Factors Explaining the Difference of Total Homocysteine Between Men and Women in the European Investigation Into Cancer and Nutrition Potsdam Study

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Interestingly, plasma total homocysteine (tHcy) concentration is consistently higher in men than in women. This observation deserves further investigations because elevated tHcy concentrations have been shown to be independently associated with coronary, peripheral, and cerebral vascular diseases. It was the aim of the present study to define major determinants of plasma tHcy in a healthy middle-aged German population under particular consideration of the gender factor. The study population was obtained from an ongoing recruitment procedure for a cohort study and comprised 336 men and women, aged 40 to 65 years. Exclusion criteria were elevated creatinine levels in blood, history of skin or atherosclerotic diseases, current use of vitamins or other supplements, and heavy smoking. Plasma tHcy, folate, vitamin B12, vitamin B6, creatinine, testosterone and estradiol, protein, and hematocrit were measured. Fat-free mass was assessed by skinfold thickness. The C677T polymorphism of the methylenetetrahydrofolate reductase (MTHFR), a key enzyme of folate and homocysteine metabolism, was determined by polymerase chain reaction (PCR) with restriction enzyme analysis. In this population, plasma tHcy ranged from 5 to 46 μmol/L. The frequency of the T allele of the MTHFR was 0.29, which is lower than in other populations. A total of 54.2% of this population was homozygote for the wild-type, 39.6% heterozygote, and 6.2% homozygote for the mutation. tHcy correlated negatively with folate and cobalamin concentration in blood and positively with creatinine. No correlation was seen with vitamin B6. From the gender-related variables, tHyc correlated significantly with fat-free mass and testosterone and inversely with estradiol. The difference between gender with regard to tHcy was mainly explained by differences in fat-free mass, but also by estradiol concentrations. The following contributions to the variation of tHcy were seen in a multivariate regression model: plasma cobalamin (11%), creatinine (11%), plasma folate (8%), fat-free mass (5%), estradiol (2%), MTHFR polymorphisms (2%), and plasma protein (1%). We concluded that tHcy in the general population has a variety of determinants ranging from nutrition, internal metabolic parameters to gender-related variables.

ELEVATED TOTAL homocysteine (tHcy) concentration is independently associated with peripheral, coronary, and cerebral vascular disease.[1-3] A recent meta-analysis with data from cohort studies concluded that an increase of 5 μmol/L homocysteine in peripheral blood is similarly associated with the risk of coronary heart disease as an increase of 0.5 mmol/L cholesterol.[4]

The relationship of levels of tHcy in the normal range with vascular diseases requires the understanding of which variables affect tHcy levels in the general population. Research has shown that tHcy is influenced by both environmental and genetic factors. Folate and cobalamin status seem to be the most important nutritional factors.[5] Renal function,[6,7] age, and gender are other important determinants of tHcy.[8-10] Genetic influence is assumed to be due to polymorphisms or mutations in key enzymes of the homocysteine metabolism, such as cystathionine-β-synthase and methylenetetrahydrofolate reductase (MTHFR). Notably, the C677T polymorphism of the MTHFR, which is associated with decreased enzyme activity,[11] is frequently found in the general population. Surveys in different populations showed prevalence rates of the homozygous mutated genotype of about 10% to 15% and an allele frequency of about 0.35.[12-15]

Indeed, gender is affecting the tHcy concentration with men having higher concentrations than women. This result was found consistently in every study including men and women. This difference has mainly been attributed to differences in sex hormones.[9] Indeed, homocysteine decreases in pregnancy, when estradiol levels increase, to about 60% of baseline concentrations,[16,17] while it increases after menopause when estradiol concentrations diminish.[18,19] Recently, gender differences of tHcy were well described in a large population-based study.[10] However, in this study, no attempt was made to measure sex hormones directly.

The aim of this study was to investigate environmental and genetic determinants for tHcy in a healthy, well-nourished, middle-aged population with particular attention to gender differences. The latter aspect used among other variables measurements of sexual hormones in blood.

SUBJECTS AND METHODS

Subjects

Participants of this study were selected from the Potsdam cohort of the European Investigation Into Cancer and Nutrition (EPIC) study.[20] The cohort population was established in 1994 to 1998 through population registration offices of Potsdam and adjacent communities. The enrollment procedures covered men aged 40 to 64 and women 35 to 64 years. In 1994 to 1995, the participation rate for the cohort study was 35% of those who had been invited by mail. Subjects for this cross-sectional analysis were recruited between March and September 1997 within the ongoing basic examination for the cohort study. A computer program identified those cohort participants who met a priori the inclusion and exclusion criteria: age 40 to 65 years, nonsmoker or smoking of less then 10 cigarettes/day,[21] and no history of skin or atherosclerotic diseases. The computer program was activated when the

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staff of the laboratory was able to perform the extra work associated with this study. None of the eligible and approached study participants refused to give additional blood for this study. Both the cohort study itself and this project were approved by the ethic committee of the federal state of Brandenburg. In total, 370 subjects gave blood for this study (206 men and 164 women).

Anthropometry and Other Data

The body mass index (BMI) (kg/m^2) was calculated from weight (with light underware) and height measured before blood sampling.[22] Skinfolds at 4 sites (biceps, triceps, subscapular, and suprailiac) on the right body site were measured with a standard caliper (Lange, Cambridge, MD). For calculation of fat-free body mass, the equations of Durnim and Womersly[23] and Siri[24] were applied. Demographic and lifestyle variables (smoking, medication) were obtained by trained interviewers using computer-assisted interviews and a self-administered questionnaire. The creatinine clearance was calculated from age, weight, and plasma creatinine using the equation of Cockraft and Gault.[25]

Blood Collection and Laboratory Analysis

A total of 30 mL venous blood was taken from each study participant during examination for the cohort study at the study center in Potsdam. The hematocrit was determined using a hematocrit centrifuge. The plasma was separated from the blood cells within 2 hours. For red blood cell (RBC) folate analysis, whole blood was mixed with freshly prepared lysis reagent (Abbott GmbH, Wiesbaden, Germany) according to the manufacturer's instructions (50 μL blood to 1 mL reagent) and frozen. Hematocrit was frozen for DNA preparation. All samples were frozen at -80°C. The samples were transferred to the Institute of Clinical Chemistry on dry ice for analysis of tHcy, plasma and RBC folate, cobalamin, pyridoxal-5-phosphate (PLP) (vitamin B6), protein, creatinine, testosterone, and estradiol. Genomic DNA was isolated from peripheral blood cells by a combined method of alkali lysis and use of phenol:chloroform:amylalcohol extraction.

Plasma tHcy was determined by an high-performance liquid chromatography (HPLC) method with fluorescence detection according to Vester and Rasmussen.[26] This assay measures free and protein-bound homocysteine without distinguishing between them (total homocysteine assay). In a first step, oxidized and protein-bound homocysteine moieties were reduced by the addition of tri-butyl-phosphine. After protein precipitation, homocysteine was derivatized using a thiol-specific fluorogenic reagent (SBD-F; Sigma, Deisenhofen, Germany), and the derivatization product was separated from other plasma thiols by reversed-phase HPLC and detected by fluorescence detection. The assay has a between-run coefficient of variation of 5%.

PLP as active metabolite of vitamin B6 was determined in plasma using a commercial HPLC assay with fluorescence detection (Immundiagnostik, Bensheim, Germany). The between-run coefficient of variation of the method was less than 10%.

Folate and cobalamin were determined using commercial test kits (IMx Abbott Lab, Weisbaden, Germany). Folate was analyzed both in plasma and RBC folate. RBC folate was analyzed in suspension of blood in ascorbic acid solution. The assay for cobalamin measured total cobalamin. The between-run coefficients of variation were 5% for folate and 7% for cobalamin.

Testosterone was determined using a radioimmunoassay labeled with ^{125}I (DPC Biermann; Bad Nauheim, Germany). Estradiol was measured by a competitive immunoassay involving chemoluminescence, highly specific for 17β-estradiol (Immulite 2000; DPC Biermann, Bad Nauheim, Germany).

The C677T genotype of the MTHFR was determined in genomic DNA by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to Kluijtmans et al.[27] Briefly, the DNA was isolated from peripheral blood cells and amplified by PCR. The PCR product (198 bp size) was incubated with HinfI and subsequently run on a 3% agarose gel stained with ethidium bromide and analyzed under ultraviolet light. The C to T mutation introduces a HinfI recognition site so that the 198-bp DNA fragment is cleaved at the presence of the mutation into 2 products of 175 bp and 23 bp.

Total plasma protein was measured on a Hitachi 917 using the Biuret method (Boehringer Mannheim, Mannheim, Germany). Creatinine was measured enzymatically using an automated assay (Boehringer Mannheim).

Statistical Analysis

The total homocysteine concentration is influenced mainly by vitamin supplementation and by impaired renal function. Therefore, subjects who used vitamin supplements (n = 32) and subjects with elevated creatinine concentrations (>120 μmol/L, n = 2, both with tHcy >15 μmol/L) were excluded from further statistical analysis. Age, BMI, fat-free mass, and the proportion of blood samples obtained in the fasting state did not differ significantly between those excluded and those remaining in the analyses (data not shown). The final study population comprised 336 subjects, 189 men and 147 women.

Study results are presented as mean and standard deviation or as median with 5th and 95th percentiles. Statistical significance of differences between groups was assessed with the Mann-Whitney rank test. Spearman's rho was used to test for correlation between 2 variables. Influence of environmental and genetic factors on tHcy was calculated by univariate and then by multivariate analysis. Analysis of variance (ANOVA) and multiple linear regression analysis were used when testing the combined effect of 2 or more independent variables on tHcy. Logarithmically transformed variables were used in the multivariate analysis. The *P* values are 2-tailed, a *P* value of $< .05$ being considered as significant.

RESULTS

In the study population (n = 336), the average age was 53 years, both in men and in women. Both sexes were similar in BMI (men, 27.1 and women, 27.0 kg/m^2). The average weight among men was 83.6 $\pm$ 11.2 kg, with 62.1 $\pm$ 6.2 kg as fat-free mass. In women, the weight was 71.1 $\pm$ 12.0 kg on average, 44.4 $\pm$ 4.9 kg of it as fat-free mass. A slightly higher proportion of women (37%) gave blood under fasting condition than

Table 1. Characteristics of Subjects by Sex (mean ± SD or median and 5th and 95th percentiles)

	Male (n = 189)	Female (n = 147)	*P*
Creatinine (μmol/L)	74 $\pm$ 13	59 $\pm$ 12	.001
Protein (g/L)	66.2 $\pm$ 5.8	68.2 $\pm$ 4.3	.01
Fat-free mass (kg)	62.1 $\pm$ 6.2	44.4 $\pm$ 4.9	$<$.001
Body mass index (kg/m^2)	27.1 $\pm$ 3.3	27.0 $\pm$ 4.6	.98
Plasma 17β estradiol (pmol/L)	113 (73.4-166)	123 (73.4-826)	$<$.01
Plasma testosterone (nmol/L)	10.7 (5.4-18.2)	0.4 (0.1-1.1)	$<$.001
Plasma folate (nmol/L)	17.1 (10.1-26.1)	16.4 (10.6-30.0)	.41
RBC folate (nmol/L)	709 (500-1249)	691 (460-1300)	.54
Cobalamin (pmol/L)	222 (131-399)	230 (125-447)	.38
PLP (nmol/L)	40.5 (12.1-97.0)	29.3 (12.1-98.2)	.001
tHcy (μmol/L)	10.8 (7.4-17.6)	9.0 (5.9-14.1)	.001

Abbreviations: RBC folate, red blood cell folate; PLP, pyridoxal-5-phosphate; tHcy, total homocysteine.

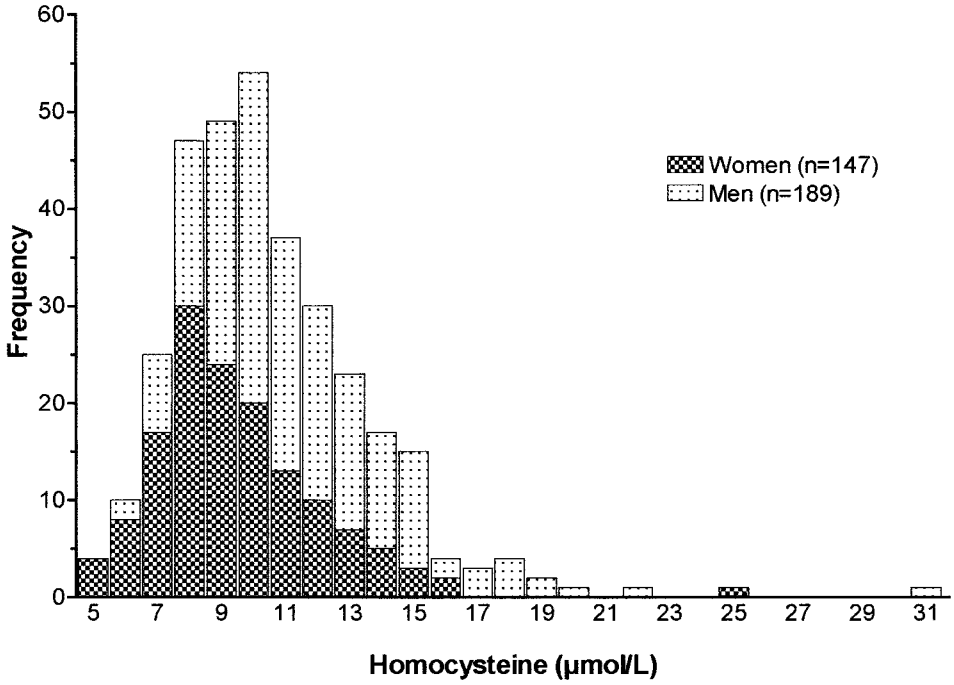

Fig 1. Distribution of tHcy in men (n = 189) and women (n = 147) of the Potsdam EPIC cohort. One male subject with tHcy of 46.1 μmol/L is not shown.

men (31%). Regarding the clinical-chemical variables, men had higher creatinine concentrations, lower protein concentrations, and higher tHcy and PLP concentrations than women (Table 1). Folate, both in plasma and RBC, and cobalamin were not significantly different between sexes. The proportion of tHcy concentrations being higher than the usual cut off of 15 μmol/L was 7.7% (26 of 336, 21 men and 5 women, Fig 1). Differences in sex hormones between men and women were found as expected.

The frequency of the C677T polymorphism and the influence on tHcy and vitamins is shown in Table 2. The overall allele frequency of the T allele was 26.0%. No differences according to gender in the allele distribution of the MTHFR gene was observed. Plasma tHcy was significantly higher in carriers of the homozygous variant in the univariate analysis (CC genotype *v* TT genotype, $P = .026$, ANOVA, Table 2). The difference became particularly obvious in subjects with folate concentrations below the median ($P < .001$, data not shown). The MTHFR genotype did not differ with respect to plasma cobalamin or PLP.

The correlation matrix between tHcy, vitamins, and other variables is given in Table 3. Significant inverse correlations were observed between tHcy and plasma and RBC folate, plasma cobalamin, and estradiol ($r = -0.15$, $P < .01$, Fig 2). Significant positive correlations were observed between tHcy and creatinine ($r = .44$, $P < .001$), between tHcy and fat-free mass ($r = .33$, $P < .01$) and between tHcy and testosterone ($r = .29$, $P < .01$, Fig 3). Of the 147 women, 34 (23%) received hormone replacement therapy or used oral contraceptives. There was no difference in tHcy or vitamin concentration between women who used hormones and those who did not (data not shown).

In the multiple regression analysis with the log-transformed data of tHcy as the dependent variable, plasma cobalamin, plasma folate, and creatinine explained most of the variation between subjects. The contribution of RBC folate to the variation of tHcy was small compared with that of plasma folate (data not shown). Replacement of creatinine by the calculated creatinine clearance did not improve the model (data not shown). The genotype of the MTHFR did not considerably contribute to the explanation of the variance of tHcy. Sex contributed to the explanation of the variance of tHcy, but was

Table 2. Vitamin and tHcy Concentrations by MTHFR Genotype (median and 5th and 95th percentiles)

	Wild-Type CC (n = 182)	Heterozygotes CT (n = 133)	Homozygous Variants TT (n = 21)	*P*
Proportion male	90 (52%)	88 (62%)	11 (57%)	.89
Plasma folate	18.0 (10.9-26.9)	15.9 (9.6-30.0)	14.4 (9.9-35.5)	.093
RBC folate (nmol/L)	716 (513-1286)	682 (457-1163)	700 (420-1842)	.056
Cobalamin (pmol/L)	220 (128-442)	234 (122-416)	245 (151-426)	.165
PLP (nmol/L)	33.3 (14.1-97.2)	38.7 (11.2-96.7)	38.3 (17.5-107)	.82
tHcy (μmol/L)	9.8 (6.7-15.0)	10.2 (6.2-17.6)	11.0 (7.7-29.4)*	.026

NOTE. *P* values by ANOVA with log-transformed data.

*Indicates group was significantly different from wild-type at .05 level by post hoc Scheffé analysis.

Table 3. Correlation Matrix (Spearman's rho correlation coefficients) for All Variables (n = 336)

	tHcy	Folate	RBC Folate	Cobalamin	PLP	Creatinine	Protein	BMI	FFM	Testosterone	Estradiol
Folate	−0.31*										
RBC folate	−0.20*	0.62*									
Cobalamin	−0.29*	0.19*	0.20*								
PLP	0.09	0.24*	0.18*	0.17*							
Creatinine	0.44*	−0.08	0.06	−0.02	0.19*						
Protein	0.09	0.09	0.11	0.16*	0.16*	0.05					
BMI	0.03	0.21*	0.20*	0.07	−0.03	0.05	0.04				
FFM	0.33*	0.02	0.09	−0.03	0.18*	0.49*	−0.14*	0.39*			
Testosterone	0.29*	−0.03	−0.03	−0.00	0.15*	0.50*	−0.18*	0.04	0.72*		
Estradiol	−0.15*	0.00	−0.06	0.04	−0.18*	−0.15*	−0.12*	−0.10	−0.15*	0.00	
Age	0.02	0.11	0.03	0.08	0.05	−0.05	−0.04	0.26*	0.02	0.01	−0.18*

Abbreviations: RBC folate, red blood cell folate; PLP, pyridoxal-5-phosphate; tHcy, total homocysteine; BMI, body mass index; FFM, fat-free mass.

*$P < .01$.

replaced by fat-free mass at the variable selection process (Table 4). Testosterone was replaced in the multiple regression analysis by either sex or fat-free mass (data not shown). Estradiol contributed to the explanation of the variance of tHcy and remained in the final model (model 2). The variables included in the final model explained about 40% of the variation of tHcy in this population in rank order, and according to explained variance put creatinine and cobalamin in the first place, followed by plasma folate, sex respectively fat-free mass, and finally C677T MTHFR polymorphism, plasma protein, and estradiol.

DISCUSSION

The present study focused on determinants of tHcy in a healthy middle-aged population rather than on the importance of tHcy as a risk factor for atherosclerotic disease. We investigated environmental and genetic determinants of tHcy with particular regard to gender differences. Vitamin status and serum creatinine appeared to be major determinants of tHcy in this population, together explaining about 34% of the variation of tHcy. We found differences between men and women being attributable to differences in fat-free mass and in estradiol.

This study is 1 of the first that relates the consistently found gender difference in tHcy to differences in fat-free mass. Men had significantly higher tHcy, fat-free mass, and creatinine than women, which were all interrelated. Creatinine is a marker for both renal function and muscle mass. It is known that tHcy, similar to creatinine, is dependent on renal function. Subjects with impaired renal function were excluded from the present investigation to minimize this effect on tHcy. The newly reported observation, relating tHcy to fat-free mass, may be explained by shared pathways of tHcy and creatinine. It was estimated that about 80% of S-adenosylhomocysteine is formed from S-adenosylmethionine during the formation of creatine

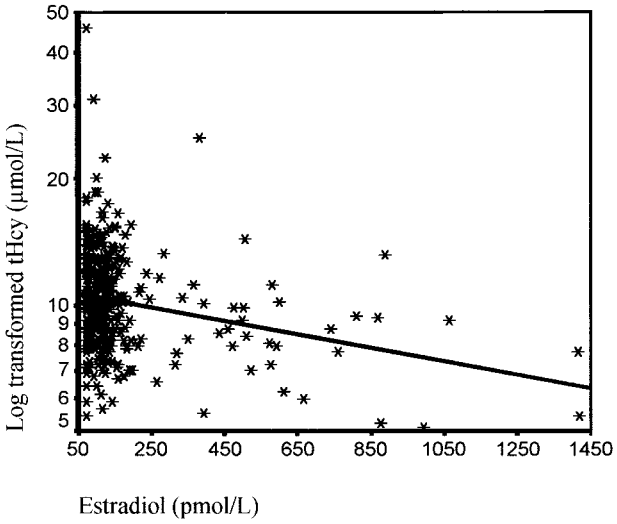

Fig 2. Linear regression of tHcy and estradiol in 336 middle-aged healthy men and women from the Potsdam EPIC cohort. tHcy was log-transformed.

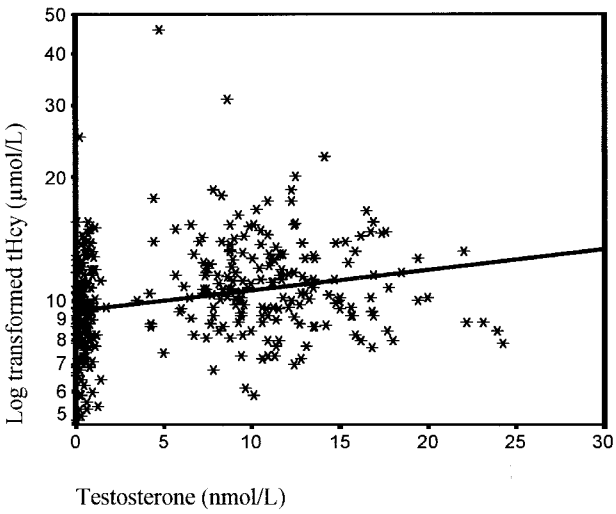

Fig 3. Linear regression of tHcy and testosterone in 336 middle-aged healthy men and women from the Potsdam EPIC cohort. tHcy was log-transformed.

Table 4. Linear Regression Models for Plasma tHcy (using log-normalized values for tHcy, plasma folate, and cobalamin)

Independent Variable	Standardized Coefficient β	Partial R^2	P
Model 1			
Creatinine	0.270	.110	<.001
Plasma cobalamin	−0.313	.109	<.001
Plasma folate	−0.241	.078	<.001
MTHFR (CC/CT = 0, TT = 1)	0.143	.019	.001
Plasma protein	0.155	.014	.001
Sex (male = 1, female = 0)	0.150	.050	.001
Estradiol	−0.102	.024	.029
Model R^2	.404		
Model 2			
Creatinine	0.268	.109	<.001
Plasma cobalamin	−0.318	.110	<.001
Plasma folate	−0.248	.081	<.001
MTHFR (CC/CT = 0, TT = 1)	0.145	.019	.001
Plasma protein	0.158	.014	.001
Fat-free mass	0.167	.052	.001
Estradiol	−0.100	.024	.030
Model R^2	.409		

from guanidinoacetate.[28] Creatine is directly related to muscle mass because creatine-phosphate is an important energy-providing molecule in the muscle. Differences in the formation of creatine might be responsible for differences in the formation of homocysteine and remethylation, and hence differences in homocysteine export from the cells into the extracellular compartment.

Regarding the association between fat-free mass and tHcy, it must be taken into account that fat-free mass was calculated from skinfold measurements in the present study. Measurement of skinfolds is known to be influenced by interobserver errors. The calculation of body composition may be less precise than the calculation from other methods, such as dual-energy x-ray absorptiometry (DEXA). However, DEXA is a method requiring cumbersome equipment, which is less suitable for a field study. In addition, the observer doing the skinfold measurements was highly trained to obtain reliable data.

Differences in tHcy between genders have also been attributed to hormonal differences. The low concentrations of tHcy in pregnancy have been associated with increased estradiol concentrations,[16] and the increase of both fasting and postmethionine load tHcy during menopause was related to the lack of estradiol.[18,19] Indeed, a small reduction of tHcy was observed after the start of hormone replacement therapy in postmenopausal women.[29] In the present study, estradiol concentrations contributed to the variance in tHcy even after adjustment for vitamins, creatinine, fat-free mass, and protein. However, the mechanism of estradiol on tHcy is unclear at present, and experimental or clinical literature on this issue is rare. Finkelstein[30] suggested that estradiol increases the activity of methionine synthase in rat tissue, thus reducing the tHcy concentration. Further studies are required to clarify the role of estradiol in homocysteine and methionine metabolism.

Some other studies investigated the underlying mechanism for the difference between men and women.[31-33] These studies suggest that the difference between men and women is due to different handling of the methionine pathway, eg, transamination of methionine,[31] remethylation of homocysteine,[33] or formation of creatine.[8]

This is the most comprehensive published study on tHcy distribution in Germany until now. We observed a low frequency of elevated tHcy, according to the current upper limit of the reference range of 15 μmol/L. The relatively low frequency of elevated tHcy can be due to the criteria eliminating, which are known to be associated with increased tHcy, eg, heavy smoking, elevated creatinine, or prevalent diseases. Other limits in terms of population representation are due to the participation rate, which was 25% taking the whole recruitment period.[20]

We estimated the frequency of the TT genotype of the MTHFR to be 6.2% in this population. A number of studies have investigated the frequency of the TT genotype, showing that there is considerable variation in the frequency of this genotype in different geographical areas and ethnic groups.[34,35] Our results are comparable to those of Gudnason et al,[35] who reported the T allele frequency in male students in different parts of Europe being low in Finland and Estonia (0.23), intermediate in Germany, Denmark, Belgium, and Switzerland (0.32) and high in Southern Europe (Portugal, Spain, Italy, and Greece 0.39).

In conclusion, it appeared from the present study that the folate concentrations in serum, renal function, fat-free mass, and the estradiol concentration are important predictors of tHcy in a population of healthy middle-aged men and women. Further studies should investigate, in particular, the association between tHcy and fat-free mass and between tHcy and estradiol.

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