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Single nucleotide polymorphisms in the transcobalamin gene: relationship with transcobalamin concentrations and risk for neural tube defects

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Homocysteine levels are elevated in mothers of neural tube defect (NTD) children, which may be due to a disturbed folate or vitamin B12 metabolism. Vitamin B12 is transported to the tissues by transcobalamin (TC). We previously showed that a low holo-TC/total-TC ratio is a risk factor for NTD, possibly due to an impaired binding of vitamin B12 to TC. The coding region of the TC gene of 12 individuals was analysed for genetic variations responsible for a disturbed vitamin B12 binding. The influence of the genetic variations observed on total-TC, holo-TC, holo-TC/total-TC, erythrocyte vitamin B12, plasma homocysteine concentrations and risk for NTD was explored in 42 mothers of a child with NTD and in 73 female controls. Direct sequencing analyses revealed five single nucleotide polymorphisms (SNPs). Three SNPs affected total-TC concentrations, whereas two SNPs seem to affect the binding of vitamin B12. None of the genotypes defined by the SNPs had a significant effect on homocysteine levels, or was associated with an increased NTD risk. Among the five SNPs observed only P259R could partly explain the reduced proportion of vitamin B12 bound to TC, which has been associated with an increased risk for having a child with NTD. Some of the variants studied affected total-TC and holo-TC/total-TC ratio but a larger study population is required to elucidate whether these SNPs influence delivery of vitamin B12 to the tissue, influence homocysteine levels and whether they are associated with an increased NTD risk.
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Introduction

In man, the neural tube closes during the fourth week of gestation and failure of closure results in a neural tube defect (NTD), among which anencephaly and spina bifida are the most common. Periconceptional folic acid supplementation reduces the risk of NTD with 50–70%.[1,2] So far, the mechanism behind this preventive effect of folic acid supplementation on the occurrence and recurrence of NTD is poorly understood. Besides its involvement in the synthesis of purines and thymidine, folate is also used in the remethylation of homocysteine to methionine. A part of the protective effect of folic acid can be explained by the metabolic effects of the 677C>T variation in the methylenetetrahydrofolate reductase (MTHFR) gene. This common variant is the first identified genetic risk factor for

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NTD, which in homozygous state is associated with increased homocysteine levels.[3] Several studies found that homocysteine levels were elevated in mothers of children with NTD.[4-6]

Homocysteine is remethylated to methionine by methionine synthase (MS) which catalyses the transfer of the methyl group of 5-methyltetrahydrofolate (5-methyl-THF), via vitamin B12, to homocysteine. Several investigators examined a possible association between vitamin B12 concentrations in blood or amniotic fluid and the risk for NTD but with ambiguous outcome.[4,7-10]

In plasma, about 20% of the vitamin B12 is bound to transcobalamin (TC), and approximately 80% is bound to haptocorrin (HC). Holo-TC is the proportion of TC that contains vitamin B12, as opposed to apo-TC to which no vitamin B12 is bound. TC transports the vitamin B12 to the cell whereas the role of HC is unclear, so far. A reduced cellular vitamin B12 availability may be detected earlier by determination of plasma holo-TC levels than by total plasma vitamin B12 levels, which is the sum of holo-TC and holo-HC.

In our recent study, we found low plasma holo-TC levels to be associated with an increased risk for having a child with NTD (OR: 2.9 95% CI: 0.9–9.2) whereas, no such association was observed with total plasma vitamin B12 levels.[11] Moreover, we found that a low percentage of vitamin B12 bound to TC (holo-TC/total-TC) is associated with an increased risk for NTD (OR: 5.0 95% CI: 1.3–19.3). Because total plasma vitamin B12 levels were not decreased in individuals with low holo-TC levels, our results suggested a disturbed binding of vitamin B12 to TC. We hypothesised that genetic variations in the TC gene may explain an impaired TC function.

In the present study we sequenced the entire coding region of the TC gene in eight individuals with a low and in four individuals with a high proportion vitamin B12 bound to TC and examined the exonic sequences for genetic variations. The influence of the genetic variations observed on total-TC, holo-TC, holo-TC/total-TC ratio, erythrocyte B12, plasma homocysteine levels and risk for NTD was assessed in 46 mothers of NTD children and 73 female controls.

Materials and Methods
Study population
The study population has been described before[11] and consisted of 46 mothers of patients with non-syndromic NTD (mean age 38 ± 4 years), mainly spina bifida, recruited by participation of the Department of Pediatrics of the University Medical Center Nijmegen and of 73 controls (mean age 35 ± 8 years). The controls were healthy female employees of our University Medical Center with no family history of NTD. All participants were Dutch Caucasians. DNA of 42 mothers was available with a NTD child and of all 73 controls. The protocol was approved by the local

ethics committee and written informed consent was obtained from all participants.

Laboratory methods
Plasma determination Holo-TC and holo-HC levels were determined in EDTA plasma according to the method described by Benhayoun et al.[12] Plasma vitamin B12 (holo-TC + holo-HC) was determined using the Dualcount Solid Phase Boil Radioassay (Diagnostic Products Corporation, Los Angeles, CA, USA). TC was removed with Heparin-Conjugated-Sepharose and the remaining holo-HC was also determined by the Dualcount Solid Phase Boil Radioassay. Holo-TC levels were calculated as the difference between holo-HC levels and plasma vitamin B12 levels. Plasma apo-TC and apo-HC levels were determined according to the method described by Nielsen et al.[13] Homocysteine concentrations were determined in EDTA plasma by HPLC with fluorescence detection.[14] Erythrocyte vitamin B12 and plasma folate levels were determined by using the Dualcount Solid Phase Boil Radioassay (Diagnostic Products).

Sequencing analyses We selected eight individuals (six mothers of NTD children and two female controls) with low holo-TC levels and a low holo-TC/total-TC ratio. In addition, four subjects (one mother of a NTD child and three female controls) with high holo-TC levels and a high holo-TC/total-TC ratio were selected for DNA analysis of their TC gene. The TC gene consists of nine exons,[15] which were sequenced using primers that were located in the flanking introns (Table 1). PCRs were carried out in a total volume of 50 μl, containing 50 ng of the sense and anti-sense primer (Table 1), 200 μM each dNTP, 10 mM Tris-HCl buffer (pH 8.3), 1.0 mM $MgCl_2$ and 0.5 units Taq polymerase (all from Life Technologies, The Netherlands) in a Perkin Elmer 9600 thermocycler (PE Biosystems, The Netherlands). PCR conditions were: initial denaturation at 92°C for 2 min, followed by 35 cycles: of 30 s at 92°C, 30 s at 47–58°C, and 30 s at 72°C, and a final step at 72°C for 7 min. Subsequently, all PCR products were sequenced on both strands using the ABI Prism BigDye Terminator cycle sequencing kit according to the manufacturer's recommendations (PE Biosystems, The Netherlands) on an ABI Prism 377 automated DNA sequencer.

SNP analyses The prevalences of the variants detected by DNA sequencing analysis were analysed by PCR–RFLP and subsequent separation on agarose gels (Table 2). For the screening of S348F in exon 7, an artificial EspI restriction side was created by using the mutagenic sense primer 5'-CCGTACAGACAGTCCGTC-3' that, in combination with the wild type allele, resulted in the EspI recognition site. The prevalences of the previously described P259R and Gln234Arg variants were also determined by ScrFI and MspI restriction enzyme analysis, respectively (Table 2). The

MTHFR 677 C>T polymorphism was determined as described by Frosst *et al*.[16]

Prediction of the putative secondary structure changes in the TC protein by each of the SNPs was performed using the Chou-Fasman[17] and GOR II (Garnier, Osguthorpe, Robson)[18] algorithms included in the OMIGA version 2.0 software package.

Statistical analyses

Odds ratios (OR) with 95% confidence interval (CI) were calculated using logistic regression analyses. Homocysteine levels showed a non-normal distribution; we therefore used logarithmically-transformed homocysteine concentrations in all calculations, and homocysteine concentrations were expressed as geometric means. Differences in mean metabolic variables between the various genotypes were assessed by linear regression analyses. All *P*-values were only adjusted for age, except those concerning homocysteine concentrations which were adjusted for age, plasma folate and the MTHFR 677 C>T polymorphism. Tests for trend were calculated using linear regression analyses. All tests were performed two-tailed, and statistical significance was accepted at $P<0.05$. All analyses were performed using SPSS software package version 10.0.

Results

Direct sequencing analyses of the coding region of the TC gene in 12 selected individuals revealed five sequence variants; each of these, including the previously described Gln234Arg variant was subsequently analysed in the entire study population of 42 NTD mothers and 73 healthy controls (Table 2). The Gln234Arg variant[19] was not detected in our study groups. Table 3 summarises the genotype distributions in mothers of NTD children and in controls. On the basis of these frequencies we calculated ORs with 95% confidence interval (CI) as estimates of the relative risk for NTD. No particular genotype was associated with an increased NTD risk.

The 67 A>G transition is located in exon 2 and results in an isoleucine by valine replacement at codon 23 (I23V). The allele frequency of the 23V allele in controls and in mothers of NTD children was 15% and 11%, respectively

(total study population 13%). The secondary structure of the TC protein did not change due to this amino acid change, as assessed by both Chou-Fasman and GOR II secondary structure prediction programs.

The 280 G>A transition is located in exon 3 and mandates a glycine by a serine substitution at codon 94 (G94S). The secondary structure of the mutated polypeptide was identical to its wild type counterpart. This variant was only observed in the heterozygous state and in two mothers of NTD children, resulting in an allele frequency of 2% (total study population 0.9%).

The third variant detected is a 1043 C>T transition in exon 7, that results in the replacement of serine by phenylalanine at codon 348 (S348F). Again, no change in secondary structure was predicted using the above-mentioned algorithms. The allele frequency of the 348F allele was 17% in controls and 14% in mothers of NTD children (total study population 16%). The distribution of the genotypes in controls and in mothers of NTD children was similar; however, the homozygous 348FF genotype was not observed among NTD mothers.

In exon 8, we observed an 1196 G>A transition, that mandates an amino acid change from arginine to glutamine at codon 399 (R399Q). Also, this variant is not expected to change the secondary structure of the TC protein. The R399Q variant was present only in the heterozygous state with an allele frequency of 3% in controls and 7% in cases (total study population 4%). The prevalence of the 399RQ genotype was higher in mothers of NTD chil-

Table 2 Sequence variations in the TC gene

	Nucleotide change	Amino acid change	RFLP[a]
Exon 2	67 A>G	Ile23Val	+*Rsa*I
Exon 3	280 G>A	Gly94Ser	+*Alw*NI
Exon 5	701 A>G	Gln234Arg	+*Msp*I
Exon 6	776 C>G	Pro259Arg	−*Scr*FI
Exon 7	1043 C>T	Ser348Phe	−*Esp*I*
Exon 8	1196 G>A	Arg399Gln	−*Bsm*AI

[a]Restriction fragment length polymorphism; *Artificially created restriction site.

Table 1 Primers used for the molecular genetic analysis of the TC gene

	Forward primer (5′→3′)	Reverse primer (5′→3′)
Exon 1	GGA AGC TGC GTC TCT CGG	GAT GTG TTA AAT GCT GAG GTC
Exon 2	GGT GTG TGC TGG GTG GAG	AGT ACC CAA GTT TCC CAT CTC
Exon 3	TGG GGT GGG TGC TGT AAG	TGG ATG GTG GGA AAG ACG
Exon 4	GGG CTG AGG CAA TGA AGG	TTC GGA GGA GCC CCA AG
Exon 5	CTC AAG CCC CTG CCT GTC	AGA GAG AGA GGA AGT CAG CCG
Exon 6	TCA GGG GTG GAG TGG TCA G	ATT TTG GCT GCT GTG TCC C
Exon 7	GGG AAG ACA AGA AGA CAA ATA ATC	TGT TCC CCG TCT CTT CTG TG
Exon 8	TTT TGC TGC TGT GGG TGA G	GCT GGG GTG AGT TGA TGA AG
Exon 9	CTG GAA GAT GAG GTT GCG G	AGG GAC ATC AGG AGG GAG G

All primers are based on intronic sequences (Genbank Acc. No: AC005006)

Table 3 Distribution of the SNPs in the TC gene in mothers of NTD children and controls

	Genotype	Mothers (n=42)	Controls (n=73)	Odds ratios	95% CI
Ile23Val*	II	34 (81%)	53 (74%)	1[a]	–
	IV	7 (17%)	17 (24%)	0.6	0.2–1.7
	VV	1 (2%)	2 (3%)	0.8	0.1–8.9
Gly94Ser	GG	40 (95%)	73 (100%)	–	–
	GS	2 (5%)	0	–	–
	SS	0	0	–	–
Pro259Arg	PP	11 (26%)	22 (30%)	1[a]	–
	PR	25 (60%)	36 (49%)	1.4	0.6–3.4
	RR	6 (14%)	15 (21%)	0.8	0.2–2.6
Ser340Phe	SS	30 (71%)	50 (68%)	1[a]	–
	SF	12 (29%)	21 (29%)	1.0	0.4–2.2
	FF	0	2 (3%)	–	–
Arg399Gln	RR	36 (86%)	69 (94%)	1[a]	–
	RQ	6 (14%)	4 (6%)	2.9	0.8–10.8
	QQ	0	0	–	–

*Number of controls=72; [a]Reference category.

dren although this did not lead to a significantly increased NTD risk (OR: 2.9 (0.8–10.8)) (Table 3).

We also encountered the previously described P259R variant, which has an allele frequency of 45% in controls and 44% in mothers of NTD children (total study population 45%). In our study population none of the genotypes defined by this polymorphism was associated with an increased NTD risk. According to the Chou–Fasman algorithm the P259R substitution will change the secondary structure of the protein: a tendency to form helix structures occurred by the substitution of proline at codon 259 by arginine. A change in secondary structure was also predicted by using the GOR II method, which revealed a tendency to form turn structures.

To establish whether the SNPs influence TC function, we investigated its effects on the total-TC, holo-TC, holo-TC/total-TC ratio, erythrocyte B12 and plasma homocysteine concentrations. Mean concentrations of these parameters were calculated for each of the genotypes defined by the SNPs and are depicted in Figure 1. The most pronounced effect was observed on total-TC concentrations (Figure 1A). The heterozygous 23IV genotype was associated with significantly lower total-TC concentrations compared with the 23II wild type. Those individuals with the homozygous 259RR and heterozygous 259PR genotypes also had lower total-TC concentrations compared with 259PP homozygotes, with the lowest concentration observed in the 259RR peers. Also the R399Q genotypes influenced the total-TC concentration: the 399RQ heterozygotes showed a higher total-TC compared with the 399RR homozygotes.

An effect on holo-TC concentrations was only observed for the P259R variant; homozygous 259RR individuals had a significantly lower holo-TC concentration compared with those with the 259PP genotype (Figure 1B).

The proportion vitamin B12 bound to TC, calculated by the holo-TC/total-TC ratio, was significantly higher in
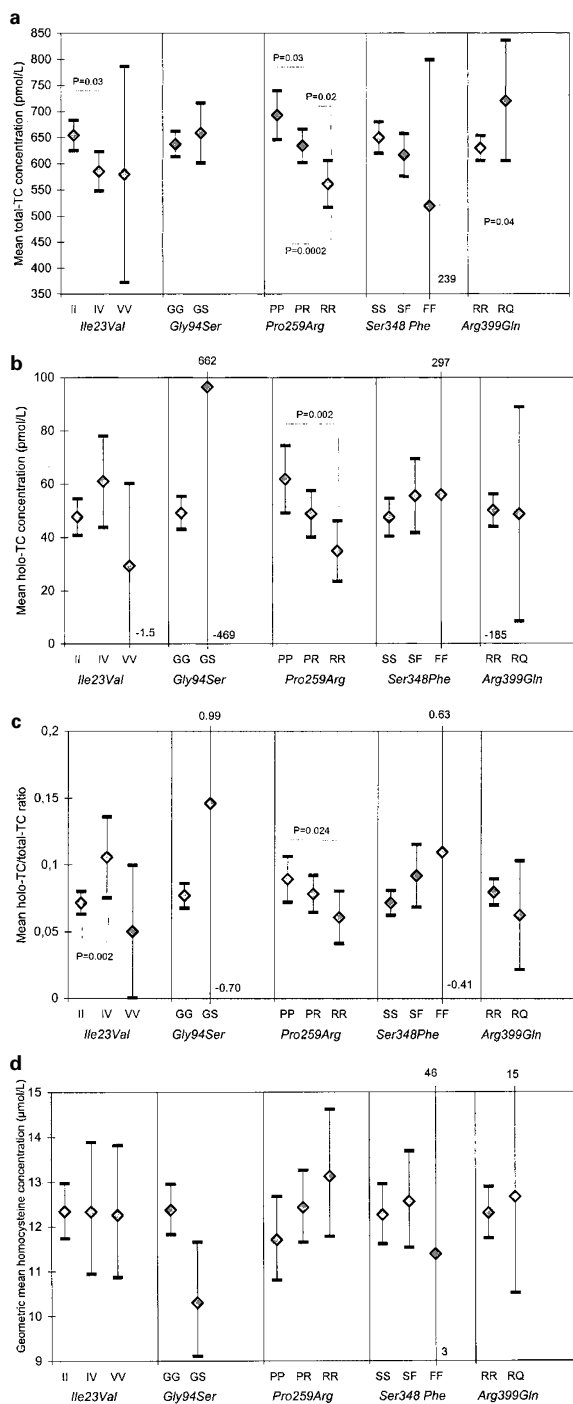

Figure 1 Associations of SNPs in the transcobalamin (TC) gene with mean total-TC (**A**), holo-TC (**B**), holo-TC/total-TC ratio (**C**) and geometric mean homocysteine (**D**) concentrations. Mean levels are shown as diamonds, 95% CI as thin vertical lines. The values of the CI's exceeding the graph are indicated in the figure. P-values are only shown when significant differences were observed ($P < 0.05$). All P-values were adjusted for age and P-values for homocysteine were also adjusted for plasma folate and the MTHFR $677C > T$ polymorphism.

heterozygous 23IV individuals compared with their wild type 23II peers. The homozygous 259RR genotype was associated with a significantly lower proportion vitamin B12 bound to TC compared with the wild type. The S348F showed a significant trend (*P*=0.03) in increasing proportion vitamin B12 bound to TC with the presence of this SNP (Figure 1C).

We subsequently studied the association between TC genotypes and plasma homocysteine concentrations. None of the TC genotypes was associated significantly with elevated plasma homocysteine concentrations, although an apparent trend was observed for the P259R variant (Figure 1D). In addition, vitamin B12 levels in erythrocytes were not influenced by the TC genotypes (data not shown).

Finally, we calculated mean apo-TC levels for each P259R genotype, to elucidate whether the decrease in total-TC concentration related to the P259R variant was merely due to a decrease in holo-TC or also due to a decrease in apo-TC. Individuals homozygous for the arginine residue showed lower apo-TC concentrations compared with individuals with the heterozygous 259PR (*P*=0.05) and wild type 259PP genotypes (*P*=0.002). In addition, individuals with the heterozygous form of this variant tended to have lower apo-TC concentrations than the wild type individuals (*P*=0.07). The association between the P259R genotypes and decreased proportion bound vitamin B12 to TC could not be explained by low vitamin B12 levels in blood because holo-HC levels, which represent 80% of vitamin B12 in plasma, were similar in the three genotype groups (data not shown).

Discussion

DNA sequencing analyses of the coding region of the TC gene revealed five variants: I23V, G94S, P259R, S348F and the R399Q. Some of the genotypes defined by these variants influenced total-TC protein content in plasma, holo-TC concentrations and the proportion vitamin B12 bound to TC: however, no effect on homocysteine and NTD risk could be detected.

The previously published P259R variant[11,19] was associated with a distinct reduction in total-TC protein and apo-TC. Moreover, it was also associated with a pronounced decreased holo-TC concentration and holo-TC/total-TC ratio. A possible explanation for the latter could be a low total vitamin B12 status. However, holo-HC and total vitamin B12 concentrations were not different between the three P259R genotypes. Therefore, the low holo-TC/total-TC ratio may be explained by a reduced binding of vitamin B12 to TC. In spite of this observation, this variant cannot explain the elevated risk for NTD associated with a low proportion vitamin B12 bound to TC as reported by us previously,[11] as the prevalence of this variant was even lower in mothers of NTD children compared with controls. No significant effect of the P259R polymorphism was found on erythrocyte vitamin B12 and plasma homocysteine

concentrations, although a tendency towards higher homocysteine levels in homozygous 259RR individuals compared with 259PP subjects was found (*P*=0.07). Larger studies are required to elucidate whether this P259R variant affects plasma homocysteine, intracellular vitamin B12 concentrations and NTD risk.

The effect of the P259R variant on TC levels may be explained by differences in charge of the protein resulting from the substitution of a proline by an arginine, i.e, a neutral by a positively charged one.[19] According to Chou-Fasman secondary structure predictions, a tendency to form helix structures was predicted as previously described by Li *et al*.[19] Although the helix structure was not confirmed using the GOR II algorithm,[19] we found that the latter method predicted a tendency to form turn structures. Both predictions point towards a change in secondary structure. The P259R is not located in one of the six hydrophobic regions, postulated to form the hydrophobic pocket for vitamin B12 binding.[20] Nevertheless, due to the intra-molecular folding, the P259R substitute may result in a disturbed binding of vitamin B12 to TC.

Our results partly corroborate those of Namour *et al*,[21] who also found an association of the P259R variant with decreased apo-TC concentrations in 159 healthy Caucasians. They observed significantly lower homocysteine levels in 259RR homozygotes compared with the heterozygous 259PR individuals. In contrast, our results point towards elevated homocysteine levels in 259RR homozygotes. In both studies the number of individuals included, may not have been sufficient to elucidate whether this SNP is associated with increased homocysteine concentrations.

Heterozygosity for the I23V variant was associated with lower total-TC concentrations, however, it lead to a higher proportion vitamin B12 bound to TC. The I23V variant was previously described by Li *et al*.[15] In that study, the coding region of the TC gene was aligned to the coding regions of intrinsic factor (IF) and one of the two haptocorrins, termed 'TCI' in that study. The authors reported that this variant was located in a conserved region of the TC protein; however, both IF and 'TCI' encode a valine instead of an isoleucine at codon 23, which may indicate that the substitution of isoleucine by valine may even improve vitamin B12 binding. In addition, the I23V SNP was not located in one of the six hydrophobic regions postulated to be involved in vitamin B12 binding.

The R399Q variant is associated with increased total-TC concentrations but does not seem to affect vitamin B12 binding and erythrocyte vitamin B12 or plasma homocysteine concentrations. In addition, the R399Q is not situated in one of the six hydrophobic regions thought to be involved in vitamin B12 binding. More individuals have to be examined to elucidate whether this SNP affects metabolic variables and whether it is associated with an increased risk for NTD.

The allele frequencies of some of the SNPs studied are very low. This applies to the G94S, for which only two individuals are heterozygous. The homozygous 23VV genotype was found in only three individuals and the homozygous 348FF was observed in two individuals. The S348F showed a trend towards a higher proportion B12 bound to TC. A consequence of the low prevalences is that this study on potential effects of these SNPs on metabolic variables and on the risk for NTD is lacking power. Examination of these SNPs in larger studies may reveal a potential effect on TC function.

In summary, none of the SNPs found in the TC gene was associated with a significantly increased risk for NTD. The P259R variant, that is expected to change the secondary structure of TC, can only partly explain the reduced proportion vitamin B12 bound to TC, which is associated with an elevated NTD risk. Some of the SNPs seem to affect TC concentrations or function; however, the relatively small number of individuals in our study precludes a conclusion whether these SNPs affect vitamin B12 and plasma homocysteine levels. Therefore, larger studies on these aspects are warranted. In addition, expression of the TC SNPs in an appropriate expression system may provide more information about their possible effects on TC function. Furthermore, in specific situations, some of these TC variants may critically effect homocysteine remethylation and risk for disease for example in individuals with a low B12 status.

The SNPs observed in the coding region of the TC gene can only partly explain the reduced binding of vitamin B12 to TC, related to a fivefold increased risk for NTD.[11] Possibly, non-coding regions of the TC gene contain variations that influence splicing, affecting TC function. Further research should focus on the non-coding region of the TC gene, using proteomics and RNA technology, to find an explanation for the disturbed binding of vitamin B12 to TC in mothers of NTD children.

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