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Chien-Hsiung Cheng
Yi-Chia Huang
Feng-Pan Chen
Ming-Chih Chou
Tsung-Po Tsai

B-vitamins, homocysteine and gene polymorphism in adults with fasting or post-methionine loading hyperhomocysteinemia

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C.-H. Cheng · M.-C. Chou · T.-P. Tsai
Institute of Medicine
Chung Shan Medical University
Taichung, Taiwan

C.-H. Cheng
Critical Care and Respiratory Therapy
Taichung Veterans General Hospital
Taichung, Taiwan

Y.-C. Huang · F.-P. Chen
School of Nutrition and Institute of Nutritional Science
Chung Shan Medical University
Taichung, Taiwan

M.-C. Chou · T.-P. Tsai (✉)
Dept. of Surgery
Chung Shan Medical University Hospital
Taichung, Taiwan
E-Mail: chchang@vghtc.vghtc.gov.tw

Abstract *Background* Although fasting and post-methionine loading (PML) homocysteine concentrations are not necessarily related, a high percentage of hyperhomocysteinemia cases would be missed if methionine loading was not performed. *Aim of the study* The influences of B-vitamins and genetic polymorphism (methylenetetrahydrofolate reductase 677C → T, MTHFR 677C → T) on fasting and PML homocysteine concentrations and the relationship between fasting and PML homocysteine were studied. *Methods* This study was a cross-sectional study. Healthy subjects were divided into either fasting hyper-homocysteinemia (≥12.2 μmol/l) (fasting hyper-hcy, $n = 51$), PML hyper-homocysteinemia (fasting homocysteine <12.2 μmol/l but PML homocysteine ≥25.6 μmol/l) (PML hyper-hcy, $n = 29$), or normo-homocysteinemia (fasting homocysteine <12.2 μmol/l and PML homocysteine <25.6 μmol/l) (normo-hcy, $n = 118$) group based on elevated fasting and PML homocysteine levels of the 75th percentile of the population. The concentrations of plasma fasting and PML homocysteine, serum folate, vitamin B-12, plasma pyridoxal 5′- phosphate (PLP) were measured. The genetic polymorphisms were determined. *Results* Fasting homocysteine, but not PML homocysteine and MTHFR 677C → T genotype, was significantly and inversely affected by serum folate concentration after adjusting for potential confounders ($\beta = -0.062$, $P < 0.01$). Fasting and PML homocysteine were highly associated in the fasting hyper-hcy and pooled groups ($P < 0.01$) but not in the PML hyper-hcy and normo-hcy groups. PML homocysteine did not interact with either serum folate ($P = 0.302$), vitamin B-12 ($P = 0.465$), plasma PLP ($P = 0.996$) or MTHFR 677C → T genotype ($P = 0.136$) to affect fasting homocysteine concentration. *Conclusions* Approximately one-third (36.3%) of hyperhomocysteinemia cases would be missed if methionine loading were not performed. Even though subjects may have a normal fasting homocysteine concentration, they need further screening for their PML homocysteine.

Key words folate – vitamin B-12 – vitamin B-6 – gene polymorphism – post-methionine loading – hyperhomocysteinemia

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Introduction

Epidemiological studies and clinical trials have shown that not only fasting hyperhomocysteinemia but also post-methionine loading (PML) hyperhomocysteinemia are significant risk factors for cardiovascular diseases [4, 13, 23, 27, 32]. Homocysteine can be remethylated to form methionine or transsulfurated to form cysteine. In the remethylation pathway, it requires methyltetrahydrofolate as a cosubstrate, 5,10-methylenetetrahydrofolate reductase (MTHFR), a folate-dependent enzyme, catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, and vitamin B-12 is a cofactor. When methionine is in excess, homocysteine is directed to the transsulfuration pathway by cystathionine β-synthase (CBS) and cystathionase, which are both pyridoxal 5′-phosphate (PLP)-dependent enzymes. B-vitamins (i.e., folate, vitamin B-6 and B-12) deficiencies and genetic defects, therefore, might be associated with elevated plasma homocysteine concentration.

Previous studies [6, 18, 34, 35] and ours [16, 17] have indicated that serum folate, vitamin B-12 and/or MTHFR 677C → T mutation significantly correlated with plasma fasting homocysteine concentration. However, there is no consistent evidence showing that plasma PLP, the biologically active form of vitamin B-6, and/or CBS mutation (CBS 844ins68) has a direct relationship with fasting plasma homocysteine concentration. In fact, plasma PLP and CBS mutation were associated with PML homocystiene concentration [5, 7, 8, 29]. Although fasting and PML homocysteine concentrations are not necessarily related, approximately 8.1–55% of hyperhomocysteinemia cases would be missed if methionine loading was not done [3, 13, 20, 28, 32].

To better understand the role that B-vitamins and genetic polymorphism play in fasting and PML homocysteine metabolism, and to examine whether PML homocysteine concentration is independently associated with or involves a synergic effect with B-vitamins and/or genetic polymorphism in the risk of fasting hyperhomocysteinemia, we studied the influences of B-vitamins and genetic polymorphism (MTHFR and CBS) on fasting and PML homocysteine concentrations. In addition, the interaction between B-vitamins, genetic polymorphism and PML homocysteine and the risk of fasting hyperhomocysteinemia was also examined.

Materials and methods

This study was designed as a cross-sectional study. Subjects were recruited from the physical check-up unit of Taichung Veterans General hospital, Taiwan by advertisements. Demographic and health data were collected Exclusion criteria included: no pregnancy, no current illness or history of cardiovascular, liver, renal or other metabolic diseases, diabetes or cancer. Inclusion criteria included: normal blood biochemical values, including fasting blood glucose <6.1 mmol/l (110 mg/dl), blood urea nitrogen (BUN) <7.9 mmol/l (22.1 mg/dl), creatinine <123.8 μmol/l (1.4 mg/dl), alkaline phosphates <190 U/l, glutamic oxoloacetic transaminase (GOT) <35 U/l, and glutamic pyruvate transaminase (GPT) <45 U/l. Informed consent was obtained from each subject. This study was approved by the Institutional Review Board of Chung Shan Medical University.

All subjects' weight and height were measured; the body mass index (BMI; kg/m^2) was then calculated. Blood pressure [systolic blood pressure (SBP), diastolic blood pressure (DBP)] was measured after a resting period of at least 5 min. Fasting venous blood samples were obtained to estimate hematological and vitamin status. Blood specimens were collected in Vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA) containing EDTA as an anticoagulant or no anticoagulant as required. Serum and plasma were prepared within 30 min after the blood was drawn and then stored frozen (−80°C) until analysis. Hematological entities [i.e., BUN, serum creatinine, GOT, GPT, total cholesterol, triacylglycerol, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol] were measured by using an automated biochemical analyzer. Plasma fasting and PML homocysteine concentrations were measured by using high-performance liquid chromatography (HPLC) according to the method of Araki and Sako [1]. The intra- and inter-assays of fasting plasma homocysteine variabilities were 1.41% (n = 5) and 3.78% (n = 6), respectively. L-methionine (100 mg/kg BW) was administered orally after the fasting blood was drawn and a second blood sample was collected 2 h after loading for the determination of PML homocysteine. Serum folate and vitamin B-12 were analyzed by using standard competitive immunochemiluminometric methods on a Chiron Diagnostics ACS: 180 Automated Chemiluminescence System (Chiron Diagnostics Corporation, East Walpole, MA, USA). Plasma PLP was determined by HPLC based on the method of Bates et al. [2], and conducted under yellow light to prevent photodestruction. The intra- and inter-assays of plasma PLP variabilities were 1.47% (n = 5) and 3.12% (n = 17), respectively. All analyses were performed in duplicate.

DNA was extracted from frozen peripheral blood lymphocytes by use of a genomic DNA purification kit (Pharmacia GFX spin column, Piscataway, NJ, USA).

The MTHFR 677C → T gene polymorphism was amplified by polymerase chain reaction. The amplified DNA fragment (198 bp) of MTHFR gene was then digested by the H*in*FI restriction enzyme (New England BioLabs, Ipswich, MA) and subsequent electrophoresis in a 4% agarose gel [36]. The amplified DNA fragment of CBS gene was subjected to polyacrylamide gel electrophoresis, ethidium bromide staining, and UV illumination for detection of the 844ins68 insertion variant [12].

Statistical analyses

Data were analyzed with SigmaStat statistical software (version 2.03; Jandel Scientific, San Rafael, CA). Differences in demographic data and hematological measurements were analyzed by one-way analysis of variance (ANOVA) or Dunn's test among three groups. For categorical response variables, differences among three groups were assessed by Chi-square test. To examine the association of B-vitamins and gene polymorphism with plasma fasting and PML homocysteine concentration, simple and multiple linear regression analyses were performed. Two-way ANOVA was used to test the interaction of B-vitamins and PML homocysteine with fasting homocysteine. Adjusted odds ratios (ORs) with 95% confidence intervals (CI) for fasting hyperhomocysteinemia (≥12.2 μmol/l) were calculated from unconditional logistic regression models. Results were considered statistically significant at $P < 0.05$. Values presented in the text are means ± standard deviation (SD).

Results

Table 1 shows the demographic data and hematological measurements of subjects. Ninety-nine men and 99 women participated in this study. Subjects' age ranged from 25 to 49 years with a mean age of 42.4 ± 5.3 years. Subjects were divided into either fasting hyper-homocysteinemia (fasting homocysteine ≥12.2 μmol/l) (fasting hyper-hcy, $n = 51$), PML hyper-homocysteinemia (fasting homocysteine <12.2 μmol/l but PML homocysteine ≥25.6 μmol/l) (PML hyper-hcy, $n = 29$), or normo-homocysteinemia (fasting homocysteine <12.2 μmol/l and PML homocysteine <25.6 μmol/l) (normo-hcy, $n = 118$) group based on elevated fasting and PML homocysteine levels of the 75th percentile of the population. Therefore, 36.3% (29/80) of subjects had normal fasting homocysteine but high PML homocysteine concentration in our population. There were no significant differences in age, sex, BMI, blood pressure, HDL-C or triacylglycerol among three groups. Total and LDL cholesterol were significantly elevated in the PML hyper-hcy group but not in the other groups. Although subjects in the fasting hyper-hcy group had significantly higher serum creatinine than subjects in the normo-hcy group, the value was within the normal range, indicating a normal renal function.

Table 1 Demographic characteristics and hematological values in normo- and hyper- homocysteinemia groups

Characteristics	Fasting hyper-hcy ($n = 51$)	PML hyper-hcy ($n = 29$)	Normo-hcy ($n = 118$)
Male/female	36/15	17/12	46/72
Age (years)	41.7 ± 5.8	43.2 ± 4.5	42.3 ± 5.5
Body mass index (kg/m^2)	24.7 ± 3.7	24.8 ± 3.3	23.6 ± 3.5
Blood pressure			
Systolic (mmHg)	122.0 ± 20.6	120.7 ± 16.9	115.1 ± 22.3
Diastolic (mmHg)	77.1 ± 13.6	77.9 ± 9.9	73.4 ± 12.7
Cholesterol (mmol/l)			
Total	5.1 ± 1.0b	5.7 ± 1.1a	5.1 ± 1.0b
LDL	3.1 ± 0.9b	3.5 ± 0.9a	3.0 ± 0.8b
HDL	1.4 ± 0.3	1.5 ± 0.4	1.5 ± 0.4
Triacylglycerol (mmol/l)	1.3 ± 0.7	1.5 ± 1.0	1.3 ± 0.9
Serum creatinine (μmol/l)	88.4 ± 17.7a	88.4 ± 17.7a, b	79.6 ± 17.7b

Values are mean ± SD. Values with different letters (a, b) are significantly different between two groups; $P < 0.05$
Fasting hyper-hcy fasting hyperhomocysteinemia, *PML* hyper-hcy, post-methionine loading hyperhomocysteinemia, *Normo-hcy* normohomocysteinemia, *LDL* low-density lipoprotein cholesterol, *HDL* high-density lipoprotein cholesterol

Table 2 shows homocysteine and B-vitamin levels and gene polymorphism in the normo- and hyperhomocysteinemia groups. Three groups were indeed divided according to the variables that differ between the groups. Serum folate and vitamin B-12 levels were significantly lower in the fasting hyper-hcy group when compared with the norm-hcy group; while there was no significant difference in plasma PLP concentration among three groups. With regard to the variants distribution of the MTHFR 677C → T and CBS 844ins68 genotypes, there were no significant differences among three groups. Only two subjects in the normo-hcy group had CBS 844ins68 genotype.

The associations of fasting homocysteine with potential confounders for hyperhomocysteinemia were examined by performing simple regression analyses. The results are listed in Table 3. Fasting homocysteine concentration was significantly and positively associated with gender, HDL-C, serum creatinine, folate, vitamin B-12 and PML homocysteine levels in the pooled group. For the individual group, fasting homocysteine was only significantly associated with PML homocysteine in the fasting and PML hyper-hcy groups; while fasting homocysteine concentration significantly correlated with gender, serum creatinine and vitamin B-12 in the normo-hcy group. Fasting

Table 2 B-vitamins, homocysteine and gene polymorphism in normo- and hyper-homocysteinemia groups

Characteristics	Fasting hyper-hcy (n = 51)	PML hyper-hcy (n = 29)	Normo-hcy (n = 118)
Fasting homocysteine (μmol/l)	15.2 ± 5.3a	9.6 ± 1.6b	9.3 ± 1.6b
PML homocysteine (μmol/l)	24.8 ± 9.5b	31.3 ± 5.4a	18.6 ± 3.5c
Serum folate (nmol/l)	19.7 ± 6.1b	29.7 ± 23.6a	27.2 ± 10.7a
Serum vitamin B-12 (*p*mol/l)	320.2 ± 105.7b	332.1 ± 131.0a, b	388.0 ± 136.7a
Plasma PLP (nmol/l)	47.3 ± 17.0	44.6 ± 17.4	53.9 ± 37.1
MTHFR 677C → T (*n*)			
CC	24	17	74
CT	25	12	41
TT	2	0	3
T allele	27	12	44
CBS 844ins68 (*n*)[d]			
Wild type	51	29	115
Insertion	0	0	2

Values are means ± SD. Values with different letters (a, b, c) are significantly different between two groups; $P < 0.05$
Fasting hyper-hcy fasting hyperhomocysteinemia, *PML hyper-hcy* post-methionine loading hyperhomocysteinemia, *Normo-hcy* normohomocysteinemia, *PLP* pyridoxal 5′-phosphate, *MTHFR* methylenetetrahydrofolate reductase, *CBS* cystathionine β-synthase
[d] There is one missing data in the normo-hcy group

Table 3 Simple linear regression β analysis of fasting plasma homocysteine with potential confounders in individual and pooled normo- and hyper-homocysteinemia groups

Characteristics	Fasting hyper-hcy (n = 51)	PML hyper-hcy (n = 29)	Normo-hcy (n = 118)	Pooled (n = 198)
Age (years)	−0.052	0.064	0.053	−0.005
Gender	1.824	0.035	1.155†	2.316†
Body mass index (kg/m^2)	0.196	0.004	−0.026	0.127
Systolic blood pressure (mmHg)	0.013	0.014	0.004	0.022
Diastolic blood pressure (mmHg)	0.034	0.010	−0.000	0.032
Total cholesterol (mmol/l)	−0.077	−0.039	0.039	−0.077
LDL cholesterol (mmol/l)	−0.039	−0.193	0.193	0.000
HDL cholesterol (mmol/l)	−3.205	−1.081	−0.463	−1.467*
Triacylglycerol (mmol/l)	0.579	0.232	0.039	0.193
Serum creatinine (μmol/l)	0.050	0.005	0.026**	0.075†
Serum folate (nmol/l)	−0.144	−0.019	−0.023	−0.079†
Serum vitamin B-12 (*p*mol/l)	−0.007	−0.003	−0.000*	−0.001*
Plasma PLP (nmol/l)	−0.038	−0.014	0.003	−0.007
PML homocysteine (μmol/l)	0.392†	−0.127*	0.032	0.237†
MTHFR 677C → T genotypes	1.388	−1.093	0.025	0.904

Fasting hyper-hcy fasting hyperhomocysteinemia, *PML hyper-hcy* post-methionine loading hyperhomocysteinemia, *Normo-hcy* normohomocysteinemia, *LDL* low-density lipoprotein cholesterol, *HDL* high-density lipoprotein cholesterol, *PLP* pyridoxal 5′-phosphate, *MTHFR* methylenetetrahydrofolate reductase
* $P < 0.05$, ** $P < 0.01$; † $P < 0.001$

homocysteine was not associated with MTHFR 677C → T genotypes in any groups.

Multiple regression analysis was further performed to study the effect of B-vitamins, homocysteine and gene polymorphism on either fasting or PML homocysteine concentration in the individual and pooled groups (Table 4). Fasting and PML homocysteine were highly associated in the fasting hyper-hcy and pooled groups. Fasting homocysteine, but not PML homocysteine and MTHFR 677C → T genotypes, was significantly and inversely affected by serum folate concentration after adjusting for potential confounders in the pooled group. Serum vitamin B-12 was significantly correlated with fasting and PML homocysteine concentration in the normo-hcy group and additionally correlated with PML homocysteine concentration in the pooled group. Plasma PLP and MTHFR 677C → T genotypes, however, had no effects on fasting and PML homocysteine concentration in the individual and pooled groups.

The risks of fasting hyperhomocysteinemia (Table 5) were calculated by the 75th percentile value of B-vitamins and PML homocysteine based on the distribution of all subjects. Serum folate, PML homocysteine and MTHFR genotypes showed significant associations with the risk of fasting hyperhomocysteinemia after the potential confounders were adjusted. When the other two B-vitamins, PML homocysteine or genotypes were additionally considered in the logistic regression model, only serum folate concentration remained to decrease the risk of fasting hyperhomocysteinemia. Serum folate above

Table 4 Multiple linear regression analysis with either fasting or post-methionine loading homocysteine concentrations as the dependent variable after adjusting the potential confounders

	Fasting hyper-hcy (n = 51) β[a]	PML hyper-hcy (n = 29) β	Normo-hcy (n = 118) β	Pooled (n = 198) β
Serum folate (nmol/l)				
Model 1[b]	−0.175 (−0.425)[e]	−0.011 (0.047)	−0.019 (−0.048)	−0.067** (−0.007)
Model 2[c]	0.007 (−0.394)	−0.001 (0.060)	−0.018 (−0.047)	−0.062** (−0.051)
Serum vitamin B-12 (*p*mol/l)				
Model 1	−0.005 (−0.005)	−0.004 (−0.003)	−0.000* (−0.001*)	−0.001 (−0.003**)
Model 2	−0.003 (−0.001)	−0.005 (0.000)	−0.000* (−0.001**)	−0.000 (−0.003*)
Plasma PLP (nmol/l)				
Model 1	−0.036 (−0.097)	−0.004 (−0.076)	−0.003 (0.009)	−0.014 (−0.027)
Model 2	0.001 (−0.092)	0.019 (−0.086)	−0.002 (0.011)	−0.005 (−0.016)
PML (Fasting) homocysteine (μmol/l)				
Model 1	0.389† (1.259†)	−0.090 (−0.878)	−0.001 (−0.006)	0.209† (0.774††)
Model 2[d]	0.382† (1.200†)	−0.096 (−1.118)	−0.040 (−0.196)	0.199† (0.763†)
MTHFR 677C → T genotypes				
Model 1	1.774 (1.735)	−0.984 (−0.845)	0.037 (−0.229)	0.848 (0.578)
Model 2[d]	0.634 (1.408)	−1.344* (−0.170)	0.008 (−0.244)	0.664 (−0.012)

Hyper-Hcy hyperhomocysteinemia, *Normo-Hcy* normohomocysteinemia, *MTHFR* methylenetetrahydrofolate reductase
[a]β, regression coefficient; * $P < 0.05$; ** $P < 0.01$; † $P < 0.001$
[b]Adjusting for age, gender, high-density lipoprotein cholesterol and creatinine
[c]As for model 1 and additionally adjusting for other two B-vitamins and genotypes
[d]As for model 1 and additionally adjusting for three B-vitamins
[e]The value in the parentheses is when post-methionine loading homocysteine as the dependent variable after adjusting the potential confounders

Table 5 Multivariate adjusted odds ratios for fasting hyperhomocysteinemia

	Factors adjusted[a]			Additional factors adjusted[b]		
	OR	95% CI	*P*	OR	95% CI	*P*
Serum folate						
<29.9 nmol/l	1			1		
≥29.9 nmol/l	0.16	(0.047–0.563)	0.004	0.17	(0.045–0.607)	0.007
Serum vitamin B-12						
<427.3 *p*mol/l	1			1		
≥427.3 *p*mol/l	0.43	(0.169–1.073)	0.070	0.51	(0.189–1.402)	0.194
Plasma PLP						
<52.5 nmol/l	1			1		
≥52.5 nmol/l	0.59	(0.254–1.378)	0.224	0.81	(0.313–2.070)	0.653
PML homocysteine						
<25.6 μmol/l	1			1		
≥25.6 μmol/l	2.15	(1.027–4.511)	0.042	1.73	(0.769–3.901)	0.185
MTHFR genotypes						
CC	1			1		
T-allele	2.07	(1.032–4.142)	0.041	1.88	(0.889–3.991)	0.098

CI 95% confidence interval, *OR* odds ratio, *PLP* pyridoxal 5′-phosphate, *PML* post-methionine loading, *MTHFR* methylenetetrahydrofolate reductase
[a]Adjusting for age, gender, high-density lipoprotein cholesterol and creatinine
[b]Additionally adjusting for B-vitamins, post-methionine loading homocysteine or MTHFR genotypes

29.9 nmol/l decreased the odds ratio of fasting hyperhomocysteinemia by 17% (OR 0.17, 95% CI 0.045–0.607). Serum vitamin B-12, plasma PLP and MTHFR 677C → T genotypes, however, had no associations with the risk of fasting hyperhomocysteinemia. To understand whether PML interacts with B-vitamins or genotypes to affect fasting homocysteine concentration, two-way ANOVA was performed. However, PML homocysteine did not interact with either serum folate ($P = 0.302$), vitamin B-12 ($P = 0.465$), plasma PLP ($P = 0.996$) or MTHFR genotypes ($P = 0.136$) to affect fasting homocysteine concentration.

Discussion

Even though the relationship between fasting or PML hyperhomocysteinemia concentrations and the risk of cardiovascular diseases has been the focus of much research, PML hyperhomocysteinemia is often

ignored in the absence of fasting hyperhomocysteinemia. Our PML hyperhomocysteinemia subjects consisted of 36.3% of total number of hyperhomocysteinemia subjects (fasting plus PML hyperhomocysteinemia), and this percentage was within the range in other studies [3, 13, 20, 28, 32]. The cutoff point for the elevated homocysteine concentration was set at exceeding the 75th percentile of the population in this study. Thus, fasting and PML hyperhomocysteinemia were 12.2 and 25.6 μmol/l, respectively. These two cutoff points were lower than those in previous studies which were either set at the 90th [3, 11, 28], 95th [20, 32] or 97.5th [31] percentile of the population for fasting and PML hyperhomocysteinemia. The 90th and 95th percentile of fasting hyperhomocysteinemia in the present study were 14.4 and 16.3 μmol/l, and of PML hyperhomocysteinemia were 30.7 and 34.8 μmol/l, respectively. Dunkelgrun et al. [9] recently defined hyperhomocysteinemia as a fasting homocysteine level ≥15 μmol/l and PML homocysteine level ≥45 μmol/l for patients with atherosclerotic risk. However, the cutoff point set by the Nutrition Committee of the American Heart Association to define fasting hyperhomocysteinemia is ≥10 μmol/l [19]. Additionally, some studies have shown that fasting homocysteine concentration >12 μmol/l increases the incidence of cardiovascular disease by 5–10% [10, 24, 25]. We, therefore, believe that using 75th percentile (a relatively lower value) as the cutoff point in the general population might have clinical benefit for the prevention of the risk of cardiovascular diseases. Comparable with previous studies [3, 20, 28, 31, 32], however, our study showed that approximately 36.7% (11/30) or 37.5% (6/16) of hyperhomocysteinemia cases would be missed if methionine loading was not done when the cutoff point was set at 90th or 95th percentile in the population, respectively. The percentages are similar to the cutoff point which was set at the 75th percentile. No matter what the cutoff point is, besides factors that contribute to fasting hyperhomocysteinemia, factors in relation to high PML homocysteine concentration should also be taken into consideration.

It has been hypothesized that S-adenosylmethionine (SAM), an activator for the enzyme CBS and an inhibitor for the enzyme MTHFR, specifically regulates remethylation of homocysteine metabolism to methionine during the fasting state [26]; folate deficiency or MTHFR genetic defect thus would affect SAM concentration and fasting homocysteine concentration would be further increased. Therefore, it is not surprising that other studies [5, 30, 35] and ours [16] are in agreement that serum folate and MTHFR 677C → T genotypes result in a significant increase in the risk of fasting hyperhomocysteinemia. However, the effect of the genetic defect (MTHFR 677C → T) on the risk of fasting hyperhomocysteinemia disappeared after folate concentration was considered. Nevertheless, folate is still an independent and significant factor that affects plasma fasting homocysteine concentration.

Although low serum folate plays an important role in determining in fasting homocysteine concentration, it is not related to an abnormal increase in plasma homocysteine after methionine loading. Serum folate concentrations fluctuate rapidly with recent changes in folate intakes while red blood cell (RBC) folate represents body folate stores. Long term (RBC folate) rather than short term (serum folate) folate status might have more influence on PML homocysteine. Since we did not measure RBC folate in this study, the association between RBC folate and PML homocysteine cannot be determined. The correlation was also not observed between two other B-vitamins (i.e., serum vitamin B-12 and plasma PLP) and PML homocysteine concentration. When methionine is in excess, homocysteine will be directed to the transsulfuration. Vitamin B-6 deficiency and/or CBS genetic defect inhibit the transsulfuration and thus increase homocysteine concentration after methionine loading. In the study by Miller et al. [21], homocysteine concentration did dramatically increase after methionine loading in the vitamin B-6 deficient rats, while the folate-deficient rats did not show any significant change in PML homocysteine concentration. However, the present results as well as those of a previous study [5] do not seem to support the hypothesis that low plasma PLP concentration is related to high PML homocysteine concentration. A possible explanation for the lack of any effect of plasma PLP on PML homocysteine concentration might be that our subjects had adequate vitamin B-6 status and thus transsulfuration was not inhibited. As long as a subject has an adequate plasma PLP level, the increased PML homocysteine concentration can not be due to vitamin B-6 status. Besides vitamin B-6 status, CBS defect may also affect the transsulfuration pathway and thus cause the elevation of PML homocysteine concentration. However, a previous study [20] and ours did not observe any relationship between CBS 844ins68 genotype and PML homocysteine. Since only two subjects had the CBS defect in the normo-hcy group, the sample size might be too small to detect any relationship. The relationship between CBS 844ins68 and PML homocysteine concentration needs to be further elucidated.

Although total and LDL cholesterol were significantly elevated in the PML hyper-hcy group when compared to the other two groups, we did not find the association of PML homocysteine concentration with total and LDL cholesterol in the individual and pooled groups (data not shown). A link between increased

fasting and PML homocysteine and lipid profiles has been discussed in previous studies [14, 15, 22, 33]; however, the role of homocysteine plays in the lipid metabolism is intriguing but still highly controversial. An effect of homocysteine on lipid metabolism is clinically important and should be further studied.

The limitation of this study was that the study subjects were a healthy population; therefore, it was not possible to study the interaction and magnitude effect of fasting and PML homocysteine concentrations on the risk of cardiovascular diseases. Fasting and PML homocysteine concentrations were only related in the fasting hyper-hcy group. Even though subjects may have a normal fasting homocysteine concentration, they may need further screening of their PML homocysteine.

In conclusion, fasting homocysteine concentration alone could not identify all hyperhomocysteinemia cases. Thus, over one-third of hyperhomocysteinemia cases would be missed if methionine loading was not performed. No interaction of the PML homocysteine concentration with any of B-vitamins had an effect on the risk of fasting hyperhomocysteinemia.

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