The American Journal of Clinical Nutrition

Relations of glutamate carboxypeptidase II *(GCPII)* polymorphisms to folate and homocysteine concentrations and to scores of cognition, anxiety, and depression in a homogeneous Norwegian population: the Hordaland Homocysteine Study[1–4]

Charles H Halsted, Donna H Wong, Janet M Peerson, Craig H Warden, Helga Refsum, A David Smith, Ottar K Nygård, Per M Ueland, Stein E Vollset, and Grethe S Tell

ABSTRACT

Background: Glutamate carboxypeptidase II (*GCPII*) encodes for intestinal folate hydrolase and brain *N*-acetylated α-linked acidic dipeptidase. Previous studies provided conflicting results on the effect of the *GCPII* 1561C→T polymorphism on folate and total homocysteine (tHcy) concentrations.

Objective: We aimed to determine the potential effects of 2 polymorphisms of *GCPII* on plasma folate and tHcy concentrations, cognition, anxiety, and depression in a large aging cohort of Norwegians enrolled in the Hordaland Homocysteine Study.

Design: DNA samples were genotyped for the *GCPII* 1561C→T and 484A→G polymorphisms, and the results were linked to plasma folate and tHcy concentrations and to scores for cognition, anxiety, and depression.

Results: The 2 polymorphisms were in linkage disequilibrium and were associated with concentrations of tHcy. After adjustment for covariates, persons in the *CT* or combined *CT* and *TT* groups of the 1561C→T polymorphism had higher plasma folate concentrations and lower tHcy concentrations than did those in the *CC* group. Subjects with the *TT* genotype had lower Symbol Digit Modalities Test (SDMT) scores than did subjects with the *CC* genotype. Compared with abstainers, moderate alcohol drinkers had higher plasma folate concentrations and higher scores on the Mini Mental State Examination. However, women abstainers with the *CT* genotype had lower SDMT scores than did abstainers with the *CC* genotype or moderate drinkers with the *CT* genotype.

Conclusions: The 1561C→T polymorphism is associated with higher plasma folate and lower tHcy concentrations and with lower SDMT cognitive scores in women who abstain from alcohol. *Am J Clin Nutr* 2007;86:514–21.

KEY WORDS Glutamate carboxypeptidase II, *GCPII*, folate, homocysteine, cognition, gene polymorphisms

INTRODUCTION

Glutamate carboxypeptidase II (*GCPII*; EC 3.4.17.21) is located at chromosome 11p11.2, incorporates 19 exons, and encodes for 751 amino acids (1, 2). Collectively, *GCPII* expresses 2 separate enzymes: intestinal folate hydrolase, which regulates folate absorption by the cleavage of glutamates from dietary polyglutamyl folates, and brain *N*-acetylated α-linked acidic dipeptidase, which produces glutamate and *N*-aspartyl acetate (NAA) and regulates neurotransmission (3, 4). Our original report (5) found a functional polymorphism at 1561C→T in human intestinal *GCPII* that gave a frequency of 0.08 for *CT* heterozygotes. This polymorphism was associated with lower folate and higher total homocysteine (tHcy) blood concentrations in 75 healthy elderly persons residing in Oxford, United Kingdom, and the activity of folate hydrolase was lower by one-half in mammalian COS-7 cells that were transfected with a homozygous mutant (5). However, this finding of the effects of the 1561C→T polymorphism on folate and tHcy concentrations was not confirmed by subsequent studies of larger populations residing in the United States or in northern Europe (6–9), including several that found that its presence had the opposite association of lower serum or red cell folate concentrations (10–13).

The Hordaland Homocysteine Study evaluated apparently healthy adults residing in Bergen and surrounding communities in western Norway, of whom 18 044 were screened initially in 1992–1993 and 7074 of the same participants were screened a second time in 1998–1999 (14, 15). The first Hordaland Homocysteine Study established separate ranges of tHcy concentrations for men and women and found that dietary folate intake, smoking status, and consumption of coffee were major determinants of elevated tHcy (14). The second Hordaland

[1] From the University of California Davis, Davis, CA (CHH, DHW, JMP, and CHW); the University of Oxford, Oxford, United Kingdom (HR and ADS); and the University of Bergen, Bergen, Norway (OKN, PMU, SEV, and GST)

[2] HR is currently at the Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway.

[3] Supported by research grants DK56085 and DK 35747 from the US National Institutes of Health to CHH and by a grant from the Norman Collisson Foundation, UK, to ADS. Data on the subject volunteers were collected as part of the Hordaland Health Study 1997–1999 in collaboration with the Norwegian National Health Screening Service and with financial support from the Norwegian Research Council and the Advanced Research Program of Norway.

[4] Reprints not available. Address correspondence to CH Halsted, Room 6323, Genome and Biomedical Sciences Facility, University of California Davis, Davis, CA, 95616. E-mail: chhalsted@ucdavis.edu.

Received June 30, 2006.

Accepted for publication April 5, 2007.

 © 2007 American Society for Nutrition

Homocysteine Study found that tHcy concentrations were associated positively with cardiovascular events and with higher serum creatinine concentrations but were significantly lowered by vitamin supplementation and quitting smoking (15). Of interest, tHcy concentrations were marginally lower in smokers who consumed moderate amounts of alcohol (16).

The goals of the present study were first to determine the potential relation of the 1561C→T polymorphism in *GCPII* to an additional polymorphism at nucleotide 484 (484A→G) in DNA samples that were available from subjects in the second Hordaland Homocysteine Study cohort. Second, we determined the relations of each polymorphism and their haplotypes with folate and tHcy concentrations and with scores of cognition, depression, and anxiety.

SUBJECTS AND METHODS

Subjects

The second round of the Hordaland Homocysteine Study was conducted as part of the Hordaland Health Study from 1997 to 1999 as a collaboration between the National Health Screening Service of Norway, the University of Bergen, and local health services. Each subject provided blood samples for DNA extractions and measurements of plasma folate and tHcy concentrations. We also collected lifestyle data and results of neuropsychiatric testing. Of the 7074 participants, 3341 were born between 1925 and 1927 (16). From this group, we selected 2734 subjects who provided comprehensive data on lifestyle variables, folate and tHcy values, and neuropsychiatric scores, as well as high-quality DNA samples. Among the final group, 43.4% were men and 56.6% women aged between 70 and 72 (**Table 1**). The study protocol was approved by the Regional Committee for Medical Research Ethics, and each participant provided informed consent to participate.

TABLE 1
Characteristics of the subjects selected for study[1]

	Value
Age	%
70 y	35.4
71 y	32.8
72 y	31.8
Sex	
Male	43.4
Female	56.6
Cardiovascular events (past 6 y)	10.5
Alcohol use	
Abstainers	51.0
Men	37.5
Women	61.7
≤2 drinks/d	47.5
Men	59.5
Women	37.9
>2 drinks/d	1.5
Men	3.0
Women	0.4
Smoking	
Current	15.5
Former	48.4
Education	
Not finished primary school (<7 y)	8.2
Primary school (7–10 y)	33.3
Middle school or 1–2 y of high school	29.2
High school	11.4
College or university (< 4 y)	10.1
College or university (>4 y)	7.9
Coffee consumption	
1–3 cups/d	58.7
4–10 cups/d	34.3
Multivitamin use	28.1

[1] Data were from 2734 subjects born between 1925 and 1927 who participated in the second 1998–1999 Hordaland Homocysteine Study (15).

DNA preparation

DNA was extracted from frozen whole-blood samples in 96-well plates with the use of a NucleoSpin Multi 96 blood extraction kit (Macherey-Nagel, Düren, Germany) and was subsequently shipped to the University of California, Davis, laboratory for genotyping. Among the DNA samples, 2734 provided satisfactory genotyping patterns for at least one polymorphism, and 2471 samples provided satisfactory patterns for both polymorphisms.

Genotyping

The 1561C→T (H475Y) polymorphism that was initially located in exon 13 of the catalytic region of *GCPII* (5) is now identified at C1684T in *Ensembl* (FOLH1, ENSG00000086205). In keeping with prior studies (5–8, 10–13), however, this polymorphism will be referred to as 1561C→T in the present report. The second polymorphism at 484A→G (H75Y) was identified through the Celera Database and is now identified through *Ensembl* as a missense mutation in exon 2 of the structural transmembrane region. Both polymorphisms were analyzed on an ABI 7900 genotyping machine using the 5' nuclease (Taqman) assay and a high-throughput genotyping method that uses allele-specific fluorogenic probes (Applied Biosystems, Foster City, CA). The primers and probes for genotyping of 484A→G and 1561C→T were provided by the Assays by Design service of Applied Biosystems (Foster City, CA). The fluorescence detection system relies on 2 allele-specific probes that contain different fluorescent reporter dyes (FAM and VIC) to differentiate the amplification of each allele when the probes are cleaved by the 5' nuclease activity of Taq DNA polymerase. Each probe consisted of an oligonucleotide with a 5' reporter dye (FAM or VIC) and a 3' nonfluorescent minor-groove-binding quencher dye that anneals specifically to complementary sequences between the forward and reverse primer sites (Applied Biosystems). The primers for 484A→G included 5'AACTTGTCCATATAAACTTTCGAG GATGT3' (sense) and 5'GCATTTTTGGATG AATTGAAAGCTGAGA3' (antisense), and the probes were *1*) VIC-CATCAA GAAGTTCTT ATAGTAAG and *2*) 6FAM-TCAAGA AGTTCTTACAG TAAG. The primers for 1561C→T included 5'GAGTTGATTGTA CACCGCTGATG3'(sense) and 5'CCACCTATGTTTA ACATAATACCTCAAG3' (antisense), and the probes were *1*) 6FAM-CTTGG TACACAACCTAA and *2*) VIC-AGCTTGGT ATACAACCT. PCR reactions were performed with the following thermal cycler conditions on a GeneAmp PCR System 9700 or ABI PRISM 7900HT Sequence Detection System: 95 °C for 10 min, followed by 40 cycles of 92 °C for 15 s and 60 °C for 1 min. The reaction components were as follows: 1X Taqman Universal PCR Master Mix, 900 nmol/L of each primer, 200 nmol/L of the probe for allele 1, 200 nmol/L of the

The American Journal of Clinical Nutrition

TABLE 2
Combined genotype distributions of the glutamate carboxypeptidase II 484A→6 and 1561C→T polymorphisms, 1998–1999[1]

	1561CC		*1561CT*		*1561TT*		Total	
	n	Frequency	*n*	Frequency	*n*	Frequency	*n*	Frequency
484AA	1104	0.446	178	0.072	12	0.005	1294	0.524
484AG	930	0.376	81	0.033	1	0.000	1012	0.410
484GG	161	0.065	3	0.001	1	0.000	165	0.066
Total	2195	0.888	262	0.106	14	0.006	2471	

[1] Pearson's chi-square test for independence, $P < 0.0001$; Hardy-Weinberg equilibrium for 1561C→T, $P = 0.080$, and for 484A→G, $P = 0.090$. Linkage disequilibrium D′ = 0.786 (0.61–0.89).

probe for allele 2, and 25 ng genomic DNA. A post-PCR plate read on the 7900HT was used to determine genotype. The 7900HT Sequence Detection System collects fluorescence data on the samples for ≈5 s, and SDS software version 2.1 (Applied Biosystems) analyzes the fluorescence signals, which can be visualized in graph form. Samples that could not be determined were repeated, and unreadable results on the second run were scored as missing. All DNA genotyping was performed and analyzed at the University of California, Davis, laboratory, and the results were linked to phenotypic variables provided from the Hordaland Homocysteine Study data bank.

Measurement of plasma folate and tHcy and lifestyle variables

All subjects provided plasma samples for measurements of tHcy by automated HPLC with fluorescence detection (17), for measurement of folate and vitamin B-12 by microbiological assays (18, 19), and for measurement of serum creatinine concentrations. Lifestyle data were obtained for smoking as cigarettes per day, for alcohol consumption as drinks per day where one drink was estimated to be equal to 12.8 g ethanol, and for the use of supplemental multivitamins. Additional information was obtained on levels of education and on the incidence of cardiovascular events during the prior 6 y. Cognitive function was tested according to a shortened form of the Mini Mental State Examination (s-MMSE) (20), the symbol digit modality test (SDMT) (21), the Kendrick Object Learning Test (22), and the block design test, for which better cognition is signified by higher scores on each test, and by the trail making test, in which high scores indicate poor cognitive performance. Anxiety and depression were assessed by the Hospital Anxiety and Depression Scale (23) in which higher scores signify worse results.

Statistical analyses

Analyses were conducted with SAS for WINDOWS release 9 (Cary, NC). Continuous variables were assessed for conformance to the normal distribution and were transformed if appropriate. Relations between the 2 polymorphisms were determined by chi-square analysis. The Hardy-Weinberg equilibrium for each polymorphism and linkage disequilibrium between the 2 polymorphisms were determined with the 3.31 version of HAPLOVIEW (Cambridge, MA; http://www.broad.mit.edu/mpg/haploview/), where $P > 0.05$ indicates compliance with Hardy-Weinberg equilibrium and $P < 0.05$ represents significant linkage disequilibrium. The strength of the linkage disequilibrium was provided by the D′ score and 95% confidence boundaries, for which a value of 1 indicates complete and 0 indicates no linkage disequilibrium. Associations between continuous outcomes and the 2 *GCPII* polymorphisms were first tested with analysis of variance, followed by analyses of covariance to control for age, sex, smoking, alcohol use, educational level, coffee consumption, serum vitamin B-12 and creatinine concentrations, and multivitamin use, as well as to test for interactions between these covariates and genotypes. Tukey's test was used for post hoc comparisons.

RESULTS

The characteristics of the 2734 subjects studied in the second Hordaland Homocysteine Study (1998–1999) whose phenotypic data and DNA genotypes were entered into the present study are provided in Table 1. Within the cohort, 10.5% reported a cardiovascular event within the previous 6 y; such events included myocardial infarction, stroke, thrombosis, or cardiovascular surgery. Alcoholic beverage intake was categorized as none (abstainer) in 51.0%, 1 to 14 drinks/wk (≤2 drinks/d; moderate) in 47.5%, and >14 drinks/wk (>2 drinks/d; excessive) in 1.5%. As shown, there were fewer abstainers and more drinkers among the men than among the women. Furthermore, the patterns of beverage consumption differed by sex ($P = 0.0004$): men consumed 28% of their drinks as beer, 40% as wine, and 32% as spirits; the values for women were 9%, 77%, and 14%, respectively. Smoking was categorized as never smoked in 36.1%, former smoker in 48.4%, and current smoker in 15.5%. High school education was completed by 11.4% and university education was completed by 7.9% of the subjects. All but 7% of the study population drank coffee, and one-third drank between 4 and 10 cups/d. Supplemental multivitamins were used by 28.1% of the subjects.

The allele pair distributions of each genotype and the frequencies of their combinations among 2471 samples for which both polymorphisms were identified are shown in **Table 2**. The allele distributions for each polymorphism were in Hardy-Weinberg equilibrium, and the D′ value indicated strong linkage disequilibrium between the 2 polymorphisms. Of 9 possible haplotype pairs, 6 were present in frequencies ≥0.5% (0.005) and are represented in the subsequent tables.

The associations of plasma concentrations of folate and tHcy with each of the 2 *GCPII* polymorphisms at 484A→G and 1561C→T and their haplotypes are shown in **Table 3**. All analyses were adjusted for age, sex, smoking, education level, alcohol use, coffee consumption, serum vitamin B-12 and creatinine concentrations, and supplemental vitamin use. The numbers of subjects available for accurate analysis of each polymorphism in

The American Journal of Clinical Nutrition

TABLE 3
Associations of glutamate carboxypeptidase II (*GCPII*) genotypes with plasma concentrations of folate and total homocysteine (tHey)[1]

484A→G	1561C→T	Folate[2]	tHcy[3]
		nmol/L	*μmol/L*
All	*CC*	8.9 ± 0.152 [2226][4]	12.2 ± 0.102 [2236]
All	*CT*	10.8 ± 0.688 [265][5]	11.5 ± 0.259 [269][5]
All	*TT*	9.6 ± 1.483 [15]	10.5 ± 0.479 [16]
All	*CC/CT*	9.1 ± 0.155 [2491]	12.2 ± 0.095 [2505]
All	*CT/TT*	10.7 ± 0.656 [280][6]	11.5 ± 0.246 [285][6]
AA	All	9.3 ± 0.217 [1402]	12.0 ± 0.097 [1408]
AG	All	9.0 ± 0.215 [1090]	12.4 ± 0.176 [1099][7]
GG	All	8.7 ± 0.523 [177]	12.0 ± 0.346 [177]
AA	*CC*	9.1 ± 0.232 [1101]	12.1 ± 0.110 [1104]
AA	*CT*	10.4 ± 0.772 [176]	11.3 ± 0.261 [178]
AA	*TT*	10.4 ± 1.955 [11]	10.3 ± 0.389 [12]
AG	*CC*	8.8 ± 0.225 [923]	12.4 ± 0.196 [930]
AG	*CT*	11.0 ± 1.127 [79]	12.0 ± 0.626 [81]
GG	*CC*	8.2 ± 0.367 [161]	12.0 ± 0.367 [161]

[1] Haplotype combinations with a frequency < 0.005 were excluded. Means were compared by ANCOVA, with control for age, sex, alcohol use, smoking, education level, coffee consumption, serum vitamin B-12 and creatinine concentrations, and multivitamin use.
[2] Main effect of 1561C→T, $P = 0.0005$; main effect of 484A→G, $P = 0.79$; interaction, $P = 0.98$.
[3] Main effect of 1561C→T, $P = 0.0004$; main effect of 484A→G, $P = 0.026$; interaction, $P = 0.85$.
[4] $\bar{x}$ ± SEM; *n* in brackets (all such values).
[5,6] Significantly different from *CC* (Tukey's test): [5] $P = 0.0003$, [6] $P < 0.0001$.
[7] *AG* significantly different from *GG*, $P = 0.05$ (Tukey's test).

TABLE 4
Associations of glutamate carboxypeptidase II (*GCPII*) genotypes with cognition, anxiety, and depression[1]

484A→G	1561C→T	SDMT[2]	Depression[3]
All	*CC*	10.2 ± 0.097 [1682][4]	3.5 ± 0.063 [1833]
All	*CT*	10.1 ± 0.310 [199]	3.5 ± 0.207 [220]
All	*TT*	7.4 ± 1.238 [8][5]	4.8 ± 0.841 [13][6]
All	*CC/CT*	10.2 ± 0.097 [1881]	3.5 ± 0.063 [2053]
All	*CT/TT*	10.0 ± 0.304 [207]	3.6 ± 0.201 [233]
AA	All	10.0 ± 0.132 [985]	3.4 ± 0.087 [1094]
AG	All	10.5 ± 0.153 [775]	3.6 ± 0.099 [837]
GG	All	10.5 ± 0.383 [129]	3.9 ± 0.246 [135]
AA	*CC*	10.0 ± 0.141 [839]	3.4 ± 0.093 [933]
AA	*CT*	10.3 ± 0.386 [139]	3.4 ± 0.248 [150]
AA	*TT*	8.3 ± 0.969 [7]	4.6 ± 0.975 [11]
AG	*CC*	10.5 ± 0.159 [716]	3.6 ± 0.103 [769]
AG	*CT*	9.7 ± 0.519 [59]	3.6 ± 0.378 [67]
GG	*CC*	10.6 ± 0.382 [127]	3.9 ± 0.249 [131]

[1] SDMT, Symbol Digit Modalities Test. Haplotype combinations with a frequency < 0.005 were excluded. Means were compared by ANCOVA, with control for age, sex, alcohol use, smoking, education, coffee consumption, serum vitamin B-12 and creatinine concentrations, and multivitamin use.
[2] Main effect of 1561C→T, $P = 0.036$; main effect of 484A→G, $P = 0.23$; interaction, $P = 0.013$.
[3] Main effect of 1561C→T, $P = 0.11$; main effect of 484A→G, $P = 0.15$; interaction, $P = 0.93$.
[4] $\bar{X}$ ± SEM; *n* in brackets (all such values).
[5] *TT* significantly different from *CC* ($P = 0.027$) and *CT* ($P = 0.031$), Tukey's test.
[6] *TT* marginally different from *CC* ($P = 0.092$) and *CT* ($P = 0.096$), Tukey's test.

separate DNA samples (Table 3) exceeded the numbers of subjects available for accurate analysis of both polymorphisms in the same DNA samples (Table 2). Consistent with prior observations (14), serum creatinine concentrations were highly associated with plasma tHcy but not with plasma folate concentrations (not shown). The main effect of the 1561C→T polymorphism was significant for both plasma folate and plasma tHcy concentrations ($P = 0.0005$, $P = 0.0004$). Compared with those with the *1561CC* wild type and with control for serum vitamin B-12 and creatinine and all other variables, plasma folate concentrations were higher and tHcy concentrations were lower in the 10.6% of individuals who were *CT* heterozygotes ($P = 0.0003$ for both comparisons) and in the 11.2% of individuals who were carriers of the *T* allele in the combined population of *1561CT* heterozygotes and *TT* homozygotes ($P < 0.0001$ for both comparisons). The main effect of the 484A→G polymorphism was significant for tHcy ($P = 0.026$), with values in *AG* heterozygotes greater than those in the *GG* homozygotes ($P < 0.05$). There were no significant interactions between the 2 polymorphisms, and there were no significant pair-wise differences between the haplotype combinations. There was no association of either genotype with cardiovascular disease risk as determined by the documented occurrence of angina, myocardial infarction, stroke, or vascular surgery in the 6-y interval between the first and second Hordaland Homocysteine Studies.

After the analysis was controlled for lifestyle variables, education level, age, sex, and serum concentrations of vitamin B-12 and creatinine, there were no associations of the *GCPII* 484A→G or 1561C→T polymorphisms with selected cognitive test scores, including scores for the Kendrick Object Learning Test, block design, trail making, and the s-MMSE. However, there was a significant main effect of the 1561C→T polymorphism on SDMT scores ($P = 0.036$), which were lower in the small group of 8 subjects with the *TT* genotype than in those with either the *CC* or *CT* genotype of 1561C→T ($P < 0.027$ and $P < 0.031$, respectively; **Table 4**). There was no effect of either genotype on total anxiety, whereas the depression score was marginally higher in subjects with the *TT* genotype of 1561C→T than in those with the *CC* or *CT* genotype ($P < 0.09$ and $P < 0.10$, respectively).

Because the Hordaland Homocysteine Study had previously shown an inverse association between moderate alcohol consumption and plasma tHcy concentrations in nonsmokers (16), we looked for interactions among alcohol intake, sex, and 1561C→T effects on folate, tHcy, and cognition while controlling for the other variables. As shown in **Table 5**, we found a main effect of alcohol use on plasma folate concentrations ($P = 0.0015$) and on the s-MMSE score ($P = 0.0053$), and the moderate use of alcohol was associated with higher plasma folate and s-MMSE scores than those in abstainers ($P = 0.0012$ and $P = 0.0045$, respectively). The effect of alcohol use on the s-MMSE score remained significant after controlling for folate and tHcy concentrations.

As shown in **Table 6**, we found a significant three-factor interaction among sex, alcohol use, and the 1561C→T genotype on the SDMT score ($P = 0.021$). Among the 804 women who abstained from alcohol, the 92 women with the *CT* genotype had a lower mean score for the SDMT cognition test than did the 707

The American Journal of Clinical Nutrition

TABLE 5
Effects of moderate alcohol use on folate and homocysteine concentrations and cognitive scores[1]

Group	Folate[2]	tHcy[3]	s-MMSE[4]	SDMT[5]
	nmol/L	*μmol/L*		
Abstainers ($n = 1283$)	9.0 ± 0.222	12.0 ± 0.108	11.4 ± 0.029	9.8 ± 0.132
≤ 2 drinks/d ($n = 1193$)	9.3 ± 0.217[6]	12.1 ± 0.109	11.6 ± 0.023[7]	10.8 ± 0.142
> 2 drinks/d ($n = 38$)	9.1 ± 1.098	13.3 ± 0.642	11.8 ± 0.094	11.5 ± 0.852
All drinkers ($n = 1231$)	9.3 ± 0.213	12.1 ± 0.108	11.6 ± 0.023	10.8 ± 0.140

[1] All values are $\bar{x}$ ± SEM. s-MMSE, shortened Mini Mental State Examination; SDMT, Symbol Digit Modalities Test. Means were compared by ANCOVA, with control for age, sex, smoking, education, coffee consumption, serum vitamin B-12 and creatinine concentrations, and multivitamin use.
[2] Main effect of alcohol use, $P = 0.0015$.
[3] Main effect of alcohol use, $P = 0.19$.
[4] Main effect of alcohol use, $P = 0.0053$.
[5] Main effect of alcohol use, $P = 0.073$.
[6,7] Significantly different from abstainers (Tukey's test): [6] $P = 0.0012$, [7] $P = 0.0045$.

women in the *CC* group ($P = 0.042$). At the same time, among women with the *CT* genotype, the 92 women abstainers had a lower mean SDMT score than did the 38 women who consumed moderate amounts of alcohol ($P = 0.016$). These effects on the SDMT score remained significant after controlling for folate and tHcy concentrations.

DISCUSSION

The present analysis of 2734 DNA samples from the 1997–1999 Hordaland Homocysteine Study had sufficient statistical power to compare the distributions of 2 *GCPII* polymorphisms with metabolic or lifestyle phenotypes. The major finding of the present study relates to the effect of the 1561C→T polymorphism on plasma folate and tHcy concentrations (Table 3). Although the actual differences were small, the data in this large population of subjects were highly significant in showing that either *CT* heterozygosity that occurred in 10.6% of the population or the presence of the *T* allele that occurred in 11.2% of the population who carried the *CT* or *TT* genotype imparted higher plasma folate and lower tHcy concentrations, irrespective of age, sex, alcohol consumption, smoking, education level, coffee consumption, serum vitamin B-12 or creatinine concentrations, or multivitamin use. In addition, our study showed a weaker but significant main effect of the 484A→G polymorphism on raising tHcy concentrations, although there were no interactions or haplotype effects of the 2 genotypes (Table 3).

TABLE 6
Effects of moderate alcohol consumption, sex, and 1561TC→T genotype on Symbol Digit Modalities Test score[1]

Sex and genotype	Abstainers	≤2 drinks/d
Men		
All	9.9 ± 0.244 [389][2]	10.5 ± 0.191 [607]
CC	9.8 ± 0.256 [340]	10.5 ± 0.203 [528]
CT	11.1 ± 0.795 [47]	10.3 ± 0.571 [75]
TT	6.0 [2]	13.0 [4]
CC/CT	10.0 ± 0.245 [387]	10.5 ± 0.191 [603]
CT/TT	10.9 ± 0.786 [49]	10.4 ± 0.563 [79]
Women		
All	9.7 ± 0.163 [804]	11.2 ± 0.235 [488]
CC	9.9 ± 0.177 [707]	11.2 ± 0.242 [447]
CT	8.7 ± 0.412 [92][3,4]	12.3 ± 0.942 [38]
TT	8.7 ± 0.882 [5]	4.3 ± 1.667 [3]
CC/CT	9.7 ± 0.164 [799]	11.2 ± 0.235 [485]
CT/TT	8.7 ± 0.396 [97]	11.4 ± 0.969 [41]

[1] Means were compared by ANCOVA, with control for age, sex, smoking, education, coffee consumption, serum vitamin B-12 and creatinine concentrations, and multivitamin use. Main effect of alcohol use, $P = 0.073$; sex × genotype × alcohol use interaction, $P = 0.021$.
[2] $\bar{x}$ ± SEM; *n* in brackets (all such values).
[3] Significantly different from *CC* among abstainers, $P = 0.042$ (Tukey's test).
[4] Significantly different from moderate drinkers with the *CT* genotype, $P = 0.016$ (Tukey's test).

Except for one report (8), all previous studies of the *GCPII* 1561C→T polymorphism found population distributions of the *CC*, *CT*, and *TT* genotypes that were similar to those shown in Table 2. As summarized in **Table 7**, the heterozygous *CT* genotype was shown to have no effect (6–9), to be associated with higher plasma or red cell folate concentrations (10–13), or, in our original study, to be associated with lower folate and higher tHcy concentrations (5). One of the studies found that the bioavailability of polyglutamyl folate was not significantly different among carriers of the heterozygote or wild-type forms of *GCPII* (12). The present and largest study is the only one to show a lowering effect of the 1561C→T polymorphism on plasma tHcy concentrations along with an elevation of plasma folate concentrations (Table 3). From these comparisons, we conclude that the findings of our original report (5) were based on an insufficient number of subjects for genetic comparisons and on an in vitro transfection experiment that excluded other physiologic effects on genetic expression.

The observed relations of the *CT* and combined *CT* and *TT* genotypes of the 1561C→T polymorphism with higher plasma folate and lower tHcy concentrations (Table 3) suggest a positive effect of the *T* allele on increasing the efficiency of intestinal folate hydrolase. Expressed as folate hydrolase in the brush border surface of the small intestine, *GCPII* regulates the initial digestion of dietary polyglutamyl folates, which is an essential step for subsequent transport of monoglutamyl folates across the intestine and which regulates plasma folate concentrations (24). Subsequently, tHcy concentrations are regulated by circulating folate, which provides substrate for the transmethylation of tHcy to methionine in the liver and other tissues (25).

The secondary findings of the present study suggest that cognitive function is affected by the 1561C→T polymorphism in a way that may be influenced by moderate alcohol consumption.

The American Journal of Clinical Nutrition

TABLE 7
Summary of published studies of the effects of the glutamate carboxypeptidase II (*GCPII*) 1561C→T polymorphism on concentrations of folate and total homocysteine (tHcy)

Author, year, and reference	No. of subjects	*CC*	*CT*	*TT*	Folate effect	tHcy effect
Devlin et al, 2000 (5)	75	0.92	0.08	0.004	Decrease	Increase
Lievers et al, 2002 (11)	791	0.892	0.102	0.006	Increase	None
Vargas-Martinez et al, 2002 (10)	1913	0.895	0.099	0.005	Increase (men only)	None
Afman et al, 2003 (13)	407	0.881	0.099	0.020	Increase	None
Morin et al, 2003 (7)	137	0.891	0.109	0.000	None	None
Chen et al, 2004 (6)	439	0.929	0.071	0.000	None	None
Melse-Boonstra et al, 2004 (12)	180	0.894	0.106	0.000	Increase	None
Relton et al, 2004 (8)	531	0.719	0.269	0.011	None	None
Devlin et al, 2006 (9)	1041	0.889	0.110	0.001	None	None
Halsted et al, 2007 (current study)	2471	0.888	0.106	0.006	Increase	Decrease

Although folate concentrations were higher and tHcy concentrations were lower in the *CT* heterozygotes and in the combined *CT* and *TT* groups than in the *CC* homozygotes (Table 3), the 1561C→T polymorphism had a main effect on the SDMT score, which was found to be significantly lower in the small number of individuals with the *TT* genotype than in those with the *CC* genotype, even when the analysis was controlled for plasma concentrations of folate and tHcy (Table 4). First described in 1968 (21), the SDMT assesses attention, visual scanning, and motor speed and is not significantly affected by age or sex (26).

When evaluating the effects of alcohol consumption and controlling for other variables, on the one hand we found no association of moderate drinking with tHcy concentrations (Table 5). On the other hand, we found main effects of moderate drinking on both plasma folate concentrations and cognition according to the s-MMSE score, whereby moderate drinkers had higher folate concentrations and s-MMSE scores of cognition than did abstainers across all groups (Table 5). Although a cross-sectional study of 801 adults aged >65 y found no effect of the level of alcohol consumption on the MMSE score (27), several prospective studies found that age-related changes in the MMSE score are favorably influenced by moderate alcohol consumption (28–30). For example, a 7-y study of elderly subjects found that light to moderate drinkers had lesser declines in the MMSE score than did nondrinkers (29), an 11.5-y study of 1488 subjects of all ages found less cognitive decline in women who drank moderately than in nondrinkers or heavy drinkers (30), and a 2-y Chinese study of elderly subjects found that light to moderate drinking of wine but not beer was associated with less risk of cognitive decline (28). Other studies have shown differing effects of moderate to excessive alcohol consumption on folate and tHcy concentrations. For example, plasma tHcy concentrations were found to be elevated in excessive alcohol users (31, 32), potentially because of an inhibitory effect of alcohol and its metabolite acetaldehyde on methionine synthase, which regulates the transmethylation and reduction of tHcy (33). Larger epidemiologic studies, including the Framingham Offspring study (34) and the third National Health and Nutrition Examination Survey (35), found an association of progressive alcohol use with elevated tHcy. However, a Dutch study found that that alcohol use was associated with lower tHcy concentrations (36), and a French study concluded that beer drinking lowered tHcy, whereas it was elevated in wine drinkers (37). Others described a protective effect of beer consumption on tHcy concentrations, presumably because of its high folate content (38). A study in Wales suggested a protective effect on ischemic heart disease of the high folate content of beer, the preferred alcoholic beverage (39). However, the present findings on the permissive effect of moderate alcohol intake on plasma folate concentrations appears to be unrelated to beverage choice, because wine accounted for most of the alcohol consumption and beer for only about one-quarter of reported alcohol intake overall. Contrasting with previous data from the Hordaland Homocysteine Study (40), the positive effect of moderate alcohol consumption on improved s-MMSE score persisted after the analysis was controlled for plasma concentrations of folate and tHcy (Table 5).

After removing a potential protective effect of moderate alcohol consumption, we found also that SDMT cognition scores were significantly lower in the abstinent women in the *CT* group than in those in the *CC* group or in moderate drinkers in the *CT* group (Table 6). This finding is consistent with a prior prospective study and suggests that the positive effect of moderate drinking on overall cognition according to the MMSE score occurs in women but not in men (30), while indicating an additional genotype effect. The observed association of the *CT* genotype with lower tHcy concentrations (Table 3) and with lower SMTP scores of cognition in a subset of women who abstained from alcohol (Table 6) conflicts with previous findings from the second Hordaland Homocysteine Study and others that reduced cognition is associated with elevated tHcy (40, 41). Although the *P* values for the association of sex, alcohol status, and the 1561C→T genotype shown in Table 6 were significant after the Tukey test, it is possible that these are spurious findings due to the type 1 error that could result from the increasing numbers of comparisons.

Assuming that the finding of reduced SDMT scores in abstinent women with the *CT* genotype despite unchanged tHcy is true (Tables 3 and 6), the data suggest that the effect of the *CT* genotype on cognition in abstinent women is unrelated to its effect on intestinal folate hydrolase and subsequent folate and tHcy concentrations. Alternatively, this observation could reflect the influence of the *T* allele on the expression of *GCPII* as *N*-acetylated α-linked acidic dipeptidase, a brain enzyme that regulates the cleavage of *N*-acetyl-L-aspartyl-L-glutamate to NAA and the neurotransmitter glutamate (42). Recent data indicate that *N*-acetylated α-linked acidic dipeptidase is widely distributed in astrocytes in the human brain, whereas its higher activity is associated with glutamate neurotoxicity in various neurodegenerative disorders (43). It is possible that regulation of

The American Journal of Clinical Nutrition

the amount of glutamate produced and released from astrocytes (44) may be influenced by the *GCPII* genotype. Other data indicate that lower concentrations of NAA, which can be measured by in vivo magnetic resonance spectrophotometry, correlate directly with reduced cognitive function in aging individuals (45, 46) and that NAA is a more specific indicator of intelligence in women than in men (47). The present study points to a need for additional investigations on the effects of the 1561C→T polymorphism of *GCPII* on the expression of brain *N*-acetylated α-linked acidic dipeptidase and on its regulation of cognitive function.

We are grateful to D Warden for DNA isolation from blood samples, K Engedal and HA Nygaard for assistance with the cognitive assessments, and AA Dahl for permission to use the data on anxiety and depression.

All authors participated sufficiently in the work to take public responsibility for appropriate portions of its content. The individual contributions of the authors were as follows—CHH: provided the original concept of the study, oversaw all aspects of the study and data analysis, and wrote the manuscript; DHW: performed all the genotyping, participated in its analysis, and wrote portions of the manuscript; JMP: provided all statistical analyses and a portion of the manuscript; CHW: provided consultations on genotyping and its analysis; ADS: participated in study design and data analysis, provided oversight of DNA collections, and provided critique of the manuscript; HR: participated in HUSK data collection, participated in study design and data analysis, and provided critical evaluation of the manuscript; PMU, SEV, and OKN: participated in HUSK data collection and provided critical evaluation of the manuscript; GST: supervised the HUSK data collection, participated in study design, and provided critique of the manuscript. None of the authors had any undisclosed financial or personal interest in any company or organization connected in any way to this article.

REFERENCES

1. Halsted CH. Folylpolygammaglutamate carboxypeptidase. In: Barrett A, ed. Handbook of proteolytic enzymes. San Diego, CA: Academic Press, 1998:1437–40.
2. O'Keefe DS, Su SL, Bacich DJ, et al. Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. Biochim Biophys Acta 1998;1443:113–27.
3. Halsted CH, Ling EH, Luthi-Carter R, Villanueva JA, Gardner JM, Coyle JT. Folylpoly-gamma-glutamate carboxypeptidase from pig jejunum. Molecular characterization and relation to glutamate carboxypeptidase II. J Biol Chem 1998;273:20417–24.
4. Luthi-Carter R, Berger UV, Barczak AK, Enna M, Coyle JT. Isolation and expression of a rat brain cDNA encoding glutamate carboxypeptidase II. Proc Natl Acad Sci U S A 1998;95:3215–20.
5. Devlin AM, Ling EH, Peerson JM, et al. Glutamate carboxypeptidase II: a polymorphism associated with lower levels of serum folate and hyperhomocysteinemia. Hum Mol Genet 2000;9:2837–44.
6. Chen J, Kyte C, Valcin M, et al. Polymorphisms in the one-carbon metabolic pathway, plasma folate levels and colorectal cancer in a prospective study. Int J Cancer 2004;110:617–20.
7. Morin I, Devlin AM, Leclerc D, et al. Evaluation of genetic variants in the reduced folate carrier and in glutamate carboxypeptidase II for spina bifida risk. Mol Genet Metab 2003;79:197–200.
8. Relton CL, Wilding CS, Pearce MS, et al. Gene-gene interaction in folate-related genes and risk of neural tube defects in a UK population. J Med Genet 2004;41:256–60.
9. Devlin AM, Clarke R, Birks J, Evans JG, Halsted CH. Interactions among polymorphisms in folate-metabolizing genes and serum total homocysteine concentrations in a healthy elderly population. Am J Clin Nutr 2006;83:708–13.
10. Vargas-Martinez C, Ordovas JM, Wilson PW, Selhub J. The glutamate carboxypeptidase gene II (C→T) polymorphism does not affect folate status in the Framingham Offspring cohort. J Nutr 2002;132:1176–9.
11. Lievers KJ, Kluijtmans LA, Boers GH, et al. Influence of a glutamate carboxypeptidase II (GCPII) polymorphism (1561C→T) on plasma homocysteine, folate and vitamin B(12) levels and its relationship to cardiovascular disease risk. Atherosclerosis 2002;164:269–73.
12. Melse-Boonstra A, Lievers KJ, Blom HJ, Verhoef P. Bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid in subjects with different genotypes of the glutamate carboxypeptidase II gene. Am J Clin Nutr 2004;80:700–4.
13. Afman LA, Trijbels FJ, Blom HJ. The H475Y polymorphism in the glutamate carboxypeptidase II gene increases plasma folate without affecting the risk for neural tube defects in humans. J Nutr 2003;133:75–7.
14. Nygard O, Refsum H, Ueland PM, Vollset SE. Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. Am J Clin Nutr 1998;67:263–70.
15. Nurk E, Tell GS, Vollset SE, et al. Changes in lifestyle and plasma total homocysteine: the Hordaland Homocysteine Study. Am J Clin Nutr 2004;79:812–9.
16. Refsum H, Nurk E, Smith AD, et al. The Hordoland Homocysteine Study: a community-based study of homocysteine, its determinants, and associations with disease. J Nutr 2006;136:1731S–40S.
17. Fiskerstrand T, Refsum H, Kvalheim G, Ueland PM. Homocysteine and other thiols in plasma and urine: automated determination and sample stability. Clin Chem 1993;39:263–71.
18. O'Broin S, Kelleher B. Microbiological assay on microtitre plates of folate in serum and red cells. J Clin Pathol 1992;45:344–7.
19. Kelleher BP, Broin SD. Microbiological assay for vitamin B12 performed in 96-well microtitre plates. J Clin Pathol 1991;44:592–5.
20. Engedal K, Haugen P, Gilje K, Laake P. Efficacy of short mental tests in the detection of mental impairment in old age. Compr Gerontol [A] 1988;2:87–93.
21. Smith A. The symbol-digit modalities test: a neuropsychologic test of learning and other cerebral disorders. In: Helmuth J, ed. Learning disorders. Seattle, WA: Special Child Publications, 1968:83–91.
22. Kendrick DC. Kendrick cognitive tests for the elderly. Windsor, United Kingdom: NFER-NELSON Publishing Company, Ltd, 1985.
23. Bjelland I, Tell GS, Vollset SE, Refsum H, Ueland PM. Folate, vitamin B12, homocysteine, and the MTHFR 677C→T polymorphism in anxiety and depression: the Hordaland Homocysteine Study. Arch Gen Psychiatry 2003;60:618–26.
24. Chandler CJ, Harrison DA, Buffington CA, Santiago NA, Halsted CH. Functional specificity of jejunal brush-border pteroylpolyglutamate hydrolase in pig. Am J Physiol 1991;260:G865–72.
25. Halsted CH. Nutrition and alcoholic liver disease. Semin Liver Dis 2004;24:289–304.
26. Sheridan LK, Fitzgerald HE, Adams KM, et al. Normative Symbol Digit Modalities Test performance in a community-based sample. Arch Clin Neuropsychol 2006;21:23–8.
27. Reid MC, Maciejewski PK, Hawkins KA, Bogardus ST Jr. Relationship between alcohol consumption and Folstein mini-mental status examination scores among older cognitively impaired adults. J Geriatr Psychiatry Neurol 2002;15:31–7.
28. Deng J, Zhou DH, Li J, Wang YJ, Gao C, Chen M. A 2-year follow-up study of alcohol consumption and risk of dementia. Clin Neurol Neurosurg 2006;108:378–83.
29. Ganguli M, Vander Bilt J, Saxton JA, Shen C, Dodge HH. Alcohol consumption and cognitive function in late life: a longitudinal community study. Neurology 2005;65:1210–7.
30. Leroi I, Sheppard JM, Lyketsos CG. Cognitive function after 11.5 years of alcohol use: relation to alcohol use. Am J Epidemiol 2002;156:747–52.
31. Cravo ML, Gloria LM, Selhub J, et al. Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12, and vitamin B-6 status. Am J Clin Nutr 1996;63:220–4.
32. Hultberg B, Berglund M, Andersson A, Frank A. Elevated plasma homocysteine in alcoholics. Alcohol Clin Exp Res 1993;17:687–9.
33. Kenyon SH, Nicolaou A, Gibbons WA. The effect of ethanol and its metabolites upon methionine synthase activity in vitro. Alcohol 1998;15:305–9.
34. Jacques PF, Bostom AG, Wilson PW, Rich S, Rosenberg IH, Selhub J. Determinants of plasma total homocysteine concentration in the Framingham Offspring cohort. Am J Clin Nutr 2001;73:613–21.
35. Ganji V, Kafai MR. Demographic, health, lifestyle, and blood vitamin determinants of serum total homocysteine concentrations in the third

The American Journal of Clinical Nutrition

National Health and Nutrition Examination Survey, 1988–1994. Am J Clin Nutr 2003;77:826–33.
36. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Lifestyle factors and plasma homocysteine concentrations in a general population sample. Am J Epidemiol 2001;154:150–4.
37. Mennen LI, de Courcy GP, Guilland JC, et al. Relation between homocysteine concentrations and the consumption of different types of alcoholic beverages: the French Supplementation with Antioxidant Vitamins and Minerals Study. Am J Clin Nutr 2003;78:334–8.
38. van der Gaag MS, Ubbink JB, Sillanaukee P, Nikkari S, Hendriks HF. Effect of consumption of red wine, spirits, and beer on serum homocysteine. Lancet 2000;355:1522.
39. Ubbink JB, Fehily AM, Pickering J, Elwood PC, Vermaak WJ. Homocysteine and ischaemic heart disease in the Caerphilly cohort. Atherosclerosis 1998;140:349–56.
40. Nurk E, Refsum H, Tell GS, et al. Plasma total homocysteine and memory in the elderly: the Hordaland Homocysteine Study. Ann Neurol 2005;58:847–57.
41. Seshadri S, Beiser A, Selhub J, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 2002;346:476–83.
42. Slusher BS, Robinson MB, Tsai G, Simmons ML, Richards SS, Coyle JT. Rat brain *N*-acetylated alpha-linked acidic dipeptidase activity. Purification and immunologic characterization. J Biol Chem 1990;265:21297–301.
43. Sacha P, Zamecnik J, Barinka C, et al. Expression of glutamate carboxypeptidase II in human brain. Neuroscience 2007;144:1361–72.
44. Montana V, Malarkey EB, Verderio C, Matteoli M, Parpura V. Vesicular transmitter release from astrocytes. Glia 2006;54:700–15.
45. Chao LL, Schuff N, Kramer JH, et al. Reduced medial temporal lobe *N*-acetylaspartate in cognitively impaired but nondemented patients. Neurology 2005;64:282–9.
46. Ross AJ, Sachdev PS, Wen W, Valenzuela MJ, Brodaty H. Cognitive correlates of ^{1}H MRS measures in the healthy elderly brain. Brain Res Bull 2005;66:9–16.
47. Jung RE, Haier RJ, Yeo RA, et al. Sex differences in *N*-acetylaspartate correlates of general intelligence: an 1H-MRS study of normal human brain. Neuroimage 2005;26:965–72.

The American Journal of Clinical Nutrition