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Genetic and Environmental Influences on Plasma Homocysteine: Results from a Danish Twin Study

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Background: **Increased plasma homocysteine has been linked to many clinical conditions including atherosclerosis and ischemic stroke. We assessed the genetic and environmental influences on homocysteine in adult twins and tested the influence of 3 candidate polymorphisms.**
Methods: **Homocysteine was analyzed in 1206 healthy twins, who were genotyped for 3 polymorphisms: *MTHFR* 677C>T, *MTR* 2756A>G, and *NNMT* (dbSNP: rs694539). To perform quantitative trait linkage analysis of the *MTHFR* locus, the genotyping was supplemented with 2 genetic markers localized on each site of the *MTHFR* locus. The twin data were analyzed using biometric structural equation models as well as a combined association and linkage analysis in 2 age cohorts.**
Results: **Age, sex, and *MTHFR* genotype have a significant impact on homocysteine concentrations, whereas the other genotypes were not associated with homocysteine concentrations. The variance in homocysteine could be solely ascribed to additive genetic and nonshared environmental factors, with an estimated additive genetic proportion of total variation at age 18–39 years of 0.63 (95% CI, 0.53–0.71) and at age 40–65 years of 0.27 (95% CI, 0.10–0.41). The impact of the *MTHFR* locus is estimated to explain 53% (95% CI, 0.07–0.67) of the total phenotypic variation in persons 18–39 years old and 24% (95% CI, 0.00–0.39) in persons 40–65 years old, i.e., almost all additive genetic variance.**
Conclusions: **Homocysteine concentrations have a high heritability that decreases with age. The *MTHFR* gene locus is responsible for almost all the variation attributable to genetic factors, leaving very little influence of other genetic variations.**

Homocysteine (Hcy)[4] is a sulfur-containing intermediate of methionine metabolism. Hyperhomocysteinemia has been linked to many clinical conditions including atherosclerosis, ischemic stroke, osteoporosis, cognitive impairment, and death *(1–7)*. The exact pathophysiological effects of Hcy are incompletely understood, and it still remains to be proven whether moderate hyperhomocysteinemia is a causative factor or a marker of other risk factors in these diseases.

The underlying causes for mild to moderate hyperhomocysteinemia are both genetic and environmental *(8)*. These influences may be mediated by other variables reflecting genetic and/or environmental effects; thus, increasing Hcy concentrations are associated with male sex, smoking, coffee consumption, increasing age, high blood pressure, unfavorable lipid profile, and high creatinine. Variables such as physical activity, moderate alcohol consumption, and a good folate or vitamin B-12 status are associated with lower Hcy concentrations *(9)*. Genetic factors are also thought to directly affect tHcy concentrations *(10, 11)*, although studies have reported very different estimates of heritability *(12, 13)*.

Several enzymes are involved in the methionine/homocysteine metabolic pathway. Cofactors include pyridoxine (vitamin B_6), vitamin B_{12}, and folate. The 3 key enzymes involved are methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), and cystathionine β-synthase (CBS). It is well known that a common polymorphism in the *MTHFR* gene[5] (677C>T) has a

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[4] Nonstandard abbreviations: Hcy, homocysteine; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; CBS, cystathionine β-synthase; DZ, dizygotic; MZ, monozygotic; OS, opposite-sex pairs; BMI, body mass index; AIC, Akaiki information criterion; and QTL, quantitative trait locus.
[5] Human genes: *MTHFR*, 5,10-methylenetetrahydrofolate reductase

pronounced impact on plasma Hcy concentration. As T allele dose increases, this functional polymorphism causes a graded increase in Hcy in the mild-moderate range, most pronounced in individuals with low dietary folate consumption *(14)*. A common polymorphism in the *MTR* gene (2756A>G) also seems to influence plasma Hcy, with the A-allele and the AA genotype associated with increased Hcy concentration *(15)*. No associations of Hcy concentrations with frequent polymorphisms in *CBS* have been found. Other genes have been linked to plasma Hcy—in a recent study a genomewide scan found a new candidate gene at chromosome 11q23, the nicotinamide N-methyltransferase gene (*NNMT*) as a major determinant of plasma Hcy *(16)*. One single-nucleotide polymorphism (dbSNP: rs694539) in intron 1 in the *NNMT* gene was significantly associated with Hcy concentrations.

We have assessed the genetic and environmental influences on plasma Hcy as measured in a large population-based sample of adult twin pairs. Furthermore, we tested the influence of 3 common polymorphisms in 3 candidate genes, *MTHFR*, *MTR*, and *NNMT*, that code for enzymes involved in the synthesis or the metabolism of Hcy, and estimated their relative contribution to the calculated heritability.

Materials and Methods

This study is part of a twin study of the metabolic syndrome and related components (GEMINAKAR) for which study participants were identified through the population-based Danish Twin Registry *(17)*. The selection of study participants for inclusion is described in detail elsewhere *(18)*. The regional ethics committee approved the study and all participants provided informed consent. In total, 603 adult twin pairs not suffering from diabetes or cardiovascular diseases [238 dizygotic (DZ), 239 monozygotic (MZ), and 126 opposite-sex pairs (OS)] participated in the present study. The participants underwent a thorough clinical physiologic examination and an oral glucose tolerance test. Plasma for Hcy measurement was collected in a sodium fluoride (NaF) tube simultaneously with the 2-h final glucose measurement. The NaF tube was centrifuged and plasma was separated within 1 h.

Plasma was kept at −80 °C until analysis. Zygosity was established by use of 9 polymorphic DNA-based microsatellite markers with the PE Applied Biosystems AmpFISTR Profiler Plus Kit (Perkin-Elmer). Hcy was measured in plasma samples from 1206 participants by use of the Imx® Hcy assay and the Imx analyzer (Abbott).

genotyping

Both members of the DZ twin pairs and 1 member from each MZ twin pair were genotyped. The TaqMan™ technology was used to genotype the 3 polymorphisms in *MTHFR* (677C>T), *MTR* (2756A>G), and *NNMT* (dbSNP ID: rs694539). *MTHFR* and *MTR* genotyping procedures were performed as previously described *(19)*. *NNMT* genotyping was performed with a TaqMan single-nucleotide polymorphism genotyping assay (ID: C_2134722_10).

To perform quantitative trait linkage analysis of the *MTHFR* locus, the genotyping was supplemented with the genetic markers D1S2740 and D1S2667 localized on each site of the locus. These markers have an observed heterozygosity of 0.62 and 0.83. The sense primers were labeled with 6-FAM. PCR products were resolved on the MegaBACE 1000 and analyzed with Fragment Profiler software (Amersham Biosciences).

statistical analysis

The statistical program package Stata (StataCorp, Stata Statistical Software: Release 8.0) was used for the statistical calculation. The distribution of genotypes and the presence of Hardy-Weinberg equilibrium were tested by a χ^2 test. Because the plasma concentrations of Hcy had a skewed distribution, all values were transformed by natural logarithm before analysis. We used ANOVA to compare mean Hcy according to age, sex, body mass index (BMI), smoking, fasting glucose, time of year, and *MTHFR*, *NNMT*, and *MTR* genotype.

estimation of intrapair correlations

In humans 2 types of twinning occur: MZ twins share all their segregating genes and DZ twins, like ordinary siblings, share, on average, 50% of their genes. In the classical twin study, MZ and DZ intraclass correlations for a trait are compared. A higher correlation in MZ twins indicates that genetic factors contribute to the variation. The intrapair correlations for the MZ and the same- and opposite-sex DZ twin pairs were pooled and estimated with Mx software *(20)*. The covariates sex and age were included.

estimation of heritability

To estimate heritability of Hcy, we used data from intact, same-sex, and opposite-sex twin pairs. The twin data were analyzed using biometric structural equation models *(20)*. It was assumed that the total variance (V) in a scale could be decomposed as:

$$V = A + D + C + E,$$

where A refers to the variance contribution of additive genetic effects, D refers to the variance contribution of genetic effects due to dominance (intralocus interaction), C refers to the variance contribution of shared environmental effects (i.e., environmental factors that are shared by twins reared together and thus a source of their similarity), and E refers to the variance contribution of nonshared environmental effects (i.e., environmental factors that are not shared by twins reared together, which in

(NADPH); *MTR*, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; *NNMT*, nicotinamide N-methyltransferase; and *CBS*, cystathionine-beta-synthase.

the analysis are considered as a source of random variation).

Variance components are estimated by the maximum likelihood method using the Mx software *(20)*. An ACE sex limitation model was fitted to evaluate whether different sets of genes influence Hcy concentration expression in males and females. Afterward the following models were fitted: ACE, ADE, AE, DE, CE, and E. Age and sex were included as covariates in the variance components models. The best-fitting model was chosen in accordance with the lowest Akaiki information criterion (AIC) ($2 \cdot \text{loglikelihood} + 2 \cdot df$) *(21)*. Thereby both the goodness of fit and the simplicity of the model were taken into account in model selection.

To account for the possibility that different genetic variations may influence plasma Hcy at different ages, the analyses were done in 2 age cohorts, young twins (18–39 years) and middle-aged twins (40–65 years). These age cohorts were selected because they each contained approximately half of the study population.

linkage analysis

The linkage analysis, the association analysis, and the combined analyses were performed as described by Beekman et al. *(22)*. The software package Merlin 1.0.1 was used to estimate the distribution of the identity-by-descent probabilities at the *MTHFR* locus *(23)*.

The linkage model for the natural logarithm of the Hcy concentration is:

$$y_{ij} = \mu + (\beta_1 \times \text{age}_{ij}) + (\beta_2 \times \text{sex}_{ij}) + e_{ij},$$

where y_{ij} is the observed log-Hcy concentration for twin j in the i-th twin pair, μ denotes the grand mean, β_1 denotes the regression coefficient for age, β_2 denotes the deviation of females, and e_{ij} denotes a residual that is not explained by age and sex. The variance of e_{ij} is decomposed into A, E, and additive genetic variance due to a quantitative trait locus (QTL) in the vicinity of the marker. No other variance components were included, because the previous twin analysis found the best-fitting model to be the AE model. We used a weighted likelihood approach to obtain maximum advantage of the full distribution of the identity-by-descent probabilities.

association analysis

The association model for the natural logarithm of the observed Hcy concentrations as a function of the *MTHFR* polymorphism is

$$y_{ij} = \mu + (\beta_1 \times \text{age}_{ij}) + (\beta_2 \times \text{sex}_{ij}) + (a_b \times A_{bi}) + (d_b \times D_{bi}) + (a_w \times A_{wij}) + (d_w \times D_{wij}) + e_{ij},$$

where y_{ij} is the observed score for twin j in the i-th twin pair and μ, β_1, and β_2 denote as above. A_{bi} is the derived coefficient for the additive genetic effect of the *MTHFR* polymorphism between twin pairs for the i-th twin pair; A_{wij} denotes the coefficient as derived for the additive genetic effects of the *MTHFR* polymorphism within twins for twin j from the i-th family; D_{bi} is the coefficient for the dominant genetic effect of the *MTHFR* polymorphism between twin pairs for the i-th twin pair; D_{wij} denotes the coefficient for the dominant genetic effects of the *MTHFR* polymorphism within twin pairs for twin j from the i-th twin pair; a_b and a_w are the estimated additive effects between and within twin pairs; d_b and d_w are the estimated dominance effects between and within families; and e_{ij} denotes a residual that is not explained by the age, sex, or allelic effects of the *MTHFR* polymorphism. The variance of e_{ij} is decomposed into A and E components. Population stratification was tested by constraining genetic effects within and between twin pairs (i.e., $a_w = a_b$ and $d_w = d_b$), and the presence of nonadditive allelic effects at the *MTHFR* locus was tested by constraining the dominance coefficients to zero ($d_w = d_b = 0$) *(22, 24, 25)*.

combined association and linkage analysis

The association model that provided the most parsimonious fit to the data (lowest AIC) was taken as a starting point for the combined association and linkage analysis. In the combined model, the variance in the natural logarithm of the Hcy concentrations that was not accounted for by age, sex, and the *MTHFR* polymorphism (e_{ij}) was decomposed into A, E, and Q components and thus was tested for the *MTHFR* polymorphism whether linkage with the log-Hcy concentration was still present when modeled simultaneously with association of the *MTHFR* polymorphism *(24, 25)*. Again we used a weighted likelihood approach to account for the full distribution of identity-by-descent probabilities.

Because the correlations between twins 1 and 2 in some of the twin pairs could have been outliers (a scatter plot of the log-Hcy concentration between twin 1 and 2 is shown in Fig. 1), the estimation of heritability, association, and linkage analysis were repeated after exclusion of the pairs with the highest difference, a total of 2%.

Results

Sample characteristics and the Hcy distribution are shown in Tables 1 and 2. The covariates age, sex, and *MTHFR* genotype had a significant impact on tHcy ($P < 0.03$), whereas BMI, season (summer or winter), smoking, fasting blood glucose, and genotypes for *NNMT* and *MTR* ($P > 0.74$) were without a significant impact. Mean (SD) is shown in Table 2 divided in groups according to age, sex, and *MTHFR* genotype.

distribution of genotypes

The genotype distributions (Table 1) are compatible with the Hardy-Weinberg equilibrium for the population as a whole [$P = 0.99$ (*MTHFR*), $P = 0.37$ (*MTR*), and $P = 0.19$ (*NNMT*)], as well as for females [$P = 0.98$ (*MTHFR*), $P = 0.89$ (*MTR*), and $P = 0.58$ (*NNMT*)]. For males the distri-

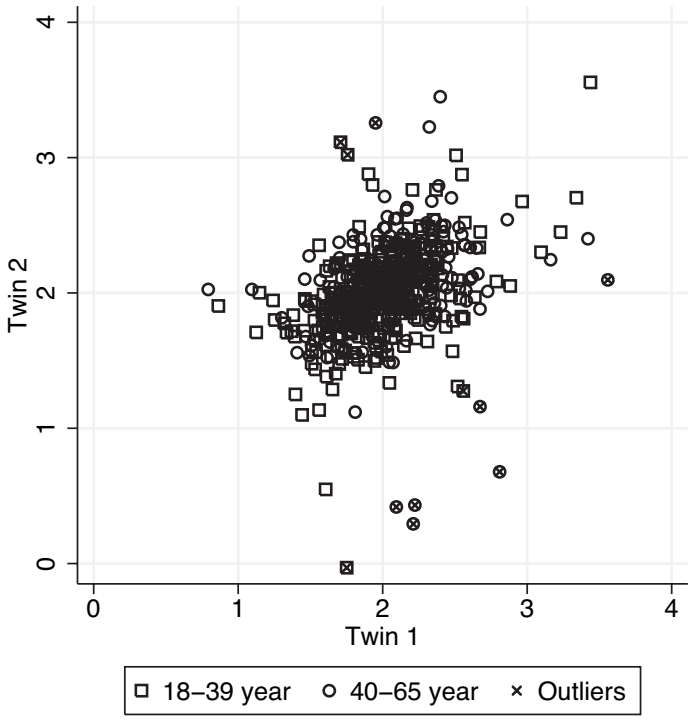

Fig. 1. A scatter plot of the log-Hcy concentration in twin 1 and 2 in a twin pair.

The 2% most deviating pairs are deleted in the confirmatory statistical analyses and are marked with an *X*.

bution was in Hardy-Weinberg equilibrium for *MTHFR* and *MTR* ($P = 0.97$ and $P = 0.15$). The distribution for *NNMT*, however, deviated significantly from Hardy-Weinberg equilibrium ($P = 0.006$) because of fewer than expected heterozygotes.

ESTIMATION OF HERITABILITY

Both age groups exhibited significantly higher intrapair correlations for MZ than DZ twin pairs (youngest group, $P = 0.002$; oldest group, $P = 0.05$). The excess similarity for MZ compared to DZ pairs indicates a significant genetic influence on log-Hcy concentration.

The sex limitation ACE model showed no sex differences in genetic relationships for the natural logarithm of Hcy concentration ($P = 0.88$ in the youngest group, $P = 0.39$ in the oldest) (results not shown). Therefore, the opposite-sexed twin pairs were included together with the same-sex DZ twin pairs in all subsequent analyses. In both age groups we found that the best-fitting model in the twin analysis (lowest AIC) was the AE model that included sex and age as covariates, a finding indicating that the proportion of variance in Hcy concentration for given sex and age is solely due to additive genetic factors (*A*) and nonshared environmental factors (*E*) (Tables 3 and 4).

Table 1. Sample characteristics.[a]

	Males (n = 592)			Females (n = 614)		
Age, mean (range)	38.1 (18–63)			38.2 (18–67)		
Body mass index, mean (range)	24.8 (17.7–40.4)			24.0 (16.4–43.7)		
Fasting glucose	4.7 (2.7–9.3)			4.9 (2.8–13.0)		
Summer, %	38.1			39.0		
Smoking, n (%)	198 (33.4)			200 (32.5)		
No. of MZ	234			244		
Estrogen use, n (%)				124 (20.1)		
Systolic BP, mmHg (SD)	120.6 (13.5)			114.0 (14.5)		
Diastolic BP, mmHg (SD)	70.9 (10.3)			68.2 (10.4)		
Total cholesterol, mmol/L (SD)	5.5 (1.2)			5.5 (1.2)		
Genotype distribution						
MTHFR	CC	CT	TT	CC	CT	TT
(n = 1136)	269	235	51	288	242	51
MTR	AA	AG	GG	AA	AG	GG
(n = 1126)	376	153	23	377	177	20
NNMT	GG	GA	AA	GG	GA	AA
(n = 1095)	380	134	25	360	178	18

[a] Summer: samples drawn between May 1 and October 31.

LINKAGE ANALYSIS

In the youngest twin group the analysis showed a significant QTL effect of the *MTHFR* locus ($P = 0.04$) compared to an AE model, but for the oldest group this effect was insignificant ($P = 0.57$). The QTL effect was thus estimated to account for a considerable proportion of the phenotypic variance and most of the additive genetic variance.

ASSOCIATION ANALYSIS

In both age groups, association had significant effect on log Hcy concentration ($P < 0.001$), and there was no evidence for the presence of population stratification ($P = 0.11$ and $P = 0.36$ in the 2 age groups). We could not exclude from the model a genetic dominant effect associ-

Table 2. Distribution of total plasma Hcy.[a]

	Age					
	<30 years			≥30 years		
Males						
Hcy μmol/L, mean (SD)	8.06 (3.80), n = 172			8.70 (3.39), n = 419		
MTHFR genotype	CC	CT	TT	CC	CT	TT
Hcy μmol/L, mean (SD)	6.98 (1.80), n = 79	7.98 (2.58), n = 59	14.98 (8.59), n = 14	8.27 (2.21), n = 189	8.67 (3.31), n = 176	11.45 (6.64), n = 37
Females						
Hcy μmol/L, mean (SD)	6.87 (2.37), n = 169			7.52 (2.93) n = 446		
MTHFR genotype	CC	CT	TT	CC	CT	TT
Hcy μmol/L, mean (SD)	6.53 (2.26), n = 71	6.68 (1.73), n = 75	9.46 (4.02), n = 16	6.90 (1.96), n = 217	7.88 (2.77), n = 165	10.16 (5.56), n = 35

[a] Genotype is missing in 37 males and 36 females due to lack of DNA material.

ated with the *MTHFR* locus ($P < 0.001$ in the youngest), although it showed only borderline significance in the oldest age group ($P = 0.03$).

COMBINED LINKAGE AND ASSOCIATION

We modeled the QTL effect together with the best-fitting association model (no population stratification). The youngest age group showed significant QTL in the presence of association ($P = 0.03$). The effect of the QTL situated in the *MTHFR* locus was estimated to explain 53% of the total variation in study participants in the 18–39 year age group, a result indicating that *MTHFR* variation apart from 677T>C is significant. In the oldest age group an estimated 24% of the total variance was due to the QTL, a value that did not differ significantly from zero ($P = 0.54$).

RETESTING AFTER EXCLUSION OF OUTLIERS

The statistical analyses were repeated after exclusion of the samples suspected to be outliers (Table 5). The esti-

Table 3. Fit statistics of nested models in young (18–39 years) Danish twins.

		q[a]	−2LL	AIC	vs	*df*	χ^2	*P*	Remarks
Model									
0	Saturated	12	321.223	345.223					r_{mz} = 0.64 (0.53;0.72)
									r_{dz} = 0.30 (0.16;0.42)
1	ACE	6	327.075	339.075	1 vs 0	6	5.85	0.44	
2	AE	5	327.075	337.075	2 vs 1	1	~0.00	~1.00	a^2 = 0.63 (0.53;0.71)
									e^2 = 0.37 (0.29;0.47)
3	CE	5	344.487	354.487	3 vs 1	1	17.41	<0.001	
4	E	4	413.432	421.432	4 vs 2	1	86.36	<0.001	
5	ADE	6	327.016	339.016	5 vs 0	6	5.79	0.45	
6	DE	5	331.110	341.110	6 vs 5	1	4.09	0.04	
Linkage + sex, age									
7	*AEQ*	6	322.983	334.983	5 vs 7	1	4.09	0.04	a^2 = 0.15 (0.00;0.63)
									e^2 = 0.35 (0.27;0.44)
									q^2 = 0.50 (0.02;0.72)
Association + sex, age									
8	$AE + a_{-b} + a_w + d_b + d_w$	9	263.057	281.057	5 vs 8	4	64.02	<0.001	
9	$AE + (a_{-b} = a_w) + (d_b = d_w)$	7	267.513	281.513	9 vs 8	2	4.46	0.11	a^2 = 0.57 (0.45;0.66)
									e^2 = 0.43 (0.33;0.55)
10	$AE + (a_{-b} = a_w) + (d_b = d_w = 0)$	6	279.980	291.980	10 vs 9	1	12.47	<0.001	
Linkage in the presence of association + sex, age									
11	$AEQ + (a_{-b} = a_w) + (d_b = d_w)$	8	262.516	278.516	9 vs 11	1	5.00	0.03	a^2 = 0.06 (0.00;0.51)
									e^2 = 0.41 (0.32;0.52)
									q^2 = 0.53 (0.07;0.67)

[a] *q*, No. of estimated parameters; −2LL, −2 · log likelihood; AIC, −2LL + 2 · q; vs, model comparison; *df*, no. of degrees of freedom; χ^2, difference in −2LL; *P*, probability of type 1 error.

Table 4. Fit statistics of nested models in middle-aged (40–65 years) Danish twins.

		q^a	−2LL	AIC	vs	*df*	χ^2	*P*	Remarks
Model									
0	Saturated	12	303.315	327.315					r_{mz} = 0.28 (0.09;0.44)
									r_{dz} = 0.12 (−0.05;0.27)
1	ACE	6	317.049	329.049	1 vs 0	6	13.73	0.03	
2	AE	5	317.049	327.049	2 vs 1	1	~0.00	~1.00	a^2 = 0.27 (0.10;0.41)
									e^2 = 0.73 (0.59;0.90)
3	CE	5	318.705	328.705	3 vs 1	1	1.66	0.20	
4	E	4	326.950	334.950	4 vs 2	1	9.90	<0.002	
5	ADE	6	316.989	328.989	5 vs 0	6	13.67	0.03	
6	DE	5	317.279	327.279	6 vs 5	1	0.29	0.59	
Linkage + sex, age									
7	*AEQ*	6	316.724	328.724	5 vs 7	1	0.33	0.57	a^2 = 0.04 (0.00;0.41)
									e^2 = 0.74 (0.59;0.90)
									q^2 = 0.23 (0.00;0.41)
Association + sex, age									
8	$AE + a_{-b} + a_w + d_b + d_w$	9	296.879	314.879	5 vs 8	4	20.17	<0.001	
9	$AE + (a_{-b} = a_w) + (d_b = d_w)$	7	297.705	311.705	9 vs 8	2	0.83	0.36	a^2 = 0.25 (0.08;0.40)
									e^2 = 0.75 (0.60;0.92)
10	$AE + (a_{-b} = a_w) + (d_b = d_w = 0)$	6	302.162	314.162	10 vs 9	1	4.46	0.03	
Linkage in the presence of association + sex, age									
11	$AEQ + (a_{-b} = a_w) + (d_b = d_w)$	8	297.137	313.137	9 vs 11	1	0.57	0.54	a^2 = 0.00 (0.00;0.39)
									e^2 = 0.76 (0.61;0.91)
									q^2 = 0.24 (0.00;0.39)

[a] *q*, No. of estimated parameters; −2LL, −2 · loglikelihood; AIC, −2LL + 2 · *q* (Akaiki's information criterion); vs, model comparison; *df*, no. of degrees of freedom; χ^2, difference in −2LL; *P*, probability of type 1 error.

mates of *A* and *E* are in the same order as before exclusion; however, the significant QTL effect of the *MTHFR* locus disappears.

Discussion

Plasma Hcy concentrations showed pronounced differences according to age, sex, and *MTHFR* genotype (Table 2). This finding is in accordance with previous studies identifying effects of lifestyle and specific genetic factors on Hcy concentrations *(26, 27)*. Although smoking, BMI, season, fasting blood glucose, and *MTR* and *NNMT* genotype, among other factors, have previously been shown to influence Hcy concentrations, the impact of these specific variables was not significant in our study. This study confirms that the *MTHFR* 677C>T polymorphism has a marked effect on Hcy plasma concentrations. The magnitude of this effect is so great that it calls into question the use of a single common reference interval for plasma Hcy concentrations.

The distribution of alleles in *NNMT* is in Hardy-Weinberg equilibrium in the population as a whole and in females, but not in males. Deviation from Hardy-Weinberg equilibrium could be due to genotyping error, chance, failure of assumptions underlying Hardy-Wein-

Table 5. Fit statistics of nested models after exclusion of outliers.

	Young twins (18–39 years)			
Model	A^2	e^2	q^2	*P* (QTL = 0)
AE	0.67 [0.58;0.74]	0.33 [0.26;0.42]		
AEQ	0.44 [0.00;0.74]	0.33 [0.26;0.41]	0.23 [0.00;0.70]	0.48
AEQ + association	0.25 [0.00;0.68]	0.38 [0.30;0.48]	0.37 [0.00;0.68]	0.18
Middle aged twins (40–65 years)				
AE	0.48 [0.35;0.60]	0.52 [0.40;0.66]		
AEQ	0.48 [0.01;0.60]	0.52 [0.40;0.66]	0.00 [0.00;0.44]	~1.00
AEQ + association	0.46 [0.00;0.58]	0.54 [0.42;0.68]	0.00 [0.00;0.46]	~1.00

berg expectations, or selection against certain genotypes. In the absence of an obvious reason for this finding, we interpret it as a chance finding.

With heritability defined as the proportion of the total variation in the trait that is due to genetic factors, our twin analyses show that Hcy concentrations were highly heritable, with a heritability of 63%, in the twins in the younger age group (18–39 years), but heritability decreased to 27% in the older twin group (40–65 years). When the component attributable to the *MTHFR* gene locus was included in the analyses, this component accounted for almost all of the heritability. This finding was significant in the younger age group but not completely significant in the older group, as indicated by inclusion of zero in the CI. One possible explanation for the drop in heritability with age could be that the total variance due to environmental factors increases with age and thereby decreases the heritability. Hcy concentrations increase with age, probably because of an age-dependent decrease in kidney function. This increase could decrease the relative importance of genetic influences.

Several correlations between the twin pairs (Fig. 1) may be outliers. Reliable statistical identification of outliers in a data set remains a challenge, and there are no clear recommendations as how to treat outliers. After exclusion of the 2% most deviating twin correlations, estimates of A and E were almost unchanged, but the QTL effect of the *MTHFR* locus was no longer significant, indicating that the results from the QTL analysis were driven by the outliers. Another explanation is that the combined association and linkage analysis, described by Beekman et al. *(22)* and used in this study, lacks power to detect loci affecting quantitative variation unless the QTL exerts a large effect or the number of study participants is very large. Given that our study had ~180 sibling pairs in each group, it is not surprising that the estimates of *Q*, or residual *Q* after allowing for association, have wide CIs (Tables 3 and 4).

The estimated heritability is in accordance with most published studies *(13)*, although a previous twin study failed to detect any heritability *(12)*, most likely due to small sample size (60 twin pairs). However, because heritability is a population-specific measurement reflecting the relative influences of genetic and environmental variation in the particular population, such findings could also be due to massive variation in environmental influence on Hcy concentrations in the selected population. For example, very large differences in folate consumption could probably obscure any genetic influence.

The finding in several previous studies that mild-to-moderate hyperhomocysteinemia is associated with cognitive dysfunction, cardiovascular diseases, and stroke *(1–7)* makes it tempting to believe that decreasing Hcy by B-vitamin supplementation may have a protective effect that arrests or slows the disease processes. The results from the Hcy-lowering trials have been conflicting, however. Two recently completed clinical trials, the Vitamin Intervention for Stroke Prevention and the Vitamin and Thrombosis trials, found no significant effects of Hcy-lowering therapy on vascular outcomes in patients with prior stroke *(28)* or venous thromboembolism *(29)*. In a subgroup of 2155 patients from the Vitamin Intervention for Stroke Prevention trial, however, an efficacy analysis derived after exclusion of participants with baseline B_{12} concentrations below the 25th or above the 95th percentile and/or glomerular filtration rates below the 10th percentile showed a 21% reduction in the risk of vascular events *(30)*. Results from the ongoing Hcy-lowering trials must be analyzed before recommendations can be made on the use of B-vitamins for prevention of vascular disease.

Whether increased Hcy mediates or is merely an indicator of metabolic abnormalities that lead to cardiovascular disease is uncertain. The finding that Hcy has a heritability of 27%–65% and that this genetic component is mostly attributable to the *MTHFR* gene locus highlights a possible association between this locus and disease progression. Several studies have looked into this possible association, however, and a recent metaanalysis did not provide evidence to support an association between the *MTHFR* 677C>T polymorphism and coronary heart disease *(31)*. The obvious conclusion is therefore that the disease risk associated with mild to moderate hyperhomocysteinemia is not an inherited risk. It is much more likely that increased Hcy is not a causal mechanism but a marker for an environmental disease risk such as inadequate vitamin supplementation or simply a phenomenon associated with existing disease.

To perform a combined association and linkage analysis to determine to what extent the polymorphisms in the *MTHFR* locus account for the observed heritability in Hcy plasma concentrations, we supplemented the genetic analysis with 2 genetic markers located on each site of the *MTHFR* locus, a procedure that enabled us to differentiate identity-by-descent from identity-by-state covariances. Combined association and linkage analysis is a powerful tool for pinpointing functional quantitative traits (QTLs) responsible for regions of significant linkage identified in genome-wide scans. The analysis revealed that the association of the *MTHFR* gene locus to Hcy plasma concentrations completely explained the linkage in the younger twin group and partly in the middle-aged twin group. The analysis also revealed that *MTHFR* genetic variation influences Hcy apart from 677C>T. Several genome-wide linkage scans have been conducted in an effort to localize genes influencing variation in plasma Hcy concentrations. One study identified an *NNMT* gene locus *(16)*, which was tested in our study and shown to be without significant influence. A recent linkage study could not confirm this linkage result, but identified 3 other regions that showed weak to suggestive linkage to Hcy concentrations

(13). This finding is not surprising, because random genomic screening of a complex trait does not have high power, and false-positive findings are common. We show that the *MTHFR* locus does account for nearly all of the heritability components in the younger twin group, thus making it very unlikely that a genomic screening would have the power to identify new loci involved in the Hcy concentration, because the impact of these regions must be very small.

In conclusion, we demonstrated that Hcy concentrations have a high heritability that decreases with age. The *MTHFR* gene locus is responsible for almost all the variation attributable to genetic factors, leaving only minor influence to other genetic variations.

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