ ) than the rate of 6.1% in the control population (odds ratio 3.47, 95% CI 1.28–9.41). This is the first specific genetic abnormality to be identified in NTDs. It explains the association between some NTDs and elevated homocysteine, given that the reductase is important in homocysteine metabolism. It also explains how folic acid supplementation prevents some NTDs, by overcoming a partial block in the conversion of 5,10 methylenetetrahydrofolate to 5 methyltetrahydrofolate. Genetic screening could identify women who will require folic acid supplements to reduce their risk of having a child with an NTD.

### Introduction

Recent studies have shown that folic acid supplements taken periconceptionally can greatly reduce a woman's risk of having a child with a neural tube defect (NTD).<sup>1,2</sup> It is clear that folic acid does not act to correct a simple nutritional deficiency, because most pregnant women carrying an affected fetus have levels of folate well above the deficient range.<sup>3–5</sup> Thus, it has been generally assumed that an abnormality in folate metabolism is responsible for a large proportion of NTDs. Steegers-Theunissen *et al.*<sup>6</sup> suggested that a cystathionine synthase abnormality was present, based on abnormal methionine loading test results in women with affected offspring. We have shown that women carrying affected fetuses have significantly higher homocysteine levels than control women.<sup>7</sup> Four enzymes regulate homocysteine levels: cystathionine synthase, SAH hydrolase, methionine

synthase and 5,10 methylenetetrahydrofolate reductase (Figure 1). It is now possible to identify functional variants in some of these enzymes by demonstrating differences in the genes encoding them. Recently, a thermolabile variant of the 5,10 methylenetetrahydrofolate reductase, in which an A→V amino acid substitution is the only apparent difference from the more common form of the enzyme, has been described.<sup>8</sup> The underlying nucleotide substitution can readily be assayed; our study examines the relationship between this genetic abnormality and NTDs.

### Methods

Blood for genetic analysis was obtained after informed consent from study subjects with NTDs,

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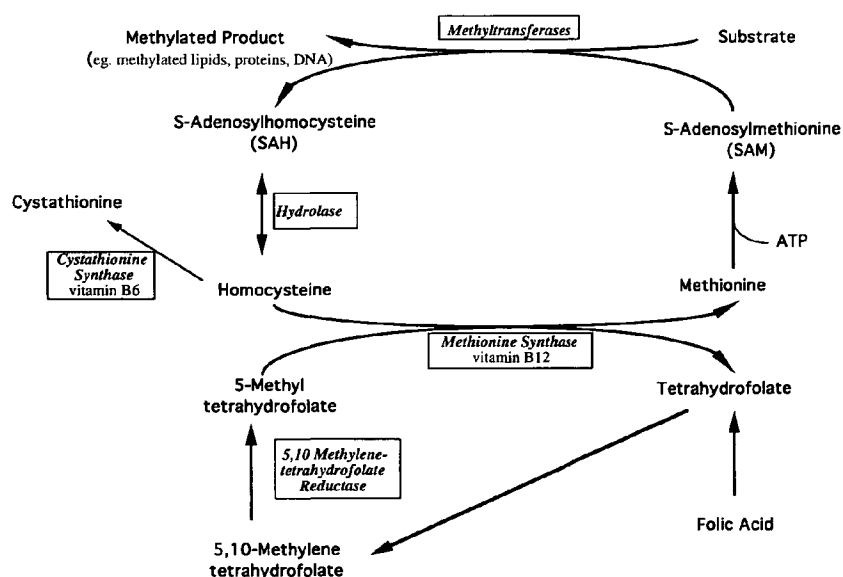
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**Figure 1.** 5,10 Methylene tetrahydrofolate reductase and other enzymes important in homocysteine metabolism.

their parents and other parents of affected persons. There were 79 subjects with spina bifida aperta and three with encephalocele. No affected subject had major birth defects other than spina bifida or encephalocele, apart from defects associated with these anomalies, e.g. hydrocephalus. Samples were collected with the assistance of the Irish Association for Spina Bifida and Hydrocephalus from various parts of Ireland and the Dublin Maternity Hospitals (Coombe, National and Rotunda).

Control DNA samples were obtained from healthy adults from various geographical locations within Ireland. They are therefore likely to represent a good approximation to the overall Irish gene pool. Blood samples or stored frozen buffy coats were processed to provide crude cell lysates suitable for PCR amplification. Briefly, white-cell pellets were lysed in 5 ml lysis buffer (50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 20 mM Tris pH 8.3, 0.45% Noridet 40, 0.45% Tween/20) with 0.2 mg per ml proteinase K. After incubation at 55 °C for at least 30 min to dissolve the cell pellet completely, lysates were boiled for 10 min to inactivate the enzyme.

Genotyping by polymerase chain reaction and allele-specific restriction digestion were performed as described by Frosst *et al.*<sup>8</sup> The normal and variant alleles give rise to diagnostic *Hinf*I fragments of 198 and 175 base pairs respectively when resolved on polyacrylamide gels.

The odds ratio (OR) and the associated two-tailed probabilities (*p*) were obtained by logistic regression for differences in frequency of the thermolabile allele T, and the TT genotype, among cases and controls. To compare the frequency of the TT genotype in

parents of cases with the frequency in controls, the excess frequency of the homozygous TT genotype expected among parents (based on the elevated allele frequency among cases) was calculated to be the frequency of allele T among children minus the frequency in controls, multiplied by the frequency of allele T among controls. The calculated OR, therefore, applies to the adjusted parental frequency compared with the control frequency.

## Results

Two tests were applied to assess the contribution of the thermolabile reductase to NTD risk. (i) By simple allele association, the frequency of the thermolabile allele (T) was significantly higher in cases than in controls (OR = 1.58, 95% CI 1.01–2.46, *p* = 0.04). This test does not, however, distinguish between homozygotes and heterozygotes. (ii) Using a more specific recessive model, the homozygous TT genotype was found in 15/82 NTD cases, and in only 6/99 controls (Table 1); this excess of the TT genotype among cases was significant (OR = 3.47, 95% CI 1.28–9.41, *p* = 0.01).

An excess of the TT genotype was also found among the parents of the NTD cases (13/56 parents). The unadjusted number of TT homozygotes was 6/32 for the mothers, and 7/24 for the fathers. This is partially attributable to their obligatory sharing of half their alleles with cases, in which an excess of the T allele was established, as outlined above. However, when the number of parents with the TT genotype was adjusted downwards to allow for the

**Table 1** Percentages for 5,10 methylenetetrahydrofolate reductase genotypes among cases and controls (number of individuals in parentheses)

		Case	Control
Thermolabile homozygote	TT	18.3% (15)	6.1% (6)
Heterozygote	TN	39.0% (32)	43.4% (43)
Normal homozygote	NN	42.7% (35)	50.5% (50)

excess mandated by the allele frequency observed in cases, there were still significantly more homozygotes in the parents compared to the controls (OR = 3.49, 95% CI 1.20–10.20,  $p=0.03$ ).

## Discussion

For decades, investigators have known that NTDs occur as a result of the interaction of environmental and genetic factors. The recent discovery that folic acid reduces the risk for NTDs provided part of the explanation: in some pregnancies, there was an increased requirement for folate to ensure normal closure of the neural tube.<sup>1,2</sup> Our previous work has shown that in NTD pregnancies the metabolism of homocysteine is affected.<sup>7</sup> This discovery enabled us to focus on specific folate-related enzymes involved in homocysteine metabolism (Figure 1). In particular, we have examined the genes that code for 5,10 methylenetetrahydrofolate reductase.

Our results indicate that homozygosity for a genetically determined thermolabile variant of the reductase is significantly more common in individuals with NTDs than in the general-population (Table 1). This is the first specific genetic risk factor to be linked to NTDs.

There are several possible mechanisms by which being homozygous for this mutation could increase the risk of having an NTD. Frosst *et al.*<sup>8</sup> demonstrated that lymphocytes from adults who are homozygous for this mutation have reduced levels of the reductase enzyme activity. The same diminished reductase activity would be likely to occur in the neural crest of the developing embryo. In such rapidly dividing tissue, reduced reductase might limit the cell's ability to provide 5 methyltetrahydrofolate (Figure 1) to manufacture methionine. This, in turn, would reduce the production of S-adenosylmethionine (SAM) and compromise the many SAM-dependent methyltransferase reactions. Critical functions that might subsequently be disrupted include DNA methylation, phospholipid biosynthesis and methylation of proteins.<sup>9</sup> Alternatively, accumulation of homocysteine

or its derivatives could be toxic to the developing neural tube.

Regardless of whether the correct hypothesis is insufficient production of SAM, or toxicity of the excess homocysteine, there is a probable explanation for how the genetic-environmental interaction works and how risk is reduced by prophylactic folic acid. If embryos with the abnormal enzyme have sufficient folate in their environment (as during folic acid supplementation), they would be able to increase the substrate available for the thermolabile reductase, i.e. 5,10 methylenetetrahydrofolate, from folic acid (see Figure 1). This would result in an increase in the rate of conversion of homocysteine to methionine, thereby eliminating or reducing the risk of non-closure of the neural tube due to either the direct cytotoxic effect of homocysteine or the reduced activity of one or more SAM-dependent methyltransferases.

This study's findings have several important implications. First, we believe that other genetic defects will be found that will explain other NTDs. Given the NTD rate in the Irish population, and the relative frequency of the thermolabile variant of the reductase allele, we estimate that 13% of NTDs are attributable to this genetic factor. Because 50 to 75% of NTDs in the Irish population are likely to be folate-related,<sup>1</sup> there could be other causative abnormalities in folate metabolism relevant to NTDs. Second, clinicians may eventually be able to use genetic screening methods to test women for all, or most, of the folate-dependent enzymes that confer added risk of NTDs. It would then be possible to identify those women who require folic acid supplements before becoming pregnant. This is a particularly important issue, because recommendations that all women of childbearing age should take folic acid to prevent NTDs have largely been unsuccessful.<sup>10,11</sup>

In summary, we have demonstrated that a genetically determined variant of the 5,10 methylenetetrahydrofolate reductase gene that specifies a product with reduced enzyme activity is associated with NTDs. This finding explains some of the association between elevated homocysteine and NTDs because of the reductase's important role in metabolising homocysteine, and it explains the efficacy of folic acid in preventing NTDs by overcoming this partial block. This study provides the first direct evidence of a genetic explanation for NTDs, and suggests the mechanism whereby a genetic factor (the abnormal enzyme) and an environmental factor (folate availability) interact to produce NTDs.

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