Association of total plasma homocysteine with methylenetetrahydrofolate reductase genotypes 677C>T, 1298A>C, and 1793G>A and the corresponding haplotypes in Swedish children and adolescents

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Abstract. We studied 692 Swedish children and adolescents (aged 9-10 or 15-16 years, respectively), in order to evaluate the effect of the methylenetetrahydrofolate reductase (MTHFR) 677C>T, 1298A>C, and 1793G>A polymorphisms on total plasma homocysteine concentrations (tHcy). Genotyping was performed with Pyrosequencing™ technology. The MTHFR 677C>T polymorphism was associated with increased tHcy concentrations in both the children and the adolescents ($P<0.001$ for both age groups) in both genders. The effect of MTHFR 1298A>C was studied separately in subjects with the 677CC and 677CT genotypes, and the 1298C allele was found to be associated with higher tHcy levels both when children were stratified according to 677C>T genotypes, and when using haplotype analyses and diplotype reconstructions. The 1793A allele was in complete linkage disequilibrium with the 1298C allele. It was still possible to show that the 1793A allele was associated with lower tHcy levels, statistically significant in the adolescents. In conclusion, a haplotype-based approach was slightly superior in explaining the genetic interaction on tHcy plasma levels in children and adolescents than a simple genotype based approach (R^2 adj 0.44 vs. 0.40). The major genetic impact on tHcy concentrations is attributable to the MTHFR 677C>T polymorphism. The common 1298A>C polymorphism had a minor elevating effect on tHcy, whereas the 1793G>A polymorphism had a lowering effect on tHcy.

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Introduction

An increased plasma total homocysteine (tHcy) is a risk factor for cardiovascular disease, neural tube defects and other birth defects (1). There is evidence that increased serum Hcy levels are associated with declining cognitive function and dementia (2). Deficiency of B vitamins, in particular folate, and/or mutations in genes coding for enzymes or proteins involved in the metabolism, are major causes of elevated concentrations of tHcy (3-7).

The homocysteine metabolism has two pathways. Hcy can be remethylated to methionine or it can undergo the irreversible transsulfuration to cystathionine (7). Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the remethylation reaction where it catalyses the reduction of methylenetetrahydrofolate to methyltetrahydrofolate, which is the methyl donor for the conversion of homocysteine to methionine (8). There are two universally common and well-investigated polymorphisms in the gene for MTHFR, 677C>T and 1298A>C. The 677C>T polymorphism was identified in 1995 (3). The T allele causes an alanine to valine amino acid substitution (Ala222Val) within the catalytical domain of the enzyme, which results in a termolabile form of the enzyme (3) with a reduced activity to ~35% compared to control values (9).

The 1298A>C polymorphism was identified in 1997 (10). This polymorphism changes a glutamate to an alanine (Glu429Ala) in the regulatory domain of the enzyme (8,11). To what extent this polymorphism affects the activity of the enzyme is somewhat unclear and may depend on the test system used. Some studies have shown that the mutation leads to decreased activity (9,11), whereas others have shown the opposite (8). There is a third common MTHFR polymorphism, 1793G>A (12), which was suggested to constitute a particular MTHFR haplotype which may protect against dementia (13).

The 677C>T polymorphism is associated with a mild increase in tHcy (14,15), but subjects with the TT genotype have normal tHcy if their folate status is optimal (16). The 1298A>C polymorphism is believed not to cause elevated

tHcy concentrations, except when present with the 677T allele in 'compound heterozygotes' (17,18). Whether the 1793G>A polymorphism has any impact on tHcy levels has not been clarified (19). Moreover, the impact of MTHFR haplotypes on plasma tHcy concentrations has not been extensively studied. We hypothesised that a haplotype-based analysis would help clarify the impact of the MTHFR 1298A>C and 1793G>A polymorphisms on tHcy levels, and we report here our findings in a random sample of healthy children and adolescents.

Materials and methods

Subjects. Blood samples were obtained from 692 children (336 girls and 356 boys) belonging to the Swedish section of the European Youth Heart Study (EYHS). EYHS is a cross-sectional school-based study of risk factors for future cardiovascular disease among children 9-10 years old and adolescents 15-16 years old. Mean ages in the Swedish sample were 9.6 years (born in 1989) and 15.6 years (born in 1983), respectively. Sampling procedures and participation rates have been described previously (20). A specific written informed consent to the present genetic study was provided by the subjects. The study was approved by the Research Ethics Committees of Örebro County Council and Huddinge University Hospital.

Homocysteine assay, DNA extraction and MTHFR genotyping. Homocysteine in acidified citrated plasma (21) was analysed using a fluorescence polarization immunoassay on an IMx® unit (Abbott Laboratories, IL, USA). Total blood DNA was extracted and purified from 200 μl of whole blood anti-coagulated with EDTA, using the QIAamp DNA blood mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The purity was assessed by the ratio of A_{280}/A_{260} which was typically 1.7-1.8. All PCR amplifications were performed with the HotStar TaqDNA polymerase kit (Qiagen) and an Eppendorf Mastercycler was used. The reaction volume was 50 μl for all polymorphisms. MTHFR 677C>T was amplified according to the Pyrosequencing assay protocol 'Genotyping of the C677T variant in the human methylenetetrahydrofolate reductase (MTHFR) gene', version 1, from Biotage AB, Uppsala, Sweden (www.biotage.com). Approximately 60 ng of genomic DNA was used as template. For the MTHFR 1298A>C and 1793G>A polymorphisms we used our own genotyping protocols using the Pyrosequencing platform as described (22).

Statistics. For test of Hardy-Weinberg equilibrium a χ^2 test was used. Plasma tHcy concentrations required transformation in order to achieve a normal distribution. After ln transformation, residuals showed a satisfactory pattern and ln tHcy was used in all statistical analyses. In the tables and the figure untransformed data are provided.

Analysis of variance (ANOVA) was used to test for differences in tHcy between age groups, gender, and the MTHFR genotypes and haplotypes. If the ANOVA showed an interaction effect, stratifications were made accordingly. For *post hoc* comparisons a Tukey's test was used. The single subject with genotype MTHFR 1793AA was not included in the statistical analysis. As removal of extreme tHcy concentrations (outliers) had no influence on presented results, all tHcy measurements were included. Statistical significance was interpreted as values of $P<0.05$. Haplotype frequencies for the three polymorphisms in the MTHFR gene were estimated using Arlequin software (http://anthro.unige.ch/arlequin) and the statistical software package PHASE, version 1.0.1 (23). Statistical analyses were performed using SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA) or Statistix (Analytical Software, Tallahassee, FL, USA).

Table I. Genotype prevalences and allele frequencies of the three studied MTHFR polymorphisms in 692 healthy children and adolescents from central Sweden.

Polymorphism			χ^2
677C>T	C/C	330 (47.7)	0.605
	C/T	302 (43.6)	
	T/T	60 (8.7)	
	p(C)	0.695	
	q(T)	0.305	
1298A>C	A/A	302 (43.6)	1.785
	A/C	322 (46.5)	
	C/C	68 (9.8)	
	p(A)	0.669	
	q(C)	0.331	
1793G>A	G/G	628 (90.8)	0.200
	G/A	63 (9.1)	
	A/A	1 (0.1)	
	p(G)	0.953	
	q(A)	0.047	

The number of subjects and percentage are shown, as well as χ^2 for Hardy-Weinberg equilibrium testing. For alleles, the number of alleles and percentage are shown.

Results

MTHFR genotypes and tHcy concentrations. The genotype prevalences and allele frequencies for all three studied MTHFR polymorphisms for the EYHS subjects are shown in Table I. All loci were in Hardy-Weinberg equilibrium. A three-way ANOVA with the factors age group (children, adolescents), gender (girls, boys), and MTHFR 677C>T genotype (at the levels CC, CT, and TT) showed that age group and MTHFR 677C>T genotype had main effects on tHcy concentrations ($P<0.01$ for both) but not gender. There was an interaction effect between age group and gender ($P=0.008$); R^2 adj of the model = 0.405. Data was therefore stratified by age group and gender, and a one-way ANOVA showed no gender effect on tHcy in children, but in adolescents the boys had higher concentrations of tHcy than the girls ($P=0.046$). The median and mean tHcy concentrations in relation to the MTHFR 677C>T genotypes are shown in

Table II. Total plasma homocysteine concentrations grouped according to MTHFR genotypes and age group.

	n	Median	Mean ± SD	n	Median	Mean ± SD	n	Median	Mean ± SD	P (ANOVA)
677C>T	CC			CT			TT			
Children										
Girls	55	6.28	6.44±1.26	70	6.53	6.50±1.23	11	7.75	8.10±2.15	0.0036
Boys	83	6.28	6.17±1.05	67	6.43	6.70±1.56	15	6.56	6.83±0.60	0.0221
All	138	6.28	6.28±1.14	137	6.50	6.60±1.40	26	6.83	7.36±1.57	0.0006
Adolescents										
Girls	94	7.90	8.06±1.41	83	8.25	8.54±2.82	18	13.54	16.81±10.19	<0.0001
Boys	93	8.38	8.49±1.96	78	9.00	9.45±2.82	16	13.83	20.69±18.01	<0.0001
All	187	8.09	8.28±1.72	161	8.50	8.98±2.85	34	13.54	18.63±14.34	<0.0001
1298A>C	AA			AC			CC			
Children										
Girls	61	6.36	6.66±1.46	66	6.56	6.52±1.34	9	6.84	6.82±1.45	0.7392
Boys	72	6.50	6.46±1.35	72	6.23	6.36±1.31	21	6.75	6.67±0.91	0.4698
All	133	6.43	6.55±1.40	138	6.30	6.44±1.32	30	6.76	6.72±1.07	0.4136
Adolescents										
Girls	90	8.29	9.91±6.10	83	8.37	8.53±2.03	22	7.72	7.70±1.10	0.0850
Boys	77	9.09	11.23±9.55	95	8.81	9.15±2.72	15	8.44	8.32±2.12	0.0731
All	167	8.56	10.52±7.88	178	8.51	8.86±2.43	37	8.07	7.95±1.60	0.0071
1793G>A	GG			GA			AA			
Children										
Girls	128	6.43	6.63±1.40	8	6.43	6.30±1.43	0	-		0.4473
Boys	148	6.41	6.49±1.30	17	5.85	6.04±1.07	0	-		0.1797
All	276	6.41	6.55±1.34	25	6.28	6.12±1.17	0	-		0.1081
Adolescents										
Girls	178	8.23	9.20±4.60	17	7.39	7.73±1.36	0	-		0.1340
Boys	166	9.07	10.22±6.85	20	7.19	7.64±1.66	1	9.49	-	[a]0.0082
All	344	8.50	9.69±5.81	37	7.23	7.68±1.51	1	9.49	-	[a]0.0034

[a]The single subject with AA homozygosity was disregarded. Median, mean, and SD (μmol/l) are shown. P-values calculated with ln-transformed tHcy values.

Table II. The MTHFR 677T allele was associated with increased tHcy concentrations in both children and adolescents.

A three-way ANOVA with the factors age group, gender, and MTHFR 1298A>C genotype (at the levels AA, AC, and CC) showed that age group and MTHFR 1298A>C genotype had main effects on tHcy concentrations (P<0.01 and P=0.049 respectively). Gender had no main effect and there was no statistically significant interaction effect between age group and gender; R^2 adj of the model = 0.250. The median and mean tHcy concentrations in relation to the MTHFR 1298A>C genotypes are shown in Table II. For consistency, stratification by gender is shown. The A allele (wild-type) appears to be associated with increased tHcy concentrations in adolescents.

A three-way ANOVA with the factors age group, gender, and MTHFR 1793G>A genotype (at the levels GG and GA) demonstrated that age group and MTHFR 1793G>A genotype had main effects on tHcy concentrations (P<0.001 and P=0.013, respectively). Gender had no main effect and there was no statistically significant interaction effect between age group and gender; R^2 adj of the model = 0.253. The median and mean tHcy concentrations in relation to the MTHFR 1793G>A genotypes for all subgroups are shown in Table II. For consistency, stratification by gender is shown. The 1793G allele (wild-type) appears to be associated with increased tHcy concentrations in adolescents (Table II).

To further elucidate the effect on tHcy of the MTHFR polymorphisms, the possible impact of linkage disequilibrium was considered. No subject was found to simultaneously have the MTHFR 677TT and 1298CC genotypes, and furthermore, no subject was found to be homozygous for the MTHFR 677T allele and simultaneously heterozygous for the MTHFR 1298A>C. Therefore, complete linkage disequilibrium was suggested between these two polymorphisms, and this needed to be considered when analyzing the effects on tHcy of the MTHFR 677T and 1298C alleles. To investigate the effect of the MTHFR 677T allele, subjects were stratified by age group and subjects with the 1298AC or 1298CC and 1793GA or 1793GG genotype were excluded

Table III. tHcy concentrations according to MTHFR 677C>T genotype.

	n	Mean ± SD	P (ANOVA)
Children (n=133)			
677CC	41	5.98±1.03	0.0002
677CT	66	6.59±1.38	
677TT	26	7.36±1.57	
Adolescents (n=167)			
677CC	49	8.21±1.96	<0.0001
677CT	84	8.58±2.53	
677TT	34	18.63±14.34	

Subjects with the 1298AC or CC and 1793GA or AA genotypes were excluded. Mean and SD (μmol/l) are shown. P-values calculated with ln-transformed tHcy values.

from the analysis. One-way ANOVA was performed and mean tHcy concentrations in relation to MTHFR 677C>T genotypes are shown in Table III. The 677T allele was associated with gradually significantly increased tHcy in both age groups.

A four-way ANOVA performed with the factors age group, gender, MTHFR 1298A>C genotype (at the levels AA, AC, and CC), and MTHFR 677C>T genotype (at the levels CC and CT) showed that age group and MTHFR 677C>T had main effects on tHcy concentrations (P<0.001 and P=0.001, respectively). There was an interaction effect between age group and gender (P=0.044) and between gender, MTHFR 1298A>C genotype, and MTHFR 677C>T genotype (P=0.021); R^2 adj of the model = 0.307. To further investigate the effect of the MTHFR 1298C allele, subjects were stratified by age group and by the two MTHFR 677C>T genotypes, CC and CT. Subjects with the MTHFR 1793GA or AA genotypes were excluded. One-way ANOVA was performed, and mean tHcy concentrations in relation to

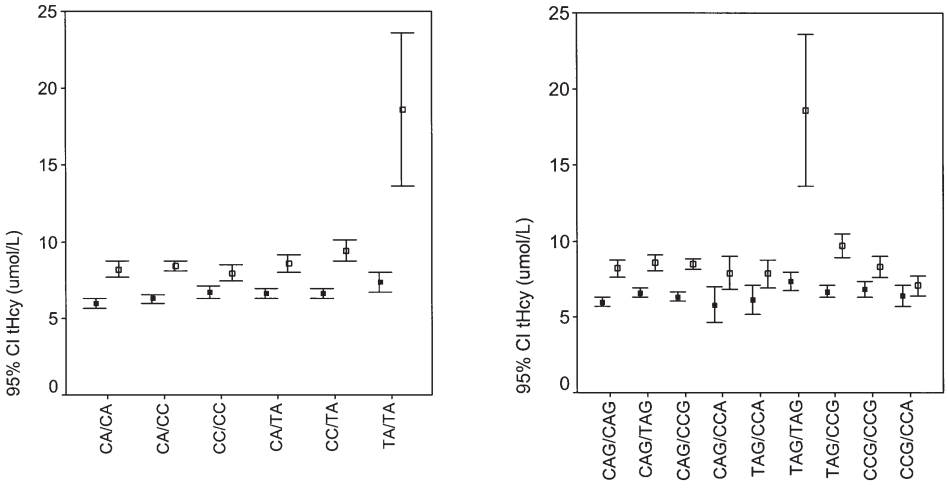

Figure 1. tHcy in relation to diplotypes of the MTHFR 677C>T, 1298A>C and 1793G>A polymorphisms. Left panel, the two-locus system (MTHFR 677C>T and 1298A>C); right panel, the three-locus system (MTHFR 677C>T, 1298A>C, and 1793G>A). ■, children; □, adolescents. Mean levels are shown, whiskers denote the 95% CI.

Table IV. tHcy concentrations according to MTHFR 1298A>C genotype in subjects with the MTHFR 677CC or CT genotype.

	MTHFR 677C>T	MTHFR 1298A>C	n	Mean ± SD	P (ANOVA)
Children (n=250)	677CC	1298AA	41	5.98±1.03	0.0162
		1298AC	60	6.32±1.18	
		1298CC	22	6.84±1.14	
	677CT	1298AA	66	6.59±1.38	0.7624
		1298AC	61	6.68±1.43	
Adolescents (n=310)	677CC	1298AA	49	8.21±1.96	0.4790
		1298AC	89	8.50±1.61	
		1298CC	24	8.33±1.67	
	677CT	1298AA	84	8.58±2.53	0.0168
		1298AC	64	9.70±3.28	

Subjects with the 1793GA or AA genotypes were excluded. Mean and SD (μmol/l) are shown. P-values calculated with ln-transformed tHcy values.

Table V. Haplotype prevalences of the MTHFR polymorphisms 677C>T, 1298A>C and 1793G>A.

	Prevalence
Haplotype 677-1298	
CA	0.364
CC	0.331
TA	0.305
Haplotype 677-1298-1793	
CAG	0.364
CCG	0.284
CCA	0.047
TAG	0.305

Upper panel, analysis as a two-locus system, nt 677-1298. Lower panel, the three-locus system, nt 677-1298-1793.

MTHFR 1298A>C genotypes are shown in Table IV. The 1298C allele was associated with gradually significantly increased tHcy in the children with the 677CC genotype, but there was no association in children with the 677CT genotype. In the adolescents, the compound heterozygotes 677CT/1298AC had ~13% higher mean tHcy concentrations than 677CT/1298AA subjects, a significant difference (P=0.0168, Table IV).

MTHFR haplotypes and tHcy concentrations. To further assess the combined contribution of these three MTHFR loci to tHcy concentrations, haplotype analyses were performed. Since molecular haplotypes could not be determined, MTHFR haplotype prevalences were inferred based on genotyped prevalences of the individual polymorphisms, using Arlequin software. The two-locus system MTHFR 677-1298 yielded three haplotypes only (Table V); CA, CC, and TA. The introduction of the third polymorphic locus, 1793G>A into the calculations, yielded only one additional haplotype. As seen from Table V, this was due to complete linkage disequilibrium of this third common polymorphism to the 1298C allele. Calculating haplotypes using the software PHASE (23) yielded identical results to those obtained with Arlequin software.

A three-way ANOVA with the factors age group, gender, and MTHFR diplotypes (haplotype combinations) from the two-locus system MTHFR 677-1298 (at the levels CA/CA, CA/CC, CC/CC, CA/TA, CC/TA, and TA/TA) showed that age group and diplotypes had main effects on tHcy concentrations (P<0.001 for both). An interaction effect was found between age group and the diplotypes (P<0.001); R^2 adj of the model = 0.407. Therefore data was stratified by age group. Fig. 1, left panel, shows the mean tHcy plasma concentrations in relation to MTHFR diplotypes, analysed as a two-locus system MTHFR 677-1298. A two-way ANOVA with the factors gender and diplotypes showed that diplotypes had a major effect in both children and adolescents (both P<0.001; R^2 adj = 0.063 and 0.267, respectively). In the adolescents, gender also had a marked effect (P=0.017). *Post hoc* analysis showed that in children, TA/TA had significantly higher tHcy levels than CA/CA (P<0.001) and CA/CC (P=0.005) but not CC/CC, CA/TA, or CC/TA. In adolescents, the TA/TA diplotype had significantly higher tHcy concentrations (P<0.001 against all other diplotypes).

A three-way ANOVA with the factors age group, gender and diplotypes from the three-locus system MTHFR 677-1298-1793 (at the levels CAG/CAG, CAG/TAG, CAG/CCG, CAG/CCA, TAG/CCA, TAG/TAG, TAG/CCG, CCG/CCG, and CCG/CCA) showed that age group and the diplotypes had major effects on tHcy concentrations (P<0.001 for both). There was an interaction effect between age group and the diplotypes (P<0.001); R^2 adj of the model = 0.442. Therefore data was stratified by age group. A two-way ANOVA with the factors gender and diplotypes showed that the diplotypes had main effects in children (P=0.001, R^2 adj = 0.062) and in adolescents (P<0.001, R^2 adj = 0.278). *Post hoc* analysis showed that children with the TAG/TAG diplotype had higher tHcy levels than children with the CAG/CAG diplotype (P=0.001) and the CAG/CCG diplotype (P=0.026). Adolescent subjects with TAG/TAG diplotype had significantly higher tHcy concentrations than subjects with the other diplotypes (P<0.001 against all other diplotypes). Fig.1, right panel, displays the mean tHcy plasma concentrations in relation to MTHFR diplotypes, analysed as a three-locus system MTHFR 677-1298-1793. Comparing subjects who had the diplotypes CCG/CCG or CCG/CCA, respectively, allows for isolating the effect of the 1793A allele. In adolescents, the 1793A allele was associated with a significantly lower tHcy level by 15% (P=0.024) (see

Table VI. Total plasma homocysteine concentrations in children and adolescents grouped according to diplotype of the three-locus system MTHFR 677C>T-1298A>C-1793G>A.

	Diplotype 677-1298-1793	n	Mean ± SD	P (ANOVA)
Children (n=30)	CCG/CCG	22	6.84±1.14	0.348
	CCG/CCA	8	6.39±0.84	
Adolescents (n=36)	CCG/CCG	24	8.33±1.67	0.024
	CCG/CCA	12	7.05±1.04	

Mean and SD (μmol/l) are shown. P-values calculated with ln-transformed tHcy values.

Table VI). In children, this difference was of a smaller magnitude and not statistically significant.

Discussion

A haplotype analysis of the MTHFR gene was performed, both as a two-locus system (677C>T and 1298A>C) and as a three-locus system (677C>T, 1298A>C, and 1793G>A). No child that was simultaneously homozygous for the 677C>T polymorphism and heterozygous for the 1298A>C polymorphism was found, and this is consistent with haplotype analysis which yielded only three 677-1298 haplotypes. Some studies report findings of the uncommon 677T-1298C haplotype (18) and it cannot be ruled out that in very large study populations this haplotype might occur even in Sweden. Findings of this haplotype might also have been due to the use of RFLP as the genotyping method in most of the studies from the 1990s. In this study, real-time DNA sequencing was used with newly developed Pyrosequencing™ assay protocols for these polymorphisms (22), providing more valid results. Inclusion of the third polymorphism, the MTHFR 1793G>A, in the haplotype analysis yielded only one more haplotype. This is due to the close linkage between the 1298A>C and the 1793G>A polymorphisms. In the white Swedish population, 9.3% were heterozygous for this recently discovered MTHFR polymorphism. The frequency of the mutated allele was 4.7% (q=0.047), a lower frequency than in American whites (6.9%) (12).

The tHcy concentrations in relation to the MTHFR 1793G>A polymorphism were either not reported in the few studies which genotyped for it (12,13), or findings were inconclusive (19,24). Employing the haplotype-based approach, we showed here for the first time that the MTHFR 1793A allele appeared to have a lowering effect by 15% on tHcy concentrations in adolescents (Fig. 1, right panel; Table VI). The 1298C allele had the opposite effect. Children with the CCG/CCA diplotype also had lower tHcy levels than children with the CCG/CCG diplotype, but in this age group the difference was not statistically significant.

Through the haplotype-based approach, the relative importance of the three studied MTHFR loci on tHcy plasma levels could also be assessed. The best explanatory power was obtained by the three-locus haplotype system MTHFR 677-1298-1793, giving an adjusted R^2 of 44.2%. The model based on the two-locus system MTHFR 677-1298 gave an adjusted R^2 of 40.7%, similar to that of the simple genotype-based model utilizing only the 677C>T genotype which reached an adjusted R^2 of 40.5%. The MTHFR 1793A allele therefore exerts a small but demonstrable lowering effect on tHcy plasma levels, accounting for about 4% of the variance in tHcy levels in Swedish children and adolescents.

Plasma tHcy concentrations for large population samples of children and adolescents have only recently been published; there are none from Sweden so far. The concentrations in Swedish children and adolescents (Table II) are appreciably higher than those reported recently from the USA (25,26) or Canada (27), more similar to those published from Northern Ireland (28), Belgium (29) and The Netherlands (30,31) but lower than those reported in Taiwanese (32) and Brazilian (33) studies. The lowest concentrations were found in children, with appreciably higher concentrations among adolescents, as in previous studies.

In conclusion, a haplotype-based approach was slightly superior in explaining the genetic interaction on tHcy plasma levels in children and adolescents than a simple genotype based approach (R^2 adj = 0.44 vs. 0.40). The MTHFR 677C>T and the 1298A>C are in complete linkage disequilibrium, as are the MTHFR 1298A>C and the 1793G>A loci. The major genetic impact on tHcy concentrations in Swedish children and adolescents is due to a tHcy-raising effect of the 677C>T polymorphism. MTHFR 1298A>C may have a minor tHcy-elevating effect limited to particular subgroups such as children, and adolescents who are simultaneously MTHFR 677CT heterozygotes. The MTHFR 1793G>A polymorphism appears to have a minor tHcy-lowering effect.

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