The association between plasma homocysteine and coronary heart disease is modified by the MTHFR 677C>T polymorphism.

The association between plasma homocysteine and coronary heart disease is modified by the *MTHFR* 677C>T polymorphism

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ABSTRACT

Objective An elevated level of total plasma homocysteine (tHcy) has been associated with risk of coronary heart disease (CHD). The level of tHcy is affected by lifestyle, in addition to genetic predisposition. The *MTHFR* 677C>T polymorphism (rs1801133) is among the strongest genetic predictors of tHcy. We examined whether the association between tHcy and CHD is modified by the *MTHFR* 677C>T polymorphism.

Design and Setting Data from two case-control studies of first-time myocardial infarction (MI), Stockholm Heart Epidemiology Programme (SHEEP), and for MI and unstable angina, INTERGENE, were analyzed in parallel.

Patients THcy was determined in a total of 1150 cases and 1753 controls.

Interventions None

Main outcome measures The outcome comprised first-time MI and unstable angina, subsumed as CHD. Logistic regression was used to investigate the association between tHcy and CHD, and its modification by genotype.

Results

High tHcy was confirmed to be a risk factor for CHD in both studies. In SHEEP, the association between tHcy and MI was observed in *MTHFR* 677 C-homozygotes (odds ratio (OR) 1.5, 95% confidence interval (CI) 1.3-1.8, for a difference by 1 standard deviation of log tHcy) and in heterozygotes (OR 1.4, 95% CI 1.2-1.6) but not in T-homozygotes, independent of smoking, physical activity and obesity. An effect modification of similar magnitude was observed but not statistically significant in the smaller INTERGENE study, and confirmed in a meta-analysis of both studies.

Conclusion

Two Swedish case-control studies showed that the association between elevated tHcy and CHD was confined to carriers of the *MTHFR* 677 C-allele, which could have implications for the efficiency of tHcy-lowering treatment.

INTRODUCTION

An elevated level of total homocysteine (tHcy) is known to be associated with a higher risk for atherosclerosis and cardiovascular disease (CVD), in a graded way without an obvious threshold.[1, 2] Although the association has been confirmed in many observational studies,[3] tHcy-lowering treatment has not been unambiguously shown to reduce the cardiovascular risk.[4, 5, 6, 7] It has been suggested that elevated tHcy may be a common by-product of atherosclerotic changes rather than a direct causal factor.[8, 9, 10] However, one prospective study showed that increased tHcy at baseline is a risk factor for myocardial infarction (MI) up to 20 years later.[11] The methylene tetrahydrofolate reductase (*MTHFR*) 677C>T polymorphism (rs1801133) has been shown to cause the strongest modulation of tHcy of all genes associated with homocysteine and folate metabolism.[12, 13] The mutation produces an altered version of the MTHFR enzyme (EC1.5.1.20) characterized by reduced activity, leading to increased plasma tHcy in association with low plasma folate. Clinically, blood values differ most between subjects with two copies of the mutant T-allele and those with at least one normal allele.[4, 12] If the effect of *MTHFR* 677 on CHD was mediated by tHcy alone, an increased risk of CHD might be expected in subjects with *MTHFR* 677 TT genotype; however, evidence is inconsistent. Some studies reported an association but mainly in subjects with low folate status,[14, 15, 16] while most studies that did not report a positive finding were not able to take the folate status into account.[7, 17] A recent prospective study of 6000 US adults even found an association of *MTHFR* 677TT with *lower* CVD mortality.[18]

The aim of the present study was to investigate if the risk for coronary heart disease (CHD) due to elevated levels of homocysteine is modified by the *MTHFR* 677C>T polymorphism, irrespective of the reason for high tHcy (genotype, diet or conventional risk factors such as smoking, physical inactivity and overweight). Previous studies suggesting that risk for CVD due to tHcy was independent of the *MTHFR* 677C>T polymorphism were rather small and might not have had the adequate statistical power to clearly demonstrate a difference of risk by genotype.[19, 20] Our analysis comprised data from two Swedish case-control studies of first-time MI, SHEEP and MI and unstable angina, INTERGENE with a total number of 1150 patients and 1753 controls.

MATERIALS AND METHODS

Stockholm Heart Epidemiology Program (SHEEP):

SHEEP is a population-based case-control study including 2246 patients aged 45–70 years who suffered a first myocardial infarction (MI). For the present study, 856 patients who survived at least 28 days after their MI were included along with 1129 controls matched to the cases on age, sex and hospital catchment area. The subjects were Swedish citizens residing in Stockholm County and recruited during 1992–1994. Cases and controls were invited to a physical examination, with participation rates given by 77% and 67%, respectively. For patients, the physical examination including both anthropometry and blood sampling was performed on average 5 months after MI, with a minimum of 3 months to ensure that the patient had regained metabolic stability. Exposure items including lifestyle, medical history, and diet were assessed using self-administered questionnaires. Smoking status was assessed as current smoking now or during the last year, former smoking, and never smoking. Physical inactivity was defined as no regular physical activity during the last 5-10 years except for occasional walks. Subjects were asked to report their habitual intake of medication and supplements. Further details about the study including the case definition can be found elsewhere.[21] Study participants had blood drawn from an antecubital vein into evacuated tubes. Blood samples were centrifuged within 30 minutes, and immediately frozen in aliquots and stored in -70 C until analysis. Total homocysteine was measured in EDTA plasma using the IMx system and fluorescence polarization immunoassay (FPIA) technology. Values above 50 μmol/L were re-measured a second time using a diluted sample. DNA was isolated from whole blood using RapidPrep Macro Genomic DNA Isolation Kit from Pharmacia Biotech. PCR amplification was performed of a specific 166 basepair long region in the MTHFR gene. Analysis of the PCR product regarding melting point and genotype (using fluorescent probes) was performed using the LightCycler instrument and the LightCycler DNA Master Hybridization Probes Kit (Roche). Primers, main location: MTHFR677 1F: 5’-TGG CAG GTT ACC CCA AAG G-3, and MTHFR677 1R: 5’-TGA TGC CCA TGT CGG TGC-3’. Both the genotyping and the analysis of tHcy were performed in 2003 on frozen blood samples. All study participants gave consent to participate in the study and the protocol was approved by the Ethical Committee at Karolinska including molecular genetic analyses to be performed on the SHEEP material (Approvals 91-259, 01-097). The study complies with the Declaration of Helsinki.

The INTERGENE (INTERplay between GENEtic susceptibility and environmental factors for the risk of chronic diseases in West Sweden) cohort and case-control study:

The INTERGENE case-control study comprises 618 patients below the age of 75, admitted in 2001-04 to three regional hospitals for acute coronary syndrome and diagnosed as myocardial infarction (ICD 10: I21.0-I21.9), with typical history, ECG and enzyme changes or unstable angina (ICD 10: I20.0). To be consistent with the SHEEP study, only patients without a history of CHD were included in this analysis (n = 294). The INTERGENE cohort consists of a population-based random sample of inhabitants of Gothenburg and the surrounding region of Västra Götaland (South-west Sweden) and between 25 and 75 years old at the time of sampling. Between April 2001 and December 2004, 3614 members of the target population sample were examined (participation rate 42%). 624 subjects without self-reported history of myocardial infarction, angina pectoris or stroke were selected as control subjects for the CHD cases. The examination of patients (on average 3 months after the event) and controls consisted of a short clinical examination including anthropometry, collection of venous 4h fasting blood samples, and the administration of a questionnaire. Participants were asked to report their smoking status (current smoking or stopped less than 1 year ago, former or never smoking), regular intake of vitamin B supplements and their habitual leisure time physical activity during the year before inclusion into the study. Physical inactivity was defined as sedentary (mainly sitting: reading, watching TV etc.) versus higher activity (any activity at least 4 hours/week or exercise at least 2 hours/week). Blood samples were collected, immediately centrifuged and stored at -20 ^{o}C for future analysis. THcy was determined in EDTA plasma by stable isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Quattro micro™ instrument (Waters Corporation, Milford, MA, USA) essentially as described by Magera *et al*.[22]. Genomic DNA was extracted from whole blood using the GenoPrep kit and the GenoM-48 apparatus (Techtum Lab AB). The *MTHFR* 677C>T polymorphism was determined by the solid-phase minisequencing method as described previously [23]. Further information can be found in references [24, 25] (case-control study), [26] (INTERGENE cohort), as well as on www.intergene.gu.se. All subjects gave their consent to the study, and the protocol was approved by the regional ethics review board, Forskningsetikkommité (Ö 237/2000). The study complies with the Declaration of Helsinki.

Statistical methods

Data from SHEEP and INTERGENE were analyzed in parallel. The characteristics of study participants are described with mean and standard deviation (SD) for continuous variables and proportions for categorical variables, and compared between patients and controls by use of t-test and chi-square test, respectively. For *MTHFR* 677C>T genotype, the p-value refers to a test of trend across number of T-allele. Since the distribution of tHcy is skewed towards large values the variable was log-transformed before analysis. The variation of log tHcy with genotype was analyzed using linear regression adjusting for age, sex, waist-to-hip ratio (WHR), smoking status (current, former, never), physical inactivity, supplementation with vitamin B, and hospital catchment area (included as random effect in SHEEP only). The analysis was performed separately for control subjects and for cases and results are presented as percent change of tHcy when comparing subjects with TT or CT versus CC genotype. Binary logistic regressions were used to investigate the association between CHD and genotype, between CHD and log tHcy, and the modification of this association by genotype. All models were adjusted for age and sex, and furthermore for WHR, smoking status, and physical inactivity. For the analysis of SHEEP data, a generalized linear model with log-link function was used to include the hospital catchment area as a random effect. The *MTHFR* 677C>T genotypes were analyzed in terms of categorical variables, and in terms of the number of T-allele assuming an additive model. Results are given as odds ratio (OR) and 95% confidence interval (CI). For tHcy, the OR refers to a difference in log tHcy by 1 SD, and the numerical value is derived from the controls in each study (SHEEP: SD = 0.32; INTERGENE: SD = 0.27; cf. Table 1). Furthermore, we used the STATA procedure metan for random-effects meta-analysis of the odds-ratios for interaction. [27] The test of Hardy-Weinberg equilibrium was performed using genhwcci (Stata).

Statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) and StataIC version 12.1 (StataCorp, Texas 77845 USA). Statistical significance was set at p-value ≤ 0.05 (2-sided test).

RESULTS

Study description

In both studies, CHD cases showed a higher prevalence of traditional risk factors (smoking, inactivity and obesity) and higher average values of tHcy than subjects from the respective control groups (Table 1). In SHEEP, cases also showed a considerably lower habitual use of vitamin B supplements than controls; this difference was similar but smaller in INTERGENE. The *MTHFR* 677C>T genotypes were in Hardy-Weinberg equilibrium, by study and by case-control status ($p > 0.3$). We saw no differences in genotype distribution between cases and controls. Although subsequent analyses adjust for WHR because of its strong association with CHD, data on body mass index (BMI) were also presented in Table 1.

Table 1. Characteristics of the 2903 participants by study and case-control status.

	SHEEP 1992 - 1994		INTERGENE 2001 - 2004	
	Controls (n = 1129)	MI cases (n = 856)	Controls (n = 624)	CHD cases (n = 294)
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Age (years)	60.4 (7.2)	59.9 (7.2)	61.5 (8.3)	60.6 (8.4)
tHcy (μmol/l)	11.7 (7.2)	12.7 (4.7)	13.1 (4.9)	14.5 (9.4)
Log tHcy	2.39 (0.32)	2.49 (0.31)***	2.53 (0.27)	2.61 (0.29)***
BMI (kg/m^2)	25.8 (3.8)	26.8 (4.1)***	26.7 (3.6)	27.5 (3.7)**
WHR	0.90 (0.089)	0.93 (0.083)***	0.91 (0.082)	0.95 (0.080)***
	n (%)	n (%)	n (%)	n (%)
Female sex	412 (36.5%)	285 (33.3%)	182 (29.2%)	86 (32.1%)
Smoking, never (ref)	455 (40.8%)	208 (25.2%)	271 (43.8%)	82 (28.2%)
Former	345 (30.9%)	218 (26.4%)***	251 (40.5%)	133 (45.9%)***
Current	315 (28.3%)	400 (48.4%)***	97 (15.7%)	75 (25.9%)***
Physical inactivity	405 (36.6%)	386 (47.3%)***	42 (6.8%)	46 (15.7%)***
Vit. B supplements	343 (30.4%)	157 (18.3%)***	119 (19.1%)	43 (14.6%)
MTHFR 677CC	586 (51.9%)	451 (52.7%)	328 (52.6%)	160 (54.4%)
677CT	464 (41.1%)	340 (39.7%)	242 (38.8%)	118 (40.1%)
677TT	79 (7.0%)	65 (7.6%)	54 (8.6%)	16 (5.4%)

Test for difference between cases and controls, ** $p<0.01$, *** $p<0.001$.

MTHFR 677C>T genotype and risk of CHD

Logistic regression was used to evaluate the association between CHD and genotype independent of homocysteine. The odds ratios of CHD for TT / CT versus CC genotype were given by 1.18 (0.82 - 1.72) / 1.03 (0.85 – 1.26) and 0.64 (0.34 - 1.19) / 0.92 (0.67 – 1.26) for SHEEP and INTERGENE, respectively (adjusted for age, sex, hospital catchment area (SHEEP only), and lifestyle variables).

MTHFR 677C>T genotype and total plasma homocysteine

Results from both studies confirmed that the *MTHFR* 677 T-allele is associated with a higher level of tHcy. In population controls from SHEEP, T-homozygotes had a 27% higher level of tHcy than subjects with CC genotype, and heterozygotes had a 4% higher level than C-homozygotes (p-value for linear trend across number of T-allele: $p < 0.0001$, adjusted for age, sex, WHR, lifestyle, and vitamin B supplementation). A somewhat weaker effect was seen in INTERGENE controls, where T-homozygotes had 10% higher tHcy and heterozygotes 4% higher tHcy than C-homozygotes (trend: $p = 0.07$). The lifestyle factors smoking and physical inactivity as well as higher values of WHR were associated with higher values of tHcy, while vitamin B supplementation was associated with reduced tHcy (data not shown). Qualitatively similar associations were observed among the cases (p-values for trend = 0.009 in SHEEP, $p = 0.1$ in INTERGENE).

Total plasma homocysteine and risk of CHD by *MTHFR* 677C>T genotype

Overall, a higher level of tHcy was associated with a higher risk of CHD in both studies (OR for 1 SD difference of log tHcy = 1.30, 95% CI 1.18-1.44 for SHEEP, OR = 1.33, 95% CI 1.14-1.56 for INTERGENE, adjusted for age, sex, hospital catchment area (SHEEP only), and lifestyle variables). In SHEEP, the association between tHcy and CHD was particularly strong in C-homozygotes and weakened with a higher number of T-allele in a dose-dependent manner (Figure 1a). No association between tHcy and CHD was observed in T-homozygotes. An effect modification of similar size was observed in INTERGENE, although it was not statistically significant (Figure 1b). The reported associations were only slightly reduced when adjusted for smoking, physical inactivity or WHR. Figure 2 shows the ORs for

statistical interaction between tHcy and *MTHFR* 677C>T genotype, separately by study and combined through meta-analysis. There is a small difference in the association between tHcy and CHD in subjects with CT or CC genotype (top), while the effect estimate for tHcy is about 30% lower in T-homozygotes compared to subjects with CC (middle). The additive model for *MTHFR* 677C>T genotype gives an average reduction of the odds of CHD by 14% per T-allele (bottom). The combined results are largely determined by the more accurate results from SHEEP in spite of similar point estimates obtained from the smaller INTERGENE study (Supplementary Table 1).

DISCUSSION

In this report, earlier findings of a positive association between higher plasma tHcy and coronary heart disease [3] were confirmed in two Swedish case-control studies of first-time CHD and consistent across both data sets. In contrast to the overall association, a reduced adverse association between tHcy and CHD was seen in *MTHFR* 677 T-allele carriers, in particular in T-homozygotes, based on data from SHEEP and on the result from a meta-analysis of both studies. This effect modification was reproduced in the more recent study (INTERGENE) as well, though not significantly so due to the smaller sample size of INTERGENE. The association between tHcy and CHD in each genotype category was hardly changed upon adjustment for lifestyle factors such as smoking, obesity and physical inactivity, indicating that the association between tHcy and CHD is independent of which non-genetic mechanism caused the elevated values of tHcy.

The reported effect modification could be clearly observed in SHEEP and in the combined study, but although the effect modification was of equal size it was not significant in INTERGENE, which included about 1/3 of the number of cases in SHEEP. It has been shown that the sample size needed to demonstrate a gene-environment interaction effect of a given size increases strongly when the smaller genotype category (here, *MTHFR677* TT) falls below 20%.[28] Although the *MTHFR* 677C>T polymorphism is considered to be a common mutation, the susceptible genotype 677TT has a rather low prevalence of around 7% in Swedish populations,[15] as is confirmed in this study. However, we also observed an interaction between tHcy and the number of T-allele (SHEEP and meta-analysis of both studies). A reduced risk of CHD for tHCY per T-allele could be related to enzyme activity, which decreases non-linearly with the number of T-allele.

We observed a similar effect size for the interaction between tHcy and *MTHFR* 677C>T genotype in both studies in spite of differences in level of tHcy and prevalence of environmental and lifestyle factors, which are important confounders for the association between tHcy and CHD. Although examined about 10 years later, subjects from the INTERGENE study had a higher average level of tHcy than subjects from SHEEP. Regarding methodology, this difference could be a consequence of a different calibration of the tHcy measurements in 2001-04 (INTERGENE) as compared with those in 1992-94 (SHEEP). However, the combination of a higher average with a smaller range of values could also indicate sub-optimal handling of whole-blood samples.[29] Among lifestyle factors potentially related to tHcy, we observed higher values of BMI and central obesity (WHR) in the later study, but less physical inactivity and smoking. However, the differences in tHcy between both studies were independent of lifestyle and anthropometry (data not shown).

While results from both studies confirmed the increase of average tHcy with number of *MTHFR* 677-T-allele described earlier,[3] we were not able to test the effect of genotype on CHD by blood folate status due to lack of data, which is a limitation of our study. Another limitation is that blood variables such as tHcy may be biased due to reverse causation, i.e. they may be modified after the coronary event in cases due to treatment or changed lifestyle. It has been shown that tHcy levels respond fast to the supply of e.g. caffeine or folic acid [30] implying that the measured levels of tHcy in coronary patients may not reflect the status before the event. While the effect estimates for tHcy and *MTHFR* 677C>T and their interaction were very similar between the studies, the comparison of numbers of cases and controls underscores the danger of under-powering when studying gene-environment interactions.

A potential interpretation of the effect modification of tHcy by genotype could be that given a higher genetically driven long-term average of tHcy in 677 T-homozygotes and the risk for CHD associated with it, other causes of high tHcy such as smoking, obesity and physical inactivity do not substantially increase the risk due to tHcy. From a clinical point of view, cardiovascular risk reduction by change of lifestyle could be expected to have a larger effect in carriers of the C-allele than in T-homozygotes. We did not find an association between *MTHFR*677C>T genotype and CHD independent of tHcy, which is consistent with results from a meta analyses of studies with a small average difference in tHcy between cases and controls [7], and with a recent meta-analysis including unpublished studies giving strong

support to a negative result.[17] The latter observation could mean that the *MTHFR*677C>T genotype does not act through tHcy alone.

In summary, the adverse effect of tHcy on CHD was modified by *MTHFR*677C>T genotype, in such a way that subjects who are genetically predisposed to mild homocysteinemia by having the TT genotype did not show a difference in risk for CHD by level of tHcy. The effect modification was observed in two independent Swedish case-control studies in spite of differences in the prevalence of lifestyle and environmental factors.

CONTRIBUTORS

All authors have substantially contributed to the manuscript in terms of conception and design, analysis and interpretation of data, drafting the article, revising it critically for important intellectual content, and final approval of the version.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

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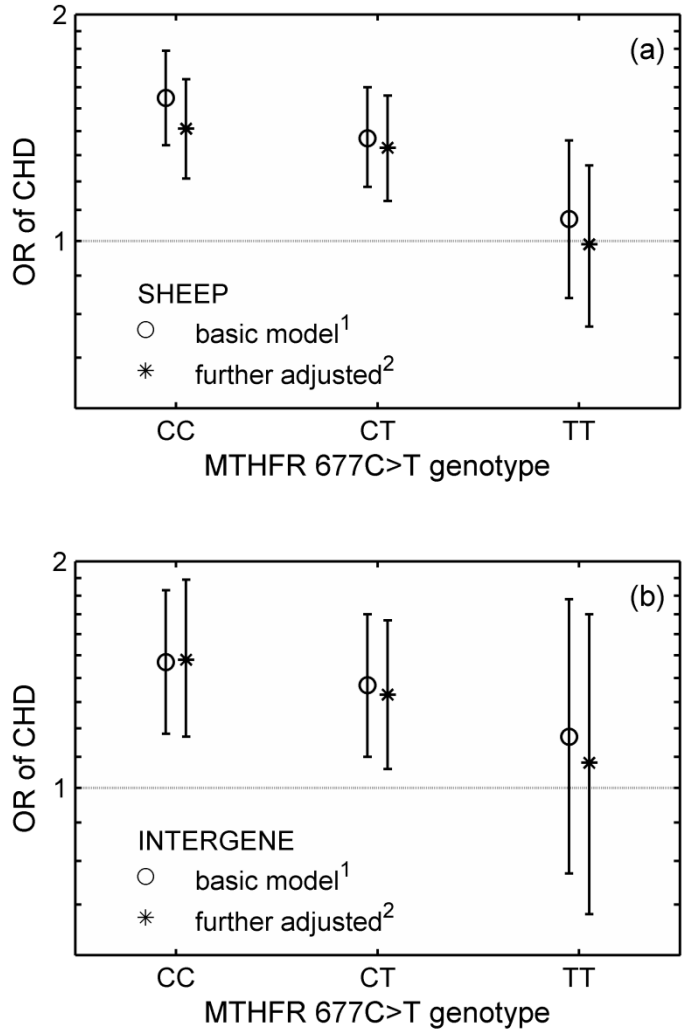
FIGURES

Figure 1 Odds ratio of CHD (with 95% CI) for a 1 SD difference of log tHcy, by genotype, showing significant associations only for carriers of the C-allele in both SHEEP (a) and INTERGENE (b). The value of SD is derived from the control subjects and differs slightly between the studies (SHEEP: SD = 0.32; INTERGENE: SD = 0.27; cf. Table 1).



[1]Basic model: genotype, log tHcy and the product term, adjusted for age, sex and hospital catchment area (for SHEEP only).

[2]Basic model with additional adjustment for WHR, smoking and physical inactivity.

Figure 2 Odds ratios for statistical interaction between log tHcy and *MTHFR*677 C>T genotype on CHD, with Forest plots displaying a random-effect meta-analysis for CT versus CC (top), TT versus CC (middle), and for an additive model in terms of the number of T-allele (bottom).