
Biological Determinants of Fasting and Postload Plasma Homocysteine Levels in Healthy Italian Subjects

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ABSTRACT

Genetic and nutritional factors are determinants of total plasma homocysteine (tHCy) whose increased levels play a pathogenic role in atherothrombosis and cardiovascular disease.

This study investigated the influence of sex, age, creatinine, serum folate, vitamin B12 and vitamin B6 (pyridoxal-5-phosphate, PLP) on fasting (FtHCy) and two hour postmethionine loading levels of tHCy in 147 apparently healthy subjects (M/F= 82/65, age range: 14-94 y).

FtHCy was higher in men than women (9.89 vs 8.00 $\mu\text{mol/L}$, $p < 0.01$). In males, the main determinants of FtHCy were age and folate levels, respectively explaining 20.5% and 19.0% of FtHCy variance. In women, besides age (22.6%), vitamin B12 (17.8%) rather than folate was a major determinant of FtHCy.

Two hour postload tHCy, expressed both as absolute value (PML) and as the difference between 2 hour postload tHCy and FtHCy (delta tHCy) was negatively correlated with folate in both sexes, and with vitamin B12 and age in women only. In males folate was the main determinant, explaining 20% of the variance of postload values, while in females vitamin B12 and PLP were predominant, explaining respectively 40% and 20% of that variance. Age was not among the main determinants of postload values in either sex. These results demonstrate that age and vitamin status differently influence both the fasting and the postmethionine plasma homocysteine levels in normal subjects.

Key Words: Homocysteine, Folate, Vitamin B12, Vitamin B6.

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INTRODUCTION

Homocysteine (HCy) is a sulfur-containing amino acid originated from demethylation of dietary methionine. The metabolism of HCy involves remethylation

and transsulfuration pathways. The remethylation pathway requires vitamin B12, as a cofactor of methionine synthase (EC 2.1.1.13), and folates as coenzymes. The transsulfuration pathway requires vitamin B6 [PLP (pyridoxal-5'-phosphate)], as a cofactor for

cystathionine β -synthase (EC 4.2.1.22) and cystathionine-g-lyase (EC 4.4.1.1). Genetic disorders and vitamin deficiencies can lead to increased concentrations of tHCy in plasma^[1-5]. Elevated tHCy plasma levels have been shown to have a causal role in the development of atherothrombosis and consequent cardiovascular disease^[6-8].

Fasting total plasma homocysteine (FtHCy) concentration has been shown to be a function of age, gender and vitamin status in healthy subjects by several Authors^[3,9-11].

The methionine-load (ML) test is a measure of tHCy concentrations after ingestion of a single dose of methionine (0.1 g/kg body wt.)^[12]. In these conditions increased tHCy synthesis occurs, thus challenging the activity of the tHCy catabolic transsulfuration pathway. ML is a screening test for detection of subjects heterozygous for cystathionine β -synthase deficiency. These subjects may have normal FtHCy but their tHCy levels may increase after ML^[13,14].

Few authors have evaluated the factors of variability of the response to a methionine load in normal subjects^[15,16]. Some data are available on the influence of folate, vitamin B12 and PLP on the response to L-methionine load in different geographical areas. The purpose of our study was therefore to evaluate the determinants of fasting and postmethionine load tHCy with special regard to age, sex, creatinine, folate, vitamin B12 and PLP.

MATERIALS and METHODS

Subjects and study criteria: We investigated 147 subjects (M/F= 82/65, age range: 14-94 y, median: M/F= 27.3/28.4 y) who volunteered from the staff working at S. Orsola-Malpighi Hospital of Bologna, Italy, and from the medical students. They were all living in the area of Bologna, northern Italy. All subjects gave their informed consent to participate in the study. The study subjects were selected according to the following criteria: Clinically healthy and free of overt disease; no history of metabolic disorders; no clinical symptoms or electrocardiographic signs of cardiovascular disease; no hypertension (either treated or untreated). Exclusion criteria were the following: Age < 15 years, known

thyroid, liver or kidney disease, diabetes or glucose intolerance, lipid disorder, gout, obesity [body mass index (BMI): ≤ 30 kg/m²], alcoholism, chronic disorders requiring medication, pregnancy and regular intake of multivitamin supplements. A standardized interview was conducted by trained personnel with regard to smoking, physical activity, use of alcohol.

Investigational procedure: After an overnight fast at 8.00 a.m., blood samples for measurement of tHCy and PLP in plasma were collected into tubes containing 4.4 mmol/L ethylenediaminetetraacetic dipotassium salt (EDTA 2K⁺) and immediately placed on ice in the dark. Platelet poor plasma (PPP) was then separated within 1 hour by centrifugation at 3000 x g for 20 min at 4°C and multiple 0.8 mL aliquots were stored at -80°. Blood samples for measurements of folate, creatinine and vitamin B12 in serum were collected into empty glass tubes.

In the subgroup of 97 patients who underwent the ML test, L-methionine (0.1 g/kg b.w) was administered orally in about 200 mL of fruit juice, followed by a light, standardized breakfast (coffee or tea and aprotic dry cookies). L-methionine, for human oral-use, was purchased by ACEF (Piacenza, Italy).

Blood sampling for postload tHCy was performed two and/or four hours after methionine administration.

Validation of abbreviate oral methionine loading test: Among the ML test subjects, 39 volunteered in a pilot study aimed at validating a 2 hour ML test versus the classical 4 hour test. In these subjects a blood sample was collected for determination both 2 and 4 hours after ML.

Laboratory analyses: tHCy concentrations were measured by high performance liquid chromatography (HPLC) according to method of Araki and Sako modified by Sassi et al (submitted)^[17]. The plasma PLP concentration was measured by HPLC according to Sassi et al^[18]. The concentration of tHCy and PLP were both calculated from the peak-area by applying the method of external standard. Serum folate and vitamin B12 were determined by using a commercial automated chemiluminescence assay system ACS-100 (Chiron Diagnostics, East Walpole, MA, USA). Serum creatinine was determined by a commercial automated as-

say according to the method of Jaffè (Roche Diagnostics; Indianapolis, IN, USA). tHCy after ML was expressed as the following:

1. Absolute increment of FtHCy [absolute postmethionine bad tHCY levelus (PML)];
2. Absolute difference between PML and FtHCy (Delta tHCy);
3. Percentage difference over FtHCy, (%Delta tHCy: [(PML-FtHCy) x 100/FtHCy]).

Statistical analyses: Log transformations were used for skewed variables and these data are presented as geometric means (GM) and 95% confidence intervals.

Pearson's *r* and Spearman's correlation coefficient were calculated to analyze the relation between 2 and 4 hour PML tHCy.

Statistical analysis of tHCy, BMI, age and vitamin concentrations was performed using log-transformed data. Group means were compared by the t-test. Correlation between variables are reported as Pearson's *r* or Spearman's coefficients. Separate stepwise multivariate regression analyses were performed for males and females. A two-sided 5% level of significance was considered significant for all statistical test; exact probability values are reported down to $p < 0.01$. Data were analyzed by using the Statistical Package SOLO (BMDP, Statistical Software Los Angeles, CA, USA).

RESULTS

Validation of the 2 hour methionine loading test:

Figure 1 shows the correlation of PML tHCy after 2 hours and after 4 hours in 39 subjects (M/F= 15/14). The 2 h tHCy concentration accounted for the 88% of the variability in the 4 hour tHCy concentration.

The correlation coefficients between delta tHCy at 2 hours (2h value, $t = 2$, minus the fasting value, $t = 0$) and delta tHCy at 4 hours (4 h value, $t = 4$, minus the fasting value, $t = 0$) for the same subjects ($n = 39$) were: $r = 0.93$, Pearson's; $r = 0.89$ Spearman's (data not shown).

On the basis of these results, for further calculations we considered the 2 h test in all 97 subjects submitted to methionine-loading.

Characteristics of subjects and fasting total plasma homocysteine and vitamin levels:

The biological cha-

acteristics, FtHCy and plasma vitamin concentrations for the whole group ($n = 147$) of study participants and separately by sex are shown in Table 1. BMI and creatinine were significantly higher in men than women ($p < 0.02$, $p < 0.01$, respectively). Current or former cigarette smoking was reported in 29% men and 33% of women (data not shown). Creatinine and PLP concentrations were determined only in 83 subjects; lack of plasma aliquots accounted for the missing values. At baseline, tHCy was significantly lower in women than in men ($p < 0.01$). Mean folate was slightly higher, and PLP and vitamin B12 slightly lower in women, but the differences were not statistically significant. The range of normality for PLP concentrations was calculated from this population and was 11.7-43.4 nmol/L (10th and 90th percentile).

Results of postmethionine load test: In the 97 subjects submitted to the 2 hours ML-test, PML tHCy and delta tHCy were not significantly different according to gender (Table 2). Only the difference expressed in percent of baseline value (% delta tHCy) was significantly higher in women than men.

Distribution of fasting and postload plasma total homocysteine and vitamins: The frequency distributions of FtHCy and PML tHCy are shown in Figure 2. The concentrations of FtHCy, covered a similarly wide range in men and women, but proportionately more women than men had low values. PML tHCy covered a similarly wide range in men and women, but proportionately more women than men had low values.

The frequency distribution of age, BMI, folate, PLP and vitamin B12 in men and women were also skewed (data not shown).

Correlates of fasting total homocysteine: Correlation coefficients between log FtHCy and other measured traits are shown in Table 3. Significant positive correlation was found between FtHCy and age both in the whole group and separately in men and in women. FtHCy was overall negatively correlated with PLP, vitamin B12 and folate, but not with PLP in women alone.

Smoking status, alcohol intake and activity level were not found to have any significant effect on tHCy concentrations (data not shown).

Age was negatively correlated with PLP, vitamin

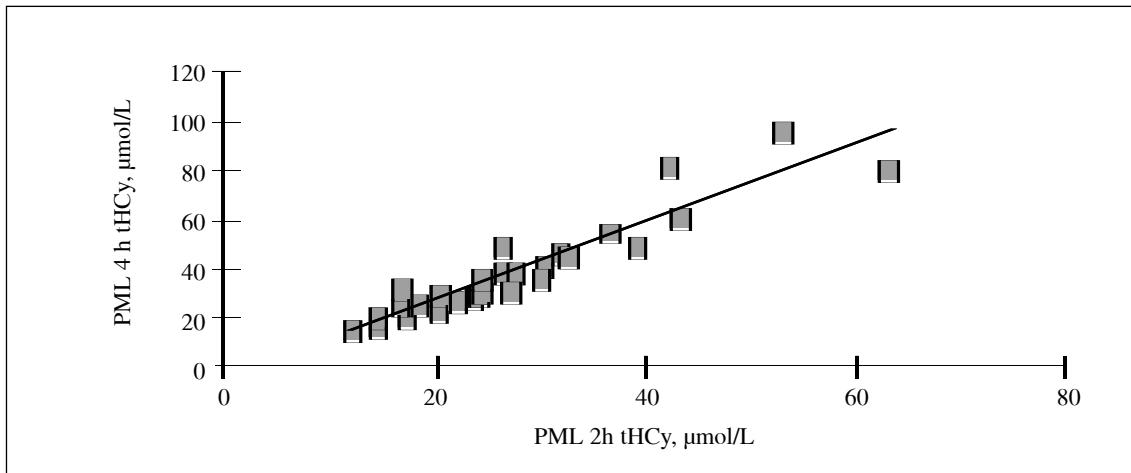


Figure 1. Correlation of 4 hour postmethionine-load vs 2 hour postmethionine-load tHcy concentrations (n= 39). Linear regression line equation: $y = 1.6238 x - 7.307$. Pearson's $r = 0.944$, Spearman's $r = 0.945$.

B12 and folate levels in the whole group (data not shown). By gender separation the correlations were confirmed in either sex, except for age with folate levels in women.

Within vitamins, the only significant correlation observed was between folate and vitamin B12 (Pearson's $r = 0.257$, $p < 0.01$; $r = 0.393$; $p < 0.01$) in the whole group and in women, but not separately in men (da-

Table 1. Characteristics of healthy subjects

	All (14-94 y) n= 147		Men (14-94 y) n= 82		Women (14-94 y) n= 65	
	Median	GM (95% CL)	Median	GM (95% CL)	Median	GM (95% CL)
Age (y)	27.50	31.93 (28.84-34.58)	28.40	32.41 (28.57-36.65)	27.30	31.32 (27.80-35.97)
BMI (kg/m ²)	23.74	24.26 (23.55-24.44)	25.26	25.02 (24.65-25.59)	22.66	23.33 * (22.92-23.97)
Creatinine (mg/dL)	0.80	0.76 (0.71-0.81)	0.86	0.82 (0.74-0.90)	0.70	0.69 ** (0.64-0.75)
FtHcy (μmol/L)	8.60 (147)	9.00 (8.30-9.57)	9.43 (82)	9.89 (9.05-11.05)	7.80 (65)	8.00 *** (7.26-8.69)
PLP (nmol/L)	29.80 (83)	29.06 (25.11-33.13)	30.40 (46)	32.13 (26.86-38.99)	28.80 (37)	25.64 (22.10-29.90)
Folate (ng/mL)	5.30 (147)	5.09 (4.83-5.44)	5.05 (82)	4.97 (4.63-5.43)	5.50 (65)	5.26 (4.80-5.74)
Vitamin B12 (pg/mL)	395.00 (145)	407.38 (383.71-432.51)	399.00 (81)	410.41 (376.01-441.37)	394.50 (64)	402.57 (363.08-435.71)

GM: Geometric mean; n: Number of subjects observed. 95% CL= 95% confidence limits.

PLP (pyridoxal-5'-phosphate); FtHcy (fasting total plasma homocysteine).

* Significantly different from men: $p < 0.02$, Student's t-test.

** Significantly different from men: $p < 0.01$, Student's t-test.

*** Significantly different from men: $p < 0.01$, Student's t-test.

ta not shown).

Correlates of total homocysteine after methionine load: Correlation coefficients between log PML and log delta tHCy and other measured traits are shown in Table 4. Age positively correlated with PML and delta tHCy in women only. No significant correlations were found for BMI and creatinine. A significant negative correlation of PML and delta tHCy was observed with folate, in both sexes. A significant negative correlation of both PML and delta tHCy with vitamin B12 and PLP was observed only in women. % delta was correlated with age in men only (data not shown).

The correlation between FtHCy and PML tHCy loading in all subjects was also calculated (data not shown). Significant correlation was observed between FtHCy and PML tHCy ($r=0.79$, $p<0.0001$) and between FtHCy and delta tHCy ($r=0.405$; $p<0.0001$).

Multiple regression analysis for fasting and postload methionine levels: Parameters with significant correlation with FtHCy were entered as independent variables into a backward multiple stepwise regression analysis. Table 5 presents the results, separately in men and women. Age appears the major determinant of FtHCy levels in both sexes and explained 20.5% and 22.6% of FtHCy variance in men and women respectively. The second major determinant was different according to gender: Folate accounted for 19.0% of FtHCy variance in men only, while vitamin B12 accounted for 17.8% of FtHCy variance but in women only. The influence of creatinine (5.3% and 7.7%), as well as PLP contribution were small and nonsignificant in either sex.

A similar procedure was used for absolute postload tHCy values (PML, Table 6). Table 6 presents the stepwise variable selection in men and women. Age had no determinant influence on postload values

(PML) in either sex. In men, folate levels explained 19.9% of PML variance, with no significant effect of PLP, creatinine and vitamin B12. In women vitamin B12 explained a great proportion of the PML variance (41.2%), followed by PLP (19.0%) and creatinine (14.6%), while a nonsignificant effect was found for folate. Similar results were obtained for delta tHCy (data not shown). However, for % delta tHCy, age explained the major percentage of variance in both sexes (data not shown).

DISCUSSION

We studied a group of healthy subjects in the area of Bologna, Italy and we determined fasting and postmethionine loading tHCy concentrations and their correlations with clinical and biological determinants.

Our results confirm the influence of age and sex on fasting tHCy concentrations observed by other investigators in European populations^[15,16,19]. Age was correlated negatively with vitamins and the increase of tHCy observed with increasing age is possibly due to a reduced efficiency of metabolic pathways^[9-11]. Little is known about other factors that affect Hcy metabolism with advancing age.

Women had significantly lower FtHCy concentrations than men. The sex difference in FtHCy has been ascribed to various factors. These include different rates of Hcy generation, the presence in women of a smaller muscle mass, limited creatine phosphate synthesis, and lowering effect of estrogens on homocysteine levels^[15,19-21]. It is however known that other conditions may increase FtHCy levels in elderly women, such as declining renal function, a critical factor for Hcy metabolism, and estrogens decrease after menopause^[6,22-24].

In our multiple regression analysis, after age, the second major determinant of FtHCy variance was folate

Table 2. Concentrations of tHCy after ML-test

	All subjects (n= 97)		Men (n= 53)		Women (n= 44)	
	Median	GM	Median	GM	Median	GM
PML (µmol/L)	22.10	22.16	22.40	23.40	21.60	20.75
Delta tHCy (µmol/L)	12.20	12.21	12.20	12.43	12.25	11.95
% delta tHCy	137.21	129.40	131.91	118.38	149.66	144.05 *

Delta tHCy: FtHCy-PML; % delta tHCy: [(PML-FtHCy) x 100/FtHCy].

* Significantly different from men ($p<0.05$, t-test).

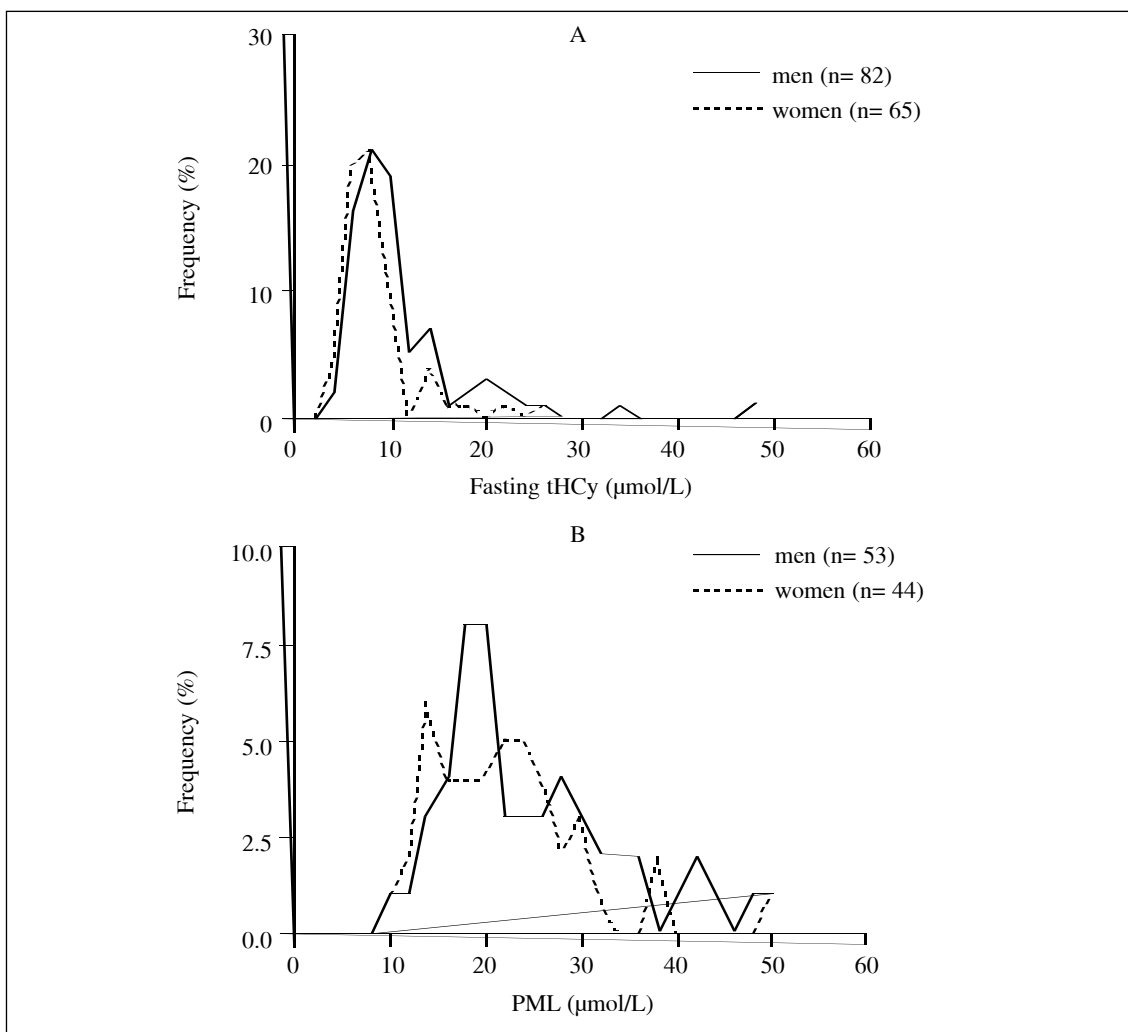


Figure 2. Panel A: Frequency distribution of Fasting tHcy concentration in healthy subjects by sex. The 90th and the 95th percentiles for tHcy were, 19.45 and 22.15 $\mu\text{mol/L}$, respectively, in men and 13.98 and 15.68 $\mu\text{mol/L}$, respectively, in women. Panel B: Frequency distribution of tHcy after ML-test, expressed as absolute increment (PML), in healthy subjects by sex. The 90th and the 95th percentiles for PML were respectively, 33.81 and 41.88 $\mu\text{mol/L}$ in men and 30.46 and 37.48 $\mu\text{mol/L}$ in women.

te in men (and not in women) and vitamin B12 in women (but not in men). This gender-related difference in the influence of vitamin B12 versus folate on FtHcy has been observed by other authors, but it remains partially unexplained^[19,24]. Men may have a higher folate requirement than women probably in relation with the different estrogen status^[23]. Estrogens seem in fact to have an up-regulatory effect on the hepatic enzyme betaine: Homocysteine methyltransferase, another pathway in Hcy remethylation^[20]. Verhoef et al observed

that this hormonal effect is mediated chiefly through effects on muscle mass^[16]. Mudd and Poole suggested that the methionine cycle may differ between men and women because of different demands for labile methyl groups^[25]. The difference between men and women may be related to the stoichiometric formation of Hcy in connection with higher levels of creatinine synthesis in proportion to the greater muscle mass in men. It was also surmised that more rapid cycling in women may result in a greater proportion of Hcy being diverted to

Table 3. Correlation between log₁₀ fasting tHCy (FtHCy) and biological variables in men and women

Variables	FtHCy								
	All subjects (n= 147)		Men (n= 82)		Women (n= 65)				
	r	p	r	p	r	p			
Log ₁₀ age	0.347	0.0000	0.389	0.0003	0.289	0.0194			
Log ₁₀ BMI	0.169	0.1338	a	0.190	0.2090	b	-0.003	0.9881	c
Log ₁₀ creatinine	0.193	0.0964	a	0.244	0.1839	b	0.025	0.8919	c
Log ₁₀ PLP	-0.241	0.0277	a	-0.427	0.0034	b	-0.187	0.2661	c
Log ₁₀ folate	-0.472	0.0000		-0.495	0.0000		-0.439	0.0002	
Log ₁₀ vitamin B12	-0.403	0.0000		-0.412	0.0001		-0.445	0.0002	

r Pearson's; n in parentheses indicate the number of subjects observed.

^a n= 83 subjects; ^b n= 46 Men; ^c n= 37 women.

Table 4. Correlation between log₁₀ total homocysteine after ML-test (expressed as PML and delta tHCy) and biological variables in men and women

Variables	PML				Delta tHCy			
	Men		Women		Men		Women	
	r	p	r	p	r	p	r	p
Log ₁₀ age	0.2041	0.1688	0.4959	0.0011	-0.0557	0.7101	0.4398	0.0045
Log ₁₀ BMI	0.3159	0.1326	0.2207	0.3788	0.1781	0.4051	0.1220	0.6296
Log ₁₀ creatinine	-0.0316	0.8863	0.1531	0.5441	-0.0515	0.8155	0.0526	0.8357
Log ₁₀ folate	-0.5042	0.0003	-0.4356	0.0050	-0.3316	0.0228	-0.3742	0.0174
Log ₁₀ vitamin B12	-0.0892	0.5554	-0.4460	0.0039	0.0005	0.9976	-0.4085	0.0089
Log ₁₀ PLP	0.1486	0.4785	-0.5188	0.016	0.2490	0.2310	-0.4928	0.0232

Men n= 46; women n= 41; r Pearson's; delta tHCy = FtHCy-PML.

cystathionine^[26,27]. In our study, the influence of creatinine did not reach statistical significance, possibly also because of the missing values.

After methionine loading, PML tHCy and delta tHCy were higher in men than in women, although the difference was not statistically significant. This result cannot be attributed to different body size (weight or body surface area) as suggested by some authors, as it was not correlated with BMI^[15,28]. It is likely that differing body composition (greater proportion of body weight as fat in women) could account for this feature.

Only when the postload values are expressed as the percent rise in tHCy levels (% delta tHCy) higher figure can be found in women than in men, as seen also by other Authors^[15,16]. However, the meaning of this find-

ing is doubtful.

Silberberg et al and Verhoef et al indicate that in future research it may be advisable to standardize the methionine dose to lean body mass rather than to body weight and protocols based on lean body mass are therefore worthy of further study^[15,16].

Coming now to correlations of the postload values, we found a significant correlation of PML and delta tHCy with age only in women. This finding could be attributed to the age-related differences in estrogen effects^[28]. Several investigators have drawn attention to the need to consider age and gender differences in the response to ML test^[14,15,27-29]. However, in our stepwise regression analysis then was no significant influence whatsoever of age on postload values in either

Table 5. Percentage of log fasting total homocysteine (FtHCy) sample variance explained by biological traits in a multiple-stepwise-regression model*

Variables	Men (n= 82)		FtHCy		Women (n= 65)	
	Percentage explained	p*		Percentage explained	p*	
Log ₁₀ age	20.5%	0.0007		22.6	0.0027	
Log creatinine	5.3%	n.s.	a	7.7	n.s.	b
Log ₁₀ PLP						
Log ₁₀ folate	19.0%	0.0010				
Log ₁₀ vitamin B12				17.8%	0.007	

^a n= 46 men; ^b n= 37 women.

* Model included: Age, creatinine, PLP, folate and vitamin B12.

Table 6. Percentage of log total homocysteine after ML-test sample variance explained by biological traits in multiple-stepwise-regression models*

Variables	Men (n= 46)		PML		Women (n= 41)	
	Percentage explained	p*		Percentage explained	p*	
Log ₁₀ age						
Log ₁₀ creatinine				14.6%	0.04	
Log ₁₀ folate	19.9%	0.03				
Log ₁₀ vitamin B12				41.2%	0.002	
Log ₁₀ PLP	3.6%	n.s.		19.0%	0.02	

* Model included: Age, creatinine, PLP, folate and vitamin B12.

sex.

With regard to associations of vitamin status and postmethionine load tHCy levels, our data show that while folate levels are determinant for postload values in males, vitamin B12 and PLP levels are determinant in females. It was seen suggested that the transsulfuration pathway could be less efficient and more dependent on vitamin B6 and vitamin B12 in females^[15,29]. Moreover, it has been also surmised that the vitamin B12 dependent remethylation pathway, activated by the transient postload increment in tHCy, could be more important in females than in males^[21].

Recently many investigators have recommended that reference ranges for tHCy be established in populations with apparently adequate vitamin status^[30-33]. However, so far the majority of authors have provided reference ranges without taking into account the biolo-

gical determinants of HCy. Normal ranges vary widely in different populations, since they are affected by several lifestyle determinants. Some have suggested that tHCy should be considered optimal or normal only in the case of an optimal supply and levels of folate and vitamin B12^[24]. Regarding the vitamin status, our data emphasize the predominant influence, on the fasting and postload homocysteine levels, of folate in males and vitamin B12 and, to some extent, vitamin B6 in females. Thus, our observations confirm that differences in age and gender should not be taken into account in giving "normal" reference ranges of fasting and postload homocysteine levels.

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S. Sassi designed the study, was responsible for the HPLC methods and HPLC analysis, data management and wrote the paper. BC designed the study, was res-

possible for data management and revised the manuscript. G P and CL supervised and recruited the participants. GG was responsible for the HPLC methods and HPLC analysis; M was responsible for statistical analysis. SC senior investigator, revised the manuscript and gave final approval for its submission.

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Abbreviations

HCy: Homocysteine,

tHCy: Total plasma homocysteine,

FtHCy: Fasting total plasma homocysteine,

PML: Absolute post-methionine load tHCy levels,

Delta tHCy: Absolute increment over FtHCy (PML-FtHCy);

%Delta tHCy: As percentage difference over FtHCy [(PML-FtHCy) x 100/FtHCy],

PLP: Pyridoxal-5'-phosphate,

BMI: Body mass index.

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