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Polymorphisms in the Transcobalamin Gene: Association with Plasma Homocysteine in Healthy Individuals and Vascular Disease Patients

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Background: **Hyperhomocysteinemia is an independent risk factor for cardiovascular disease (CVD). Intracellular vitamin B_{12} deficiency may lead to increased plasma total homocysteine (tHcy) concentrations and because transcobalamin (TC) is the plasma transporter that delivers vitamin B_{12} to cells, genetic variation in the TC gene may affect intracellular vitamin B_{12} availability and, consequently, tHcy concentrations.**

Methods: **We examined five sequence variants, i.e., I23V, G94S, P259R, S348F, and R399Q, in the TC gene as possible determinants of tHcy and, concordantly, as possible risk factors for CVD in 190 vascular disease patients and 601 controls. We also studied potential effect-modification of vitamin B_{12} by genotype.**

Results: **In individuals with high vitamin B_{12}, 259PP individuals had lower tHcy concentrations than 259PR and 259RR individuals. Homozygous 23VV individuals had lower fasting tHcy concentrations than their 23IV and 23II peers. None of the genotypes defined by the three other sequence variants showed an association with tHcy concentrations, nor was any TC genotype associated with an increased CVD risk.**

Conclusions: **In individuals in the highest quartile of the vitamin B_{12} distribution (>299 pmol/L), tHcy concentrations are lower in 259PP homozygotes than in 259PR and 259RR individuals. Therefore, 259PP individuals, who represent >25% of the general population, may be more susceptible to reduction of plasma tHcy concentrations by increasing the vitamin B_{12} status.**

Molecular defects in genes encoding enzymes involved in homocysteine metabolism may account for hyperhomocysteinemia, an independent and graded risk factor for cardiovascular disease (CVD)[5] *(1, 2)*. Homocysteine can either be catabolized in the transsulfuration pathway to cysteine or be remethylated to methionine by methionine synthase. Methionine synthase requires vitamin B_{12} as a cofactor to transfer the methyl group of 5-methyltetrahydrofolate via vitamin B_{12} to homocysteine. Intracellular vitamin B_{12} availability may therefore influence plasma total homocysteine (tHcy) concentrations.

Low vitamin B_{12} concentrations in the cell can be the result of low vitamin B_{12} intake, but they can also be attributable to a disturbance in the absorption, transport, or cellular uptake of this vitamin. Transcobalamin (TC) is the transporter of vitamin B_{12} in the circulation and delivers vitamin B_{12} to the cells *(3)*. After binding by haptocorrin in the stomach and intrinsic factor in the duodenum, vitamin B_{12} is transferred to TC within the enterocyte and released into the blood. Subsequently, the vitamin B_{12}-TC complex is taken up by receptor-mediated endocytosis via the receptor TC-R *(3)*. Variations in the TC protein could affect the binding characteristics of vitamin B_{12} to TC or recognition of the vitamin B_{12}-TC complex by TC-R, with possible repercussions on vitamin B_{12} availability in the cells. Therefore, genetic variation in the TC gene may produce altered plasma tHcy concentrations and CVD risk.

The TC gene has been mapped to chromosome 22, between bands 22q12 and 22q13 *(4)*, and encodes a

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[5] Nonstandard abbreviations: CVD, cardiovascular disease; tHcy, total homocysteine; TC, transcobalamin; OR, odds ratio; CI, confidence interval; and RFLP, restriction fragment length polymorphism.

polypeptide of 43–45 kDa *(5)*. The TC gene spans ~20 kb, contains nine exons and eight introns *(4)*, and shows considerable heterogeneity *(4, 6–9)*. Li et al. *(6)* described four sequence variants of the human TC gene: M198T, I219L, P259R, and S376L. In 1994, Li et al. *(7)* published an additional polymorphic site at position 234 (Q→R). Subsequently, a substitution of isoleucine by valine at codon 23 was reported *(4)*. Both mammalian intrinsic factor and TC I (one of the forms of haptocorrin), two other vitamin B_{12}-transporting proteins, are highly homologous in their amino acid sequences to TC (33% overall and up to 80% in certain, mostly hydrophobic, regions) *(6)*.

Very recently, Namour et al. *(10)* reported that the blood apo-TC (the proportion of TC that does not contain vitamin B_{12}) concentration of 259PP homozygotes was significantly higher than that of homozygous 259RR and heterozygous individuals. In addition, heterozygotes had higher tHcy concentrations compared with both homozygous genotypes.

In the present study, we examined five sequence variants in the TC gene, which were found by sequencing analyses (Afman et al., in press), as possible genetic determinants of plasma tHcy concentration and, concordantly, as possible genetic risk factors for CVD in 190 vascular disease patients and in 601 apparently healthy individuals.

Materials and Methods

PATIENTS AND CONTROLS

We studied 190 patients with coronary, peripheral, or cerebral vascular disease, of whom 130 cases were recruited from a cohort of patients who underwent coronary angiography in the Zuiderziekenhuis Hospital (Rotterdam, The Netherlands) *(11)*. The other 60 patients had documented premature CVD: 10 had experienced a myocardial infarction, 32 had been diagnosed with cerebral arterial occlusive disease, and 18 had been diagnosed with peripheral arterial occlusive disease *(12)*. The control group consisted of 601 controls, of whom 101 were from the general population in Rotterdam *(11)* and 500 were recruited from a general practice in The Hague, The Netherlands *(13)*. The protocol was approved by the local ethics committee, and written informed consent was obtained from each participant.

HOMOCYSTEINE AND VITAMIN B_{12} ANALYSES

All cardiovascular disease patients and controls underwent a standardized oral methionine loading test (0.1 g L-methionine/kg of body weight) *(14)*. Plasma tHcy was measured by reversed-phase separation HPLC with fluorescence detection, as described by te Poele-Pothoff et al. *(15)*. All tHcy measurements were conducted in our laboratory at the University Medical Center Nijmegen, The Netherlands. Fasting and postload plasma tHcy concentrations were obtained from 675 and 650 individuals, respectively. Serum vitamin B_{12} concentrations were measured with the Dualcount SPNB (solid-phase, no boil) radioassay (Diagnostic Product Corporation) in samples stored at −70 °C. Vitamin B_{12} concentrations were available for some of the control group (n = 500).

STATISTICS

The distributions of plasma tHcy and vitamin B_{12} concentrations showed positive skewness; therefore, natural log-transformed tHcy and vitamin B_{12} values were used in all statistical analyses. Differences between patient and control groups were tested with the Student *t*-test for continuous variables and the Pearson χ^2 test for dichotomous frequency measures. *P* values were derived from age- and sex-adjusted linear regression models. Age- and sex-adjusted odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated by logistic regression analysis to estimate the relative risk of CVD associated with the different genotypes. One-way ANOVA was used to assess the differences in continuous variables between different genotypes, followed by Bonferroni-corrected *t*-tests. In addition, we calculated Pearson correlation coefficients for the correlation between vitamin B_{12} and plasma tHcy. All reported *P* values are two-tailed, and $P < 0.05$ was considered statistically significant.

The haplotype frequencies and linkage disequilibrium were estimated by the EH program *(16)*. The extent of the linkage disequilibrium (D') is reported as the ratio between the actual value of D and the maximum value it could have for the given allele frequencies, where D is the departure from linkage disequilibrium. The sign in front of the coefficients indicates whether the rare alleles are associated (+) or whether the rare allele at one locus is associated with the most common allele at the other locus (−).

MUTATION DETECTION

In our ongoing effort to understand the molecular basis of hyperhomocysteinemia, we analyzed the coding region of the TC gene in 12 females (Afman et al., in press). Of those 12 females, 8 were selected on the basis of their low holo-TC concentrations and low holo-TC/total-TC ratio: 7 were mothers who had given birth to a child with a neural tube defect, and 1 was a control. The remaining four females included one mother who had a child with a neural tube defect and three female controls, all with high holo-TC concentrations. Using DNA sequencing analyses with intron-based oligonucleotides, we found five variants: I23V (67A→G; exon 2), G94S (280G→A; exon 3), P259R (776C→G; exon 6), S348F (1043C→T; exon 7), and R399Q (1196G→A; exon 8; Table 1). We also examined whether the Q234R variant *(7)* was present in a portion of our study population (161 cases and 100 controls; Table 1). Restriction fragment length polymorphism (RFLP) analyses were used to determine the genotypes defined by these six TC variants; PCR primers and conditions are described in Afman et al. (in press). We obtained a complete set of genetic data for 596 individuals.

Table 1. Sequence variants in the TC gene.

DNA variant	Amino acid substitution	Location	Screening method	Allele frequency of mutant allele,[a] %
67A→G	I23V[b]	Exon 2	RFLP + *Rsa*I	12.6
280G→A	G94S	Exon 3	RFLP + *Alw*NI	0.8
701A→G	Q234R[c]	Exon 5	RFLP + *Msp*I	Not found
776C→G	P259R[c]	Exon 6	RFLP − *Bst*NI	46.9
1043C→T	S348F	Exon 7	RFLP-PIRA[d] − *Esp*I	11.1
1196G→A	R399Q	Exon 8	RFLP − *Bsm*AI	2.0

[a] Patients and controls combined.
[b] Li et al. *(8)*.
[c] Li et al. *(11)*.
[d] PIRA, primer-introduced restriction analysis.

Results

The main characteristics of the patient and control groups are shown Table 2. The percentage of males was higher among patients than controls ($P < 0.001$). Mean age did not differ between both groups. The patient group had a significantly higher geometric mean fasting tHcy ($P = 0.005$); postload tHcy also tended to be higher in patients ($P = 0.10$). Unfortunately, creatinine and folate concentrations were not available; therefore, we could not adjust for them.

We examined the coding region of the TC gene for possible genetic variation by sequencing analysis (Afman et al., in press) and found five possibly functional variants (Table 1). These five variants all led to an amino acid substitution, with possible impact on TC function. To assess the effects of these variants on plasma tHcy and vitamin B_{12} and their potential contribution to CVD risk, we analyzed samples from 191 CVD patients and 601 controls. We also examined a portion of our study population (161 cases and 100 controls) for the Q234R variant *(7)*, but did not find it. Genotype distributions for all sequence variations were in Hardy–Weinberg equilibrium in both controls and patients. The allele frequencies for the five genetic variants were very divergent, varying from 0.8% to 46.9% (Table 1). Allele frequencies and genotype distributions for all five variants did not differ between cases and controls (Table 3). Thus, none of the genotypes defined by the sequence variants was associated with an increased risk for CVD.

Table 2. Characteristics[a] of CVD patients and controls.

	Cases (n = 190)	Controls (n = 601)	*P*
Age, years	49.0 ± 10.2	50.6 ± 12.7	0.11[b]
Sex, % male	75.3	47.1	<0.001[c]
Fasting tHcy, μmol/L	14.4 (13.7–15.0)	13.1 (12.7–15.1)	0.005[d]
Postload tHcy, μmol/L	41.1 (39.7–43.2)	40.1 (39.1–41.1)	0.10[d]

[a] Age is expressed as mean ± SD, and tHcy is expressed as geometric mean (95% CI).
[b] Student *t*-test.
[c] Pearson χ^2 test.
[d] Adjusted for age and sex.

Because separate analysis of the relationships between TC genotypes and tHcy in patients and in controls produced the same results (data not shown), we combined both groups to increase statistical power. Homozygous 23VV individuals (n = 6) had lower fasting tHcy concentrations than their 23IV (n = 153) and 23II (n = 507) peers ($P = 0.05$ and 0.07, respectively; Fig. 1). None of the genotypes defined by the other variants showed an association with fasting or postload tHcy or vitamin B_{12} concentrations.

To examine the effect of having more than one variation in the TC gene, we combined different genotypes to generate composite genotypes. As described above, it appeared that individuals homozygous for the I23V variant had low plasma tHcy concentrations. Individuals who were also heterozygous for the P259R variant had even lower tHcy concentrations; the three individuals with the 23VV/259PR genotype had a geometric mean tHcy concentration of 7.4 (95% CI, 3.4–15.8) μmol/L (Table 4).

We also assessed potential gene–environment interactions by subdividing a portion the control group (n = 500; see *Materials and Methods*) into quartiles according to vitamin B_{12} status. This stratification showed a significantly lower tHcy concentration in 259PP homozygotes (geometric mean, 10.9 μmol/L; n = 49) compared with 259PR (12.7 μmol/L; n = 82) and 259RR (12.6 μmol/L; n = 36) individuals (ANOVA, $P = 0.010$; Bonferroni-corrected *t*-test, $P = 0.012$ and 0.063, respectively; Fig. 2) in the fourth quartile of the serum vitamin B_{12} distribution (>299 pmol/L). We subsequently calculated the correlation coefficients between vitamin B_{12} and plasma tHcy for each 259P→R genotype. In both the 259PP homozygotes and the 259PR heterozygotes, we observed a clear negative correlation between vitamin B_{12} and tHcy ($r = -0.39$ and -0.30, respectively; both $P < 0.001$), whereas this correlation was absent in 259RR individuals ($r = -0.07$; $P = 0.5$).

On the basis of the three most frequent polymorphisms, i.e., I23V, P259R, and S348F, we constructed TC haplotypes and calculated their relative frequencies with the EH program *(16)* (Table 5). The allele frequencies of the other two TC variants were very low and were

Table 3. Prevalence and ORs (95% CI) for CVD risk according to TC polymorphisms in patients and controls.

Genotype	Controls, n (%)	Patients, n (%)	OR[a]	95% CI	P[b]
23II	388 (75.3)	127 (77.0)	1[c]		0.37
23IV	121 (23.5)	38 (23.0)	0.87	0.57–1.34	
23VV	6 (1.2)	NO[d]			
94GG	498 (98.4)	179 (98.4)	1[c]		0.95
94GS	8 (1.6)	3 (1.6)	0.85	0.22–3.33	
94SS	NO	NO			
259PP	141 (27.0)	49 (27.1)	1[c]		0.77
259PR	276 (52.8)	91 (50.3)	0.94	0.62–1.42	
259RR	106 (20.2)	41 (22.6)	1.04	0.81–1.33	
348SS	409 (78.8)	134 (77.5)	1[c]		0.36
348SF	105 (20.2)	39 (22.5)	1.03	0.67–1.58	
348FF	5 (1.0)	NO			
399RR	497 (96.1)	164 (95.3)	1[c]		0.65
399RQ	20 (3.9)	8 (4.7)	1.12	0.47–2.64	
399QQ	NO	NO			

[a] ORs are adjusted for age and sex.
[b] χ^2 test.
[c] Reference category.
[d] NO, not observed.

therefore omitted. The two most common haplotypes, A and C, accounted for >80% of all the chromosomes defined by these three polymorphisms (Table 5). We found no differences in haplotype frequencies between cases and controls (data not shown).

In addition, we calculated linkage disequilibrium coefficients (D') between these variants. A high degree of linkage disequilibrium was observed between the three most common polymorphisms; D' was −0.42 between the I23V and P259R variants, −0.97 between the P259R and S348F variants, and 0.97 between the I23V and S348F variants (all $P < 0.001$).

Discussion

Using an exon-based DNA sequencing strategy, we examined the TC gene for possible genetic variants and found five (see Afman et al., in press): I23V (67A→G; exon 2), G94S (280G→A; exon 3), P259R (776C→G; exon

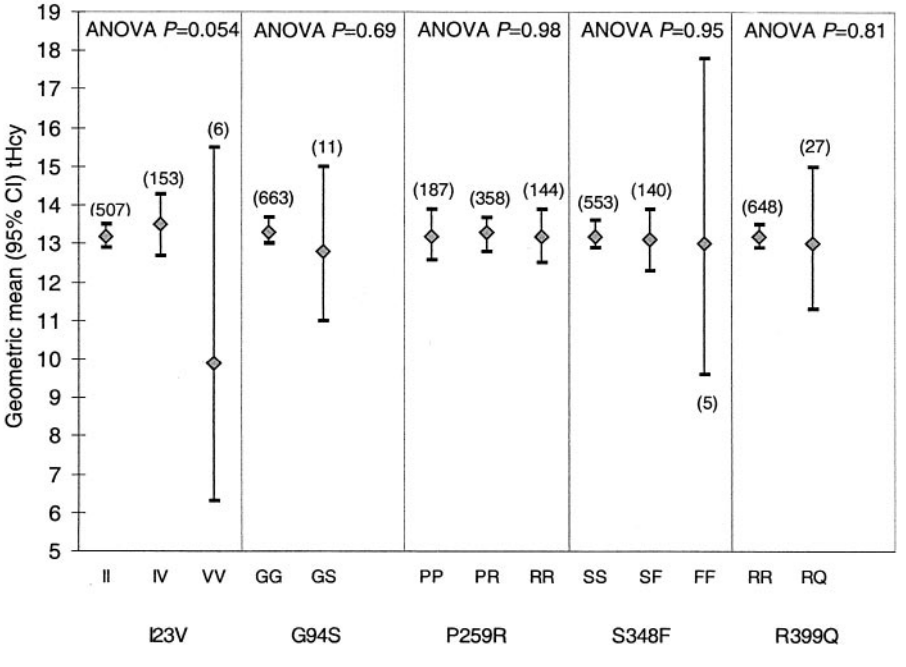

Fig. 1. Relationship between TC genotypes and fasting tHcy in the entire study population.

Values in *parentheses* indicate the number of individuals in each genotype group.

Table 4. Relationship between I23V and P259R TC composite genotypes and fasting tHcy.[a]

	tHcy,[b] μmol/L		
Genotype	**23II**	**23IV**	**23VV**
259PP	13.3 (12.5–14.0) (n = 120)	13.2 (12.0–14.4) (n = 57)	11.8 (2.8–395) (n = 2)
259PR	13.3 (12.8–13.7) (n = 260)	13.4 (12.3–14.6) (n = 74)	7.4 (3.4–15.8)[c] (n = 3)
259RR	13.1 (12.4–13.8) (n = 116)	13.6 (11.8–15.7) (n = 19)	Not observed

[a] Overall ANOVA, $P = 0.016$.
[b] Expressed as geometric mean (95% CI).
[c] $P < 0.05$ vs 23II/259PP, 23IV/259PP, 23II/259PR, 23IV/259PR, 23II/259RR, and 23IV/259RR (Bonferroni-corrected *t*-test).

6), S348F (1043C→T; exon 7), and R399Q (1196G→A; exon 8; Table 1). We also looked for the Q234R variant, reported previously by Li et al. *(7)*, but did not find this TC variant in our study population. None of the five variants affected vitamin B_{12} concentrations, nor was one of the five sequence variants associated with CVD risk. The six 23VV individuals had lower tHcy concentrations compared with those with the 23IV and 23II genotypes; 23VV individuals who were also heterozygous for the P259R substitution had even lower tHcy concentrations (Table 4), but this subset consisted of only three individuals. The 259PP homozygotes had decreased tHcy concentrations compared with 259PR and 259RR individuals when vitamin B_{12} concentrations were in the highest quartile of the distribution (>299 pmol/L; Fig. 2).

Genetic variations in the TC gene may influence the transport of vitamin B_{12} to the cells by altering the vitamin B_{12} binding site or impairing recognition by TC-R. Because vitamin B_{12} is crucial in the transfer of the methyl group of 5-methyltetrahydrofolate to homocysteine, decreased intracellular concentrations could lead to an increase in plasma tHcy. Unfortunately, holo-TC and total TC concentrations were not measured in the current study population; we could therefore not assess the potential influence of TC variants on vitamin B_{12} binding.

When we stratified a portion of the control group (n = 500) into quartiles according to their vitamin B_{12} status, we found that 259PP homozygotes in the fourth quartile for serum vitamin B_{12} (>299 pmol/L) had a lower tHcy concentration than 259PR and 259RR individuals. These data indicate that 259PP individuals in particular benefit from high vitamin B_{12} concentrations with respect to tHcy lowering, in contrast to their 259PR and 259RR peers, and suggest a gene–environment interaction between this TC variant and vitamin B_{12}. We therefore also investigated whether the P259R genotype influences the slope of the curve relating plasma tHcy and vitamin B_{12} by calculating the correlation coefficient between these two variables for

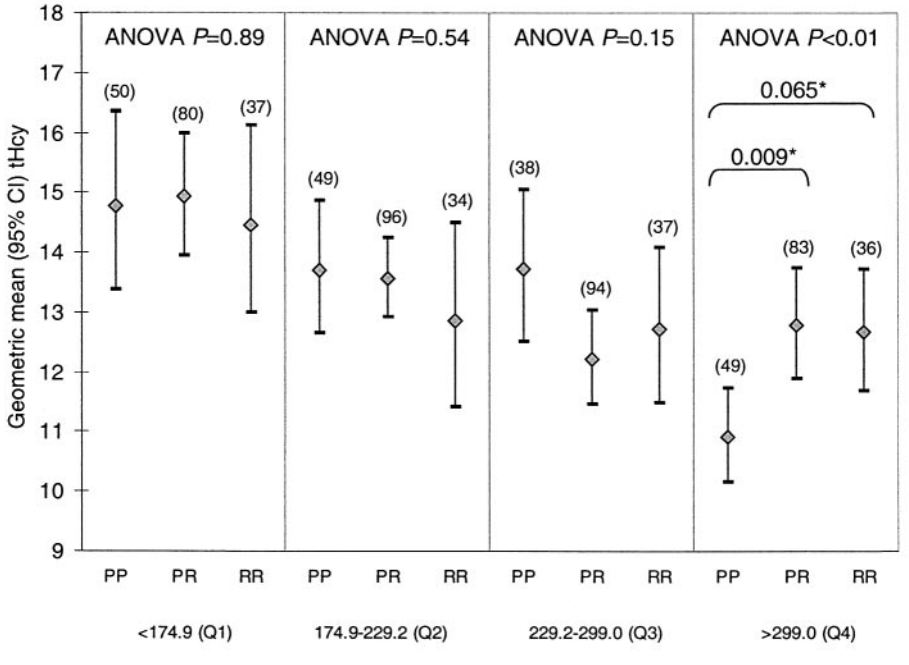

Fig. 2. Association between the P259R genotype and fasting tHcy after stratification for vitamin B_{12} concentrations (quartiles, *Q1–Q4*).

Vitamin B_{12} concentrations are in pmol/L. *Values* in *parentheses* indicate the number of individuals in each genotype group. *Values* above *brackets* in *Q4* are *P* values; * indicates statistical significance.

Table 5. Main haplotype frequencies of the TC gene and their relative frequencies.

Haplotype	Polymorphism			Relative frequency
	I23V	P259R	S348F	
A	I	P	S	0.416
B	I	P	F	0.029
C	I	R	S	0.433
D	I	R	F	<0.001
E	V	P	S	0.005
F	V	P	F	0.082
G	V	R	S	0.033
H	V	R	F	0.001

each P259R genotype group separately. We observed a clear negative correlation between vitamin B_{12} and plasma tHcy concentrations in 259PP individuals as well as in 259PR heterozygotes, whereas individuals with the 259RR genotype demonstrated no such association. The 259P allele thus may affect TC transcription or the binding and transport of vitamin B_{12} by TC, with positive repercussions on the availability of vitamin B_{12} in the cell. This raises the interesting hypothesis that more than one-fourth of the general population has a TC genotype that significantly increases susceptibility to tHcy-lowering intervention by vitamin B_{12}.

Our results correspond well with the data of Namour and coworkers *(9, 10)*, who were the first to report an association between the P259R variant and vitamin B_{12} and apo-TC concentrations in a small group of 39 healthy Caucasians *(9)*. They reported that the P259R variant affects the serum concentrations of both apo-TC and vitamin B_{12}; compared with the 259RR homozygotes, 259PP homozygotes had 1.7- and 1.4-fold higher serum apo-TC and vitamin B_{12} serum concentrations, respectively. This group was unable to reproduce their results showing an association between the 259PP genotype and increased vitamin B_{12} in a somewhat larger study that included 159 healthy Caucasians, but they did find, similar to their previous results, a 1.4-fold higher apo-TC concentration in 259PP homozygotes compared with 259RR homozygotes *(10)*.

Very recently, we measured the plasma concentrations of total vitamin B_{12}, tHcy, and the apo and holo forms of TC and haptocorrin in 46 mothers who had delivered a child with a neural tube defect and in 73 female controls *(17)*. We observed that tHcy concentrations were significantly higher among individuals with low holo-TC and total vitamin B_{12} concentrations and low holo-TC/total-TC ratios. It appears that these low holo-TC concentrations were not attributable to low plasma vitamin B_{12}, but were likely the result of reduced binding of vitamin B_{12} to TC because we observed no correlation between holo-TC and vitamin B_{12} concentrations when the holo-TC concentration was below the 50th percentile *(17)*. These low holo-TC/total-TC ratios may be explained by the reduced affinity of TC for vitamin B_{12}, possibly attributable to allelic heterogeneity in the TC gene. In another study, we found both lower holo-TC and a lower holo-TC/total-TC ratio in 259PR and 259RR individuals compared with 259PP individuals (Afman et al., in press). This suggests that the 259P protein has an increased affinity for vitamin B_{12} and consequently can influence tHcy.

In conclusion, in individuals in the highest quartile of vitamin B_{12} distribution (>299 pmol/L), the tHcy concentration was lower in 259PP homozygotes compared with 259PR and 259RR individuals. We plan to elucidate the exact mechanism for this observation in future studies. The 259P allele probably is associated with increased vitamin B_{12} binding or transport ability of TC, which is in line with other of our data [Ref. *(17)* and Afman et al., in press]. Therefore, individuals with the 259PP genotype, a group that constitutes >25% of the general population, may be more susceptible to reduction of plasma tHcy concentrations by increasing the vitamin B_{12} status.

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