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Altered folate and vitamin B₁₂ metabolism in families with spina bifida offspring

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Summary

Folic acid intake reduces the risk of neural tube defects (NTDs). Although the 677C→T mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene is a risk factor for NTDs, it only partly explains the elevated homocysteine levels in mothers of children with NTDs. We measured vitamin B₁₂, folate and homocysteine in patients with spina bifida (SB), their parents, and in controls, to investigate which other enzymes of homocysteine metabolism might be defective. Because homozygosity for the 677C→T mutation causes decreased plasma folate and increased red-cell folate (RCF) and plasma homocysteine levels, we excluded individuals homozygous for that mutation. The remaining SB patients and their parents still had lowered plasma folate and elevated total homocysteine levels, and a small subset had decreased vitamin B₁₂ levels. Red-cell folate was

the same in all groups, suggesting that dietary folate intake and its uptake was normal. Risk of SB was increased at the 25th percentile of plasma folate and at the 75th percentile of homocysteine values in SB patients and their parents, and at the 5th and 25th percentiles of vitamin B₁₂ in mothers with SB-affected offspring. This underlines the functional importance of homocysteine remethylation to methionine. There was no correlation between vitamin B₁₂ and homocysteine or RCF. In combination with the lowered plasma folate (80–90% 5-methyltetrahydrofolate), our data do not support a major involvement of methionine synthase in the aetiology of SB. Our data rather favour the involvement of genetic variation at loci coding for the formation of 5-methyltetrahydrofolate, such as MTHFR, methylenetetrahydrofolate dehydrogenase or serine hydroxymethyltransferase.

Introduction

Neural tube defects (NTDs) can arise from incomplete closure of the cranial part of the neural tube, resulting in anencephaly, or imperfect closure of the caudal part of the neural tube, resulting in spina bifida (SB). The clinical expression of this disability varies from mild to very severe abnormal-

ities, which can be lethal. The aetiology of NTD is considered to be multifactorial, i.e. the combined action of defective genes and nutritional factors.

Recently, our group identified the first genetic risk factor for SB,¹ i.e. the 677C→T mutation in the

5,10-methylenetetrahydrofolate reductase (MTHFR) gene. These findings were confirmed by others.² MTHFR catalyzes the formation of methyltetrahydrofolate, which is a substrate in the homocysteine remethylation to methionine (Figure 1). Homozygosity for the 677C→T mutation results in elevated homocysteine levels and a redistribution of folates, viz. elevated red-cell folate (RCF) and lowered plasma folate levels.¹

Periconceptional folate administration reduces the NTD birth prevalence by 60–100%.^{3–8} Although the biochemical effects of the 677C→T mutation can be corrected by folate therapy, this mutation can account at the most (after combining the genotype of the mother and child) for 25% of the observed protective effect of folate.^{1,9} Other defective enzymes may thus be present in the folate-dependent homocysteine metabolism of family members (patients and their parents) with SB offspring.

We studied the RCF levels and the vitamin B₁₂, folate and total homocysteine levels in plasma of SB patients and their parents, and compared them to the values in controls. Plasma folate is 80–90% 5-methyltetrahydrofolate, the circulatory form of folate. The other folate derivatives are mainly inside the cell. Abnormal vitamin and homocysteine patterns could suggest other defective enzymes involved in homocysteine metabolism (i.e. methionine synthase, methylenetetrahydrofolate or serine hydroxymethyltransferase) in these families with SB offspring. The 677C→T mutation in the MTHFR gene leads to lowered plasma folate levels and elevated plasma homocysteine levels in individuals homozygous for this mutation,^{1,9} indicating the plausibility of this present approach. Because these abnormal folate and homocysteine levels would have confounded

the present investigation, these individuals were excluded from the study. Heterozygosity for the 677C→T mutation does not affect the homocysteine and folate status of these individuals.^{1,9} Therefore we regarded individuals heterozygous for this common mutation and individuals with a 'wild-type' genotype as one group.

Methods

Families with SB offspring (SB patients and their parents) were recruited in collaboration with a Dutch society for patients with central nervous system defects and their parents (BOSK).¹ The study group included 63 mothers (mean age 45.7, ±SD 11.7 years); 56 fathers (mean age 47.6 ±11.6 years) and 55 of their children with SB (mean age 23.3 ±11.6 years). Children younger than 3 years were excluded, as were individuals homozygous for the 677C→T mutation in the MTHFR gene. The control group of 95 unrelated Dutch individuals (23 men and 72 women), with no history of SB (mean age 41.0, SD 9.0 years) was recruited by public advertisement.^{10,11} The study was approved by the ethics committee, and written informed consent was obtained.

Total homocysteine concentrations were measured in EDTA plasma.¹² Folate and vitamin B₁₂ levels of heparinized plasma, and folate levels in red blood cells, were determined by Dualcount Solid Phase Boil Radio assay (Diagnostic Products).¹³

Statistics

Because the distribution of the data was skewed, results are expressed as medians ± range and as percentiles of the total. Crude odds ratios (OR) and

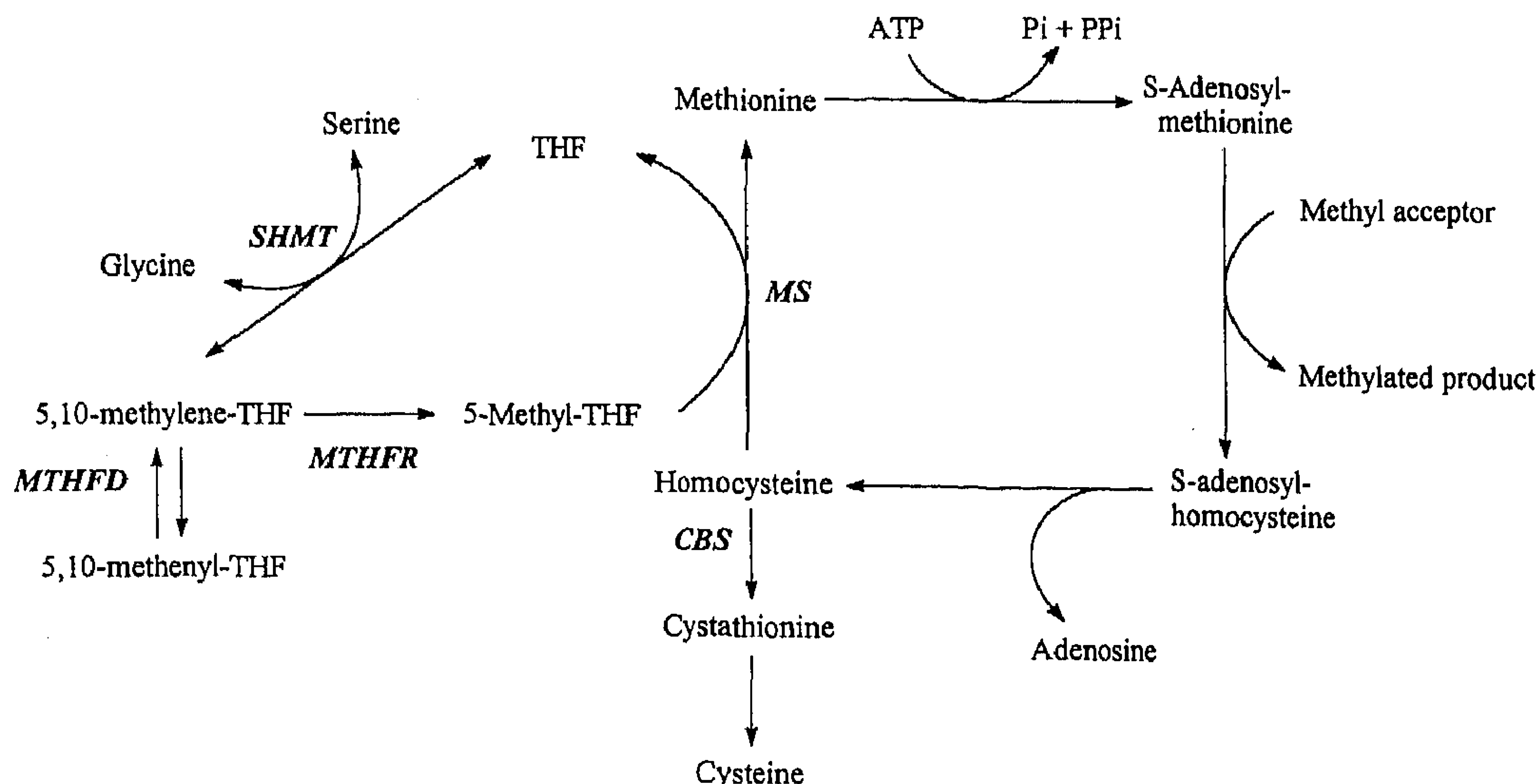


Figure 1. A simplified scheme of folate-dependent homocysteine metabolism. Key enzymes are given. MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; MTHFD, 5,10-methylenetetrahydrofolate dehydrogenase; SHMT, serine hydroxymethyl transferase; CBS, cystathionine β -synthase.

95% CIs for various cut-off points, 5%, 25%, 75% and 95%, respectively, were calculated to estimate the relative risk of the different vitamin levels. The association between variables was measured by a Spearman's rank correlation test. Statistical significance was tested by Wilcoxon Rank Sum-test. $p < 0.05$ was considered statistically significant. p values were two-tailed.

Results

Virtually all individual vitamin B₁₂, RCF, plasma folate and homocysteine levels of patients with SB and their parents were within the normal range. However, the median homocysteine values of patients with SB and their parents were significantly elevated when compared to controls, and the plasma folate levels of the patients and their fathers were significantly lower when compared to controls (Table 1). Median plasma vitamin B₁₂ and RCF levels did not differ from control values. After subdividing into groups with different increasing vitamin and homocysteine levels (Figure 2a–d), some 30–45% of the family members had plasma folate levels belonging to the lowest category of the controls (Figure 2a). Almost 50–75% of the SB patients and their parents had homocysteine levels within the two highest homocysteine categories of controls (Figure 2b). Only a small subset of the family members had decreased vitamin B₁₂ levels (Figure 2c). The distribution of RCF values in families with SB offspring resembled that in the controls (Figure 2d).

The 5% and 25% cut-off points of plasma folate, RCF and vitamin B₁₂ were 7.4 and 11.0 nmol/l, 323 and 415 nmol/l and 127 and 200 pmol/l, respectively.

The 75% and 95% cut-off points of plasma homocysteine were 12.5 and 17.9 μ mol/l. Table 2 shows the calculated crude ORs for these cut-off points of plasma folate, vitamin B₁₂, and homocysteine of mothers, fathers and SB patients vs. controls. We observed a significantly increased risk for SB offspring at the 25th percentile of the plasma folate levels and at the 75th percentile of the plasma homocysteine levels in SB patients and their parents. At the 5th and 25th percentile of vitamin B₁₂ levels, only mothers of a child with SB showed an increased risk of SB-affected offspring.

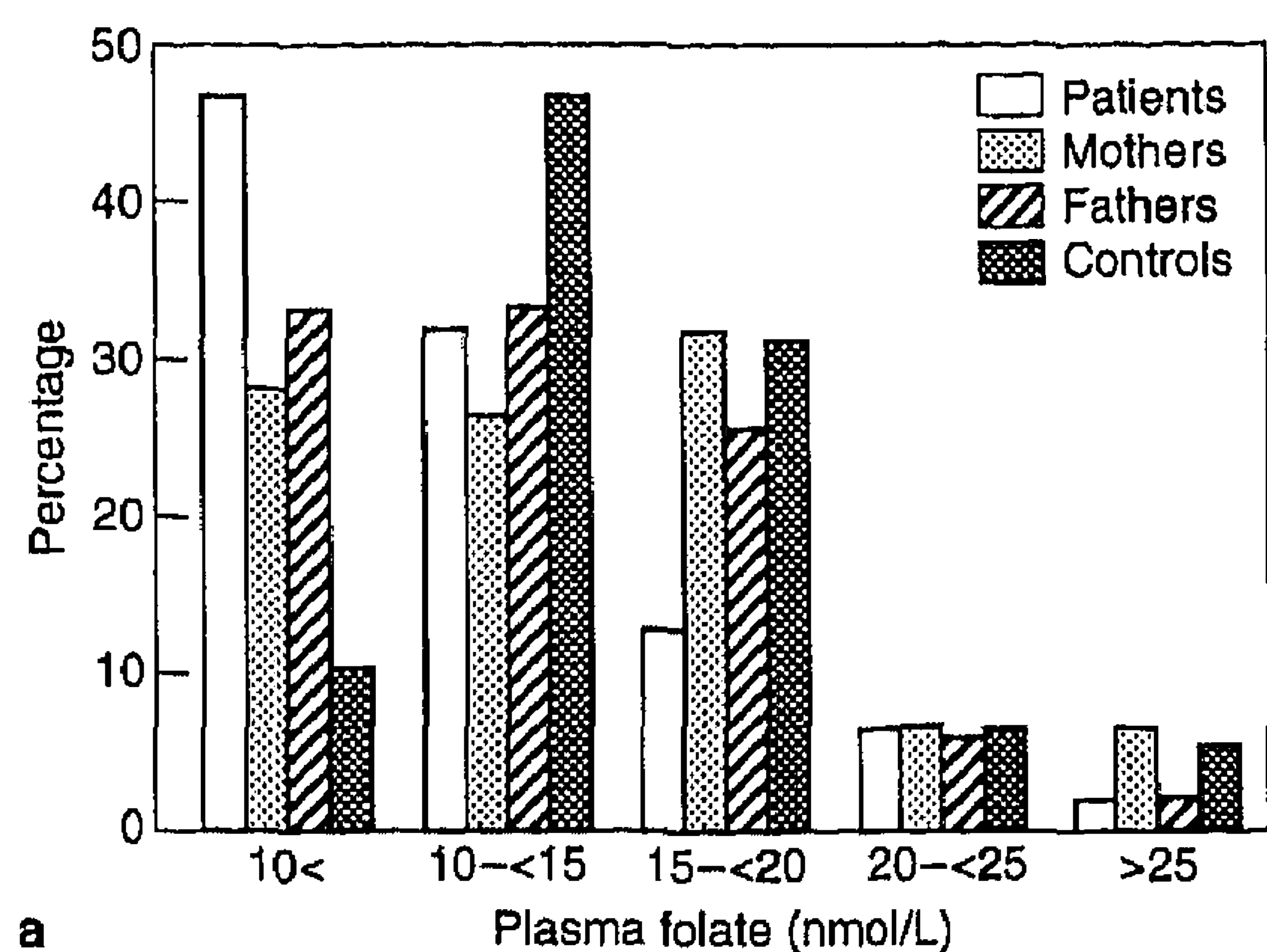
To score for associations between the different vitamins and homocysteine levels of family members and controls, we calculated the Spearman correlation coefficients (Table 3). Plasma folate correlated with RCF and showed an inverse correlation with total plasma homocysteine. There was no correlation between plasma or RCF with vitamin B₁₂. Homocysteine levels of family members showed an inverse correlation with RCF levels. The vitamin B₁₂ levels of SB patients showed an inverse correlation with plasma homocysteine values, and the vitamin B₁₂ levels of the fathers correlated with the RCF levels. No other correlations were observed.

Discussion

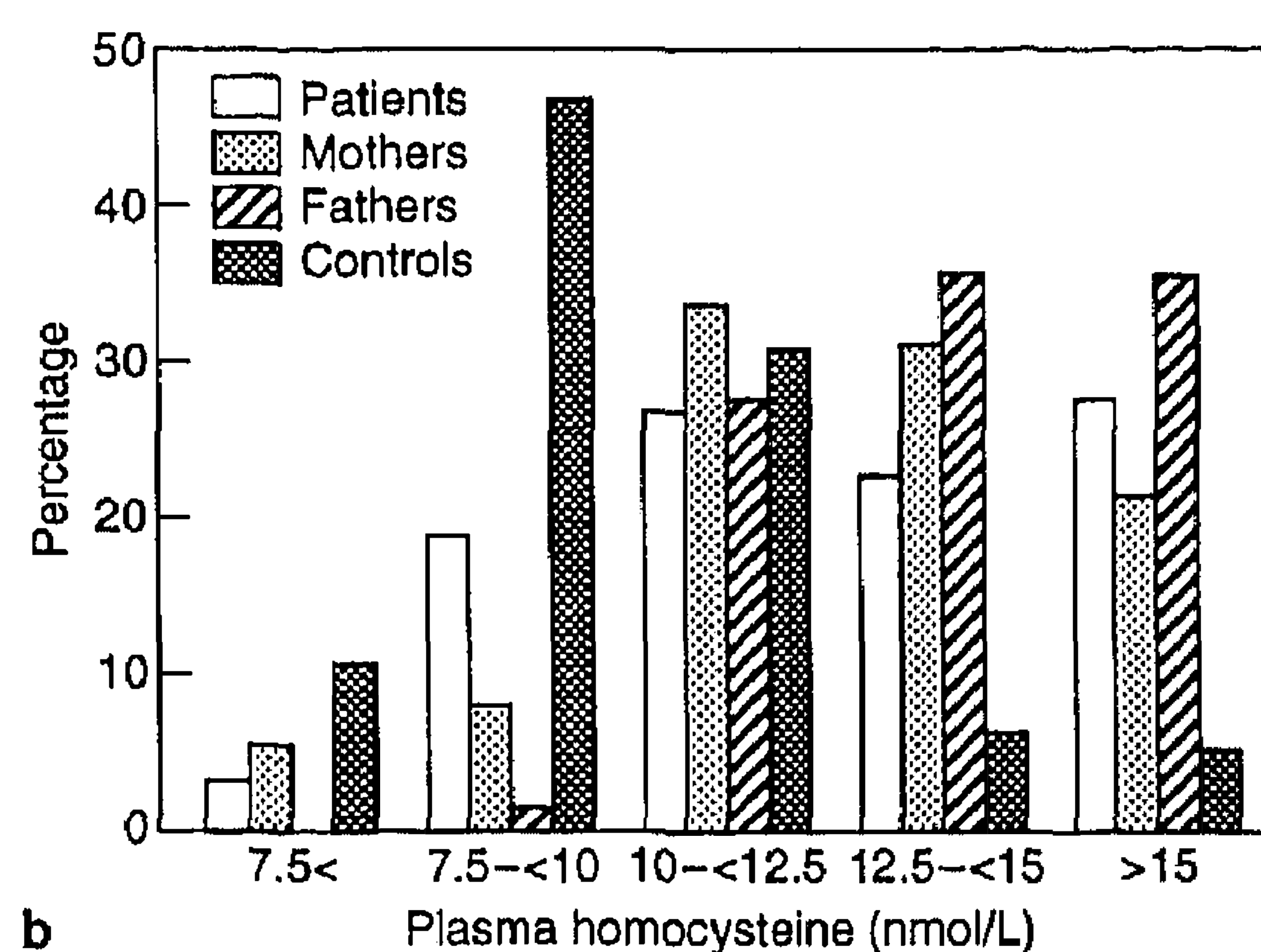
We examined the levels of vitamin B₁₂, folate and homocysteine in families with SB-affected offspring to elucidate possible alterations in their metabolism. The median plasma homocysteine levels of SB patients and their parents were significantly higher than in controls. SB patients and their fathers had significantly lower median plasma folate levels. This

Table 1 Vitamins and plasma homocysteine levels of patients with SB and their parents without individuals homozygous for the 677C→T mutation, compared to control levels

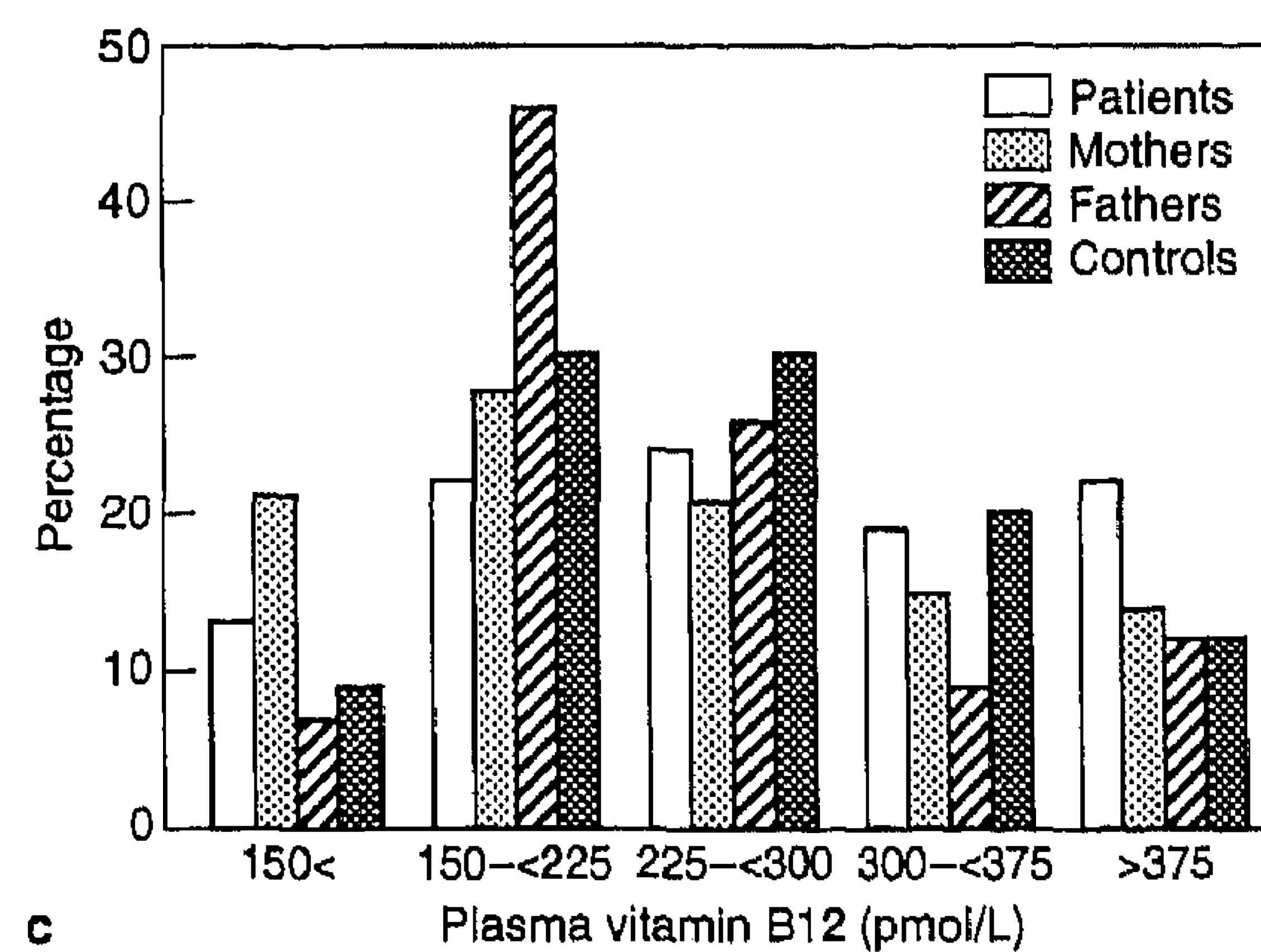
Vitamin	Group	<i>n</i>	Median [range]	<i>p</i>
Plasma folate (nmol/l)	Patients	47	10.0 [5.1–26.0]	0.00001
	Mothers	60	12.5 [6.4–52.0]	0.30
	Fathers	51	12.0 [4.9–28.0]	0.012
	Controls	94	14.0 [6.6–20.4]	–
Plasma homocysteine (μ mol/l)	Patients	55	12.5 [6.1–23.3]	0.0002
	Mothers	63	12.5 [4.0–20.7]	0.0001
	Fathers	56	13.4 [7.5–25.7]	0.00001
	Controls	95	10.1 [6.4–23.0]	–
Plasma vitamin B ₁₂ (pmol/l)	Patients	48	275 [77–920]	0.55
	Mothers	60	245 [43–620]	0.25
	Fathers	51	220 [81–430]	0.11
	Controls	94	255 [64–580]	–
RCF (nmol/l)	Patients	45	470 [230–990]	0.07
	Mothers	57	540 [280–1200]	0.58
	Fathers	49	520 [300–1100]	0.51
	Controls	72	520 [280–1000]	–



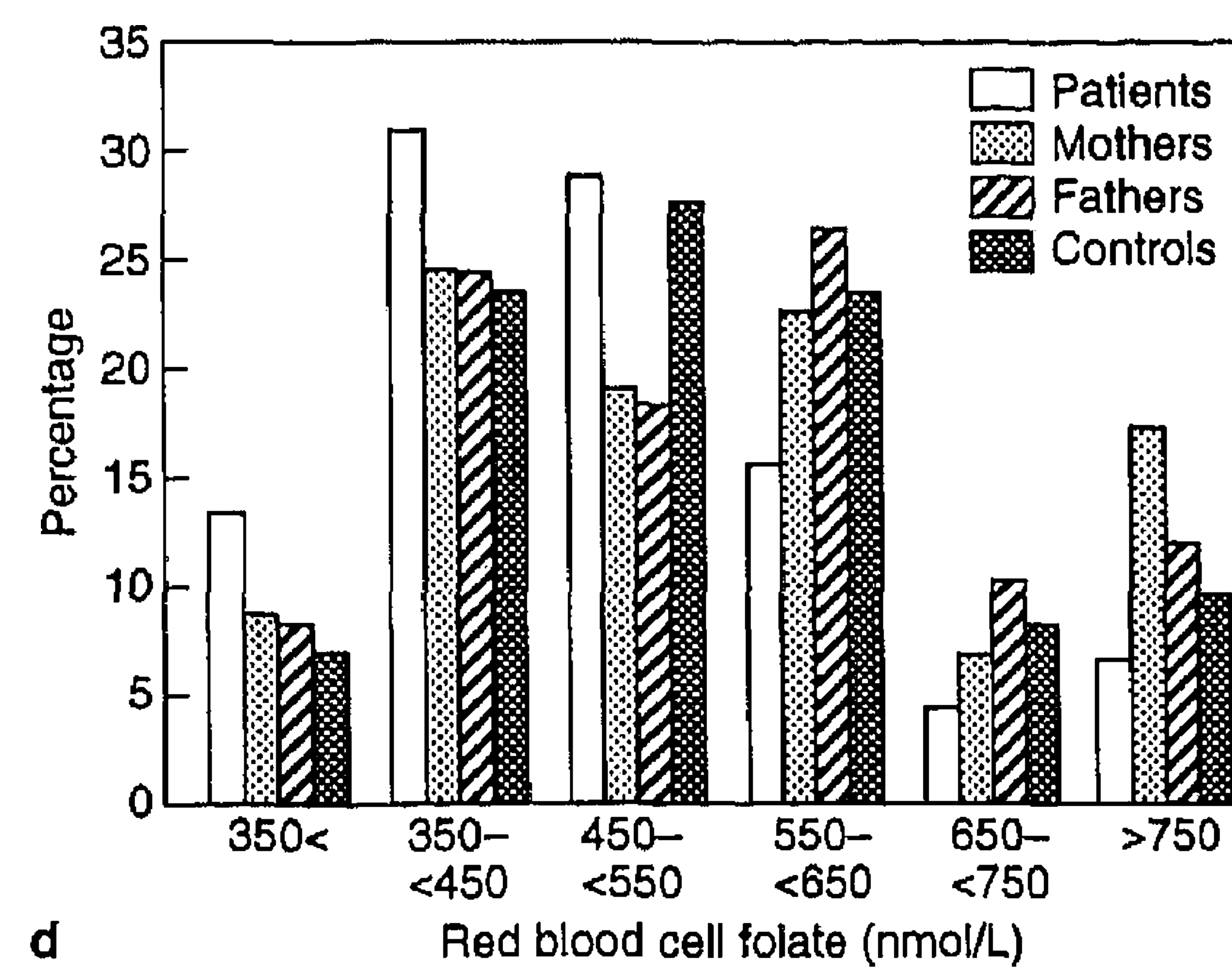
a



b



c



d

was not observed in the mothers, but after subdividing into different categories, the mothers also showed an significantly increased risk of SB-affected offspring at the 25th percentile of plasma folate. The median vitamin B₁₂ levels were not decreased in SB family members, but after subdividing into different categories, the mothers of a child with SB had a increased risk of SB-affected offspring at the 5th and 25th percentiles. These findings support our previous hypothesis of a role for folate-dependent homocysteine metabolism in the aetiology of SB.¹³

NTD may partly be caused by nutritional folate deficiencies.^{14,15} RCF is an indicator for the stored folate derivatives in the cell. Some studies have reported decreased RCF levels in mothers with NTD offspring, indicating a possible nutritional folate deficiency in these individuals.^{16,17} In our study, the RCF levels of family members were comparable to those of controls, arguing against nutritional folate deficiency or a defect in folate uptake in our study group. However, families with SB offspring may have higher nutritional folate requirements, due to a defective folate metabolism.¹⁵ Especially during pregnancy, a relatively mild biochemical defect in folate metabolism, requiring a higher nutritional folate intake, may become of major importance. In our study group, additional folate intake would be overcoming a metabolic folate deficiency than supplementing a nutritional folate deficiency.

Several studies suggest that methionine synthase (MS) might be involved in the aetiology of NTD.^{13,16,18} In the presence of vitamin B₁₂, MS catalyzes the transmethylation of 5-methyltetrahydrofolate and homocysteine to methionine and tetrahydrofolate (Figure 1). Therefore a MS or vitamin B₁₂ deficiency should theoretically lead to increased plasma folate and plasma homocysteine, and possibly to decreased RCF levels, a combination not observed in the present study. Although a small subset of mothers with SB-affected offspring had decreased vitamin B₁₂ levels, no correlations with homocysteine or folate were observed. Instead, decreased plasma folate and normal RCF levels were present in patients with SB and their parents. If the availability of vitamin B₁₂ were inadequate, one would also expect to find a correlation of vitamin B₁₂ with RCF and an inverse correlation of vitamin B₁₂ with plasma folate, which was not observed (Table 3). These findings do not support the presence of reduced MS activity in the aetiology of SB in our study group.

Figure 2. Vitamin and plasma homocysteine values divided into 5–6 different categories and presented as percentages of the total number contained in each group. **a** Plasma folate in nmol/l. **b** Total plasma homocysteine in μ mol/l. **c** Plasma vitamin B₁₂ in pmol/l. **d** Red-blood-cell folate in nmol/l.

Table 2 Odds ratios (OR) and 95% CIs at different cut-off points of families with SB offspring versus controls. The cut-off points of plasma folate, vitamin B₁₂ and RCF are 5% and 25% and the cut-off points of plasma homocysteine are 75% and 95%

Vitamin	Group	n	25/75% OR [CI]	5/95% OR [CI]
Plasma folate	Patients	45	2.1 [1.5–3.1]	5.0 [1.7–15.1]
	Mothers	60	1.4 [1.0–2.2]	1.2 [0.3–5.1]
	Fathers	51	1.7 [1.1–2.5]	2.3 [0.6–8.2]
Homocysteine	Patients	55	2.1 [1.4–3.3]	2.6 [0.8–8.8]
	Mothers	63	2.1 [1.4–3.3]	0.8 [0.2–4.0]
	Fathers	56	2.9 [1.9–4.3]	3.0 [0.9–9.7]
Vitamin B ₁₂	Patients	48	1.1 [0.7–1.9]	2.5 [0.7–8.7]
	Mothers	60	1.2 [1.0–2.4]	3.9 [1.3–11.9]
	Fathers	51	1.4 [0.8–2.2]	0.5 [0.1–4.0]
RCF	Patients	45	1.2 [0.7–2.3]	2.1 [0.5–9.1]
	Mothers	57	1.2 [0.7–2.1]	0.8 [0.1–4.9]
	Fathers	49	0.6 [0.3–1.3]	0.5 [0.1–4.6]

Table 3 Correlation between folate, vitamin B₁₂ and plasma homocysteine of SB patients and their parents, and controls. The correlations are represented by Spearman correlation coefficients.

Correlation	Number of pairs	Correlation coefficient	p	
<i>Patients</i>				
Plasma folate vs	homocysteine	47	−0.43	0.002
	red-cell folate	45	0.47	0.001
	vitamin B ₁₂	47	0.02	NS
Homocysteine vs.	red-cell folate	48	−0.33	0.02
	vitamin B ₁₂	45	−0.32	0.03
Vitamin B ₁₂ vs.	red-cell folate	45	0.29	NS
<i>Mothers</i>				
Plasma folate vs.	homocysteine	60	−0.59	0.0001
	red-cell folate	57	0.62	0.0001
	vitamin B ₁₂	60	−0.04	NS
Homocysteine vs.	red-cell folate	57	−0.41	0.002
	vitamin B ₁₂	60	−0.02	NS
Vitamin B ₁₂ vs.	red-cell folate	57	0.01	NS
<i>Fathers</i>				
Plasma folate vs.	homocysteine	50	−0.49	0.0001
	red-cell folate	49	0.72	0.0001
	vitamin B ₁₂	51	0.26	NS
Homocysteine vs.	red-cell folate	48	−0.31	0.03
	vitamin B ₁₂	50	−0.18	NS
Vitamin B ₁₂ vs.	red-cell folate	49	0.33	0.02
<i>Controls</i>				
Plasma folate vs.	homocysteine	94	−0.44	0.0001
	red-cell folate	72	0.57	0.0001
	vitamin B ₁₂	94	0.03	NS
Homocysteine vs.	vitamin B ₁₂	94	−0.15	NS
	red-cell folate	72	−0.03	NS
Vitamin B ₁₂ vs.	red-cell folate	72	0.02	NS

Kirke *et al.* observed decreased RCF, plasma folate and vitamin B₁₂ levels in mothers with NTD offspring. Because RCF levels of these mothers showed a correlation with vitamin B₁₂, they concluded that MS function is directly or indirectly impaired in these mothers.¹⁸ Increased plasma folate levels would support this hypothesis, but they in fact observed

decreased plasma folate levels, which argues against their hypothesis.

The lower vitamin B₁₂ levels in a small subset of family members may be due to reduced intake, or disturbed metabolism of vitamin B₁₂. The lower plasma folate levels observed by us and others¹⁸ are probably not caused by folate malabsorption,¹⁹ so

may reflect mutations in genes encoding the enzymes involved in the formation of 5-methyltetrahydrofolate. In this respect, next to MTHFR, methylenetetrahydrofolate dehydrogenase and serine hydroxymethyl transferase (Figure 1) are candidate enzymes.

Vitamin B₁₂ as a cofactor, and 5-methyltetrahydrofolate as a cosubstrate, are essential for the remethylation of homocysteine to methionine. Methionine can be activated by ATP to S-adenosylmethionine (AdoMet). AdoMet donates its methyl group in over a hundred different methyltransferase-catalyzed reactions, including protein and DNA methylation.²⁰ Thus reduced availability of vitamin B₁₂ and 5-methyltetrahydrofolate might cause AdoMet depletion. Our study shows the presence of elevated plasma total homocysteine levels in families with SB offspring, correlating with lowered plasma folate levels, indicating a reduced conversion of homocysteine to methionine. We recently showed that addition of methionine and even homocysteine to cultured rat embryos prevented the development of NTD.²¹ Both the lowered plasma folate in SB patients and their parents, and the lowered vitamin B₁₂ levels in mothers with SB-affected offspring observed in the present study, could cause a lowered methionine and AdoMet availability. This in turn may disturb gene expression via reduced DNA methylation and/or other important methylation reactions in the cells, interfering with the proper closure of the neural tube.

In conclusion, we found increased homocysteine and decreased plasma folate levels in SB patients and their parents, indicating defects in the enzymes involved in the synthesis of 5-methyltetrahydrofolate. Reduced function of MTHFR, methylenetetrahydrofolate dehydrogenase and serine hydroxymethyl transferase may be involved in the aetiology of NTDs.

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