

Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida

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Periconceptional folate supplementation reduces the risk of neural-tube defects. We studied the frequency of the 677C→T mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene in 55 patients with spina bifida and parents of such patients (70 mothers, 60 fathers). 5% of 207 controls were homozygous for the 677C→T mutation compared with 16% of mothers, 10% of fathers, and 13% of patients. The mutation was associated with decreased MTHFR activity, low plasma folate, and high plasma homocysteine and red-cell folate concentrations. The 677C→T mutation should be regarded as a genetic risk factor for spina bifida.

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Periconceptional folate supplementation reduces the occurrence and recurrence risk of neural-tube defects (NTD), but the protective mechanism remains unknown.^{1,2} Mothers of NTD offspring do not have deficient folate concentrations, but the values are at the lower end of the control range. We³ and others⁴ have shown higher than normal plasma homocysteine concentrations in mothers of NTD offspring. Since plasma homocysteine is a sensitive marker of folate status, we suggested that folate metabolism was abnormal in these mothers.³

Reduced activity of 5,10-methylenetetrahydrofolate reductase (MTHFR) is associated with high plasma homocysteine concentrations.^{5,6} We have isolated the human MTHFR cDNA and localised the gene to chromosome 1p36.3.⁷ We identified a 677C→T mutation in the MTHFR gene, resulting in decreased activity, increased thermolability of the enzyme, and raised plasma homocysteine concentrations.⁸ This mutation was studied as a risk factor for spina bifida.

Patients with spina bifida and their parents were recruited in 1993 in collaboration with a Dutch society for patients with central nervous system defects (BOSK). The protocol was approved by the local ethics committee and written informed consent was obtained. The study group consisted of 70 mothers (mean age 45.7 [SD 11.7] years), 60 fathers (47.6 [11.6] years), and 55 children and adults with spina bifida (23.3 [11.6] years). Children younger than 3 years were excluded.

A control group of 207 unrelated Dutch subjects (53.0 [12.1] years) was recruited in 1993 from a general practice in The Hague.⁹ Subjects with spina bifida themselves or in an offspring were excluded.

The prevalence of the 677C→T mutation was investigated by PCR of genomic DNA and restriction enzyme digestion with *Hinf*I.⁸ Specific and residual (after heat inactivation for 5 min at 46°C) MTHFR activities were measured by a radiochemical assay in lymphocytes isolated from heparinised blood.⁶ Homocysteine concentrations were measured in EDTA plasma.¹⁰ Folate and vitamin B12 concentrations in heparinised plasma,

	Genotype		
	-/-	+/-	+/+
% of group (number of subjects)			
Controls (n=207)	54 (111)	42 (86)	5 (10)
Mothers (n=70)	46 (32)	39 (27)	16 (11)
Fathers (n=60)	48 (29)	42 (25)	10 (6)
Patients (n=55)	40 (22)	47 (26)	13 (7)
Mean (SD) metabolite concentration			
Homocysteine (μmol/L)	13.4 (3.4)	13.2 (3.1)	17.1 (11.5)*
Vitamin B12 (pmol/L)	246 (130)	271 (121)	233 (94)
Red-cell folate (nmol/L)	541 (188)	517 (182)	643 (186)*
Plasma folate (nmol/L)	12.8 (6.5)	12.8 (6.7)	9.5 (3.1)*

*Significantly different from -/- and +/- groups by ANOVA; p (two-tailed) <0.02.

Table: Prevalence of 677C→T mutation (+) and biochemical measurements

and red-cell folate were measured by Dualcount Solid Phase Boil Radioassay (Diagnostic Products, Los Angeles, CA, USA).

Odds ratios (with 95% CI) were calculated to estimate the relative risk of the homozygous mutation.

The odds ratios for the homozygous mutation were 3.7 (95% CI 1.5-9.1) for the mothers, 2.2 (0.8-6.3) for the fathers, and 2.9 (1.0-7.9) for the patients versus the controls (table).

The presence of the 677C→T mutation was strongly related to MTHFR activity and thermolability (figure). The 677C→T mutation was associated with decreased activity in homozygous individuals and even in heterozygous individuals (ANOVA, p<0.005 between all three genotypes). MTHFR activities were independent of sex and age (data not shown).

The 677C→T mutation was associated with increased plasma homocysteine and red-cell folate concentrations and decreased plasma folate in comparison with the heterozygotes and the wild-type (table). Vitamin B12 concentrations were similar in all three groups.

The 677C→T mutation is the first identified genetic risk factor for spina bifida. Not only for mothers but also for patients a homozygous +/+ genotype is a risk factor for spina bifida. The odds ratios are only modestly raised but the risk factor is important because the frequency of homozygous mutants is 5% in the general population.

The product of MTHFR, 5-methyltetrahydrofolate, is the predominant form of folate in plasma, whereas other folate derivatives such as the enzyme substrate, methylenetetrahydrofolate, are found mainly within cells. The effect of decreased MTHFR activity on folate metabolism is reflected by raised red-cell and decreased plasma folate concentrations in individuals homozygous for the mutation.

The body can only convert 5-methyltetrahydrofolate by methyl-group donation to homocysteine, resulting in formation of tetrahydrofolate and methionine. Raised plasma homocysteine can be explained by reduced availability of 5-methyltetrahydrofolate in homozygous

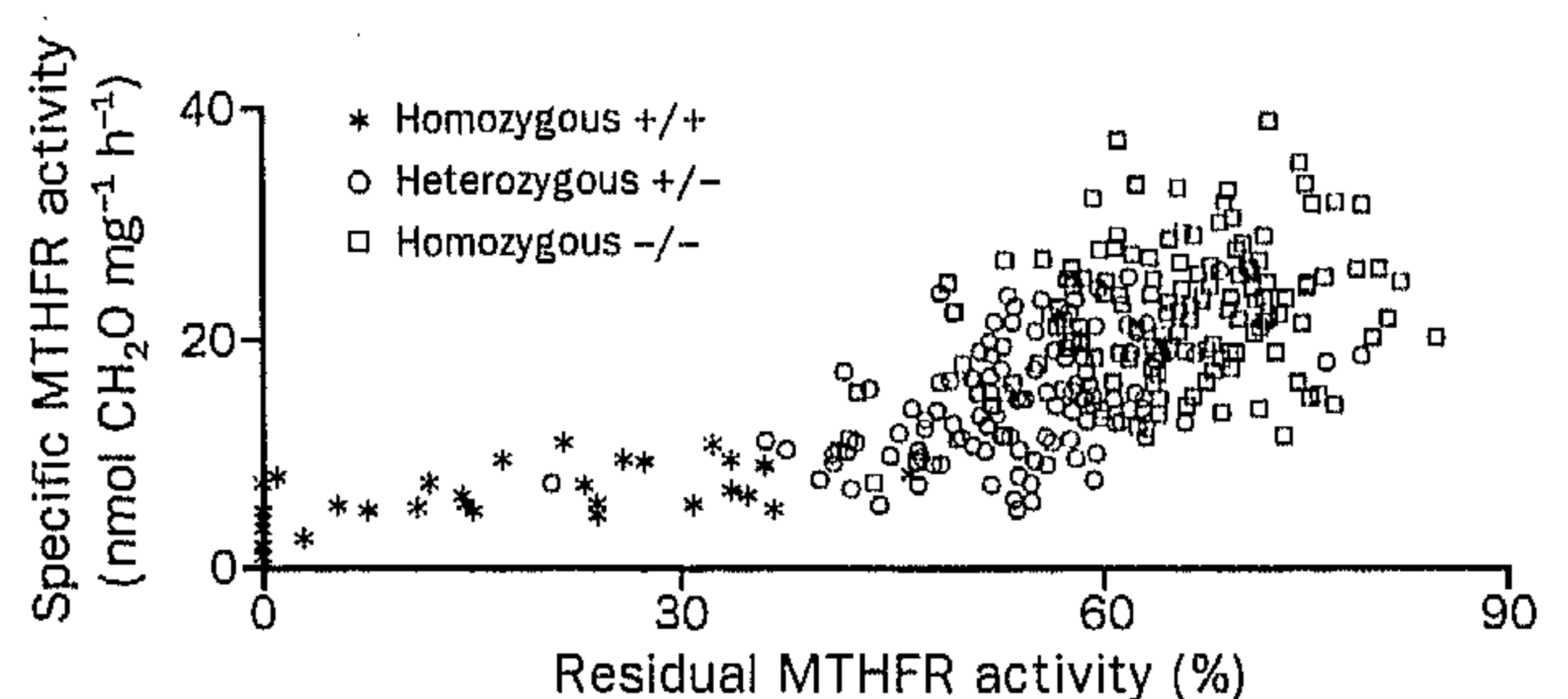


Figure: Specific versus residual MTHFR activity of controls and families with spina bifida offspring

Specific-activity=nmol CH₂O per mg protein per hour.

mutants. Our findings support the hypothesis of defective folate metabolism in at least a subset of mothers with NTD offspring.¹⁰ The genotypes of the mother and of the father contribute to the risk of having a child with spina bifida. Since folate administration may overcome the effects of reduced MTHFR activity, our findings provide a mechanism for the protective role of folate in the aetiology of spina bifida in individuals homozygous for the 677C→T mutation.

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Role of thrombin in pulmonary fibrosis

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Pulmonary fibrosis commonly develops in systemic sclerosis. We assessed the role of thrombin in promoting fibroblast proliferation in the lungs in this disorder. Bronchoalveolar lavage fluid (BALF) thrombin concentrations were higher in ten patients with systemic sclerosis than in 12 healthy controls (14.6 vs 3.6 nmol/L, $p < 0.02$), but values in patients with cryptogenic fibrosing alveolitis ($n=10$) or sarcoidosis ($n=10$) were not increased. BALF from all patients induced fibroblast proliferation. This proliferation was attenuated by thrombin inhibitors for BALF from systemic sclerosis patients only. We suggest thrombin contributes to lung fibroblast proliferation in this disorder.

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Systemic sclerosis is a multisystem disease; a major characteristic is the uncontrolled deposition of extracellular matrix components in the skin and internal organs. The lungs are involved in most patients, and pulmonary fibrosis is a common cause of death.¹ One hypothesis for the pathogenesis is that after endothelial injury, bloodborne mediators or their precursors move from the circulation into adjacent tissues and activate fibroblasts to proliferate or produce excess extracellular matrix.^{2,3} One candidate mediator is thrombin, a key enzyme in the coagulation cascade, which is also a potent mitogen and chemoattractant for fibroblasts.^{4,5}

To investigate the role of thrombin in promoting fibroblast proliferation in the lungs, we measured thrombin concentrations in bronchoalveolar lavage fluid

(BALF) from patients with systemic sclerosis, healthy controls, and two other groups of patients with pulmonary fibrosis—cryptogenic fibrosing alveolitis and sarcoidosis. The contribution of thrombin to BALF-induced fibroblast proliferation was assessed with specific inhibitors of thrombin activity, hirudin, and PPACK (D-phenylalanine-proline-arginine-methylchloride), which block the catalytic site.

We studied ten patients with systemic sclerosis (with no clinical evidence of pulmonary hypertension), ten with cryptogenic fibrosing alveolitis, ten with sarcoidosis, and 12 healthy volunteers. BALF (from bronchoalveolar lavage) was centrifuged, and the cell-free supernatant concentrated ten-fold by ultrafiltration. Thrombin was measured spectrophotometrically.⁶

Cell proliferation was assessed in human fetal and adult lung fibroblasts by a rapid spectrophotometric assay.⁷ Cells were seeded (6×10^3 per well) in Dulbecco's modified Eagle's medium plus 0.4% newborn calf serum. After 24 h incubation at 37°C in humidified air with 10% carbon dioxide, the medium from each well was replaced with fresh medium plus BALF at final dilutions between 1 in 16 and 1 in 128 or purified human α -thrombin at concentrations up to 1 nmol/L. Fibroblasts exposed to medium plus newborn calf serum alone were used as controls. Optimum concentrations of thrombin inhibitors (hirudin 6 nmol/L, PPACK 5 pmol/L) were preincubated with BALF or thrombin for 20 min before addition to cells. Changes in cell number were assessed 48 h later. Results were expressed as percentage change in mean absorbance compared with controls containing medium only. Important results were confirmed by direct cell counting.

Group data are expressed as median and range. Initial statistical analysis was by non-parametric one-way analysis of variance (Kruskal-Wallis test). If this test suggested that the groups were not derived from populations with the same median, two-sample Mann-Whitney tests (unless stated otherwise) were used to identify differences. The effects of thrombin and inhibitors on fibroblast proliferation were compared by t test.

Thrombin concentrations in BALF were higher than the highest control value in all the systemic sclerosis patients (figure 1); this median value was four-fold greater than that in controls ($p < 0.02$). When expressed with respect to albumin the difference was two-fold (13.1