Influence of age, sex and vitamin status on fasting and post-methionine load plasma homocysteine levels

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Background and Objectives. To investigate the effects of age, sex and vitamin status on total plasma homocysteine (tHCy), both after fasting (FtHCy) and two hours post-methionine load (PML-tHCy). The secondary aim was to determine the reference values for FtHCy and PML-tHCy.

Design and Methods. A cohort of apparently healthy volunteers underwent blood sampling for FtHCy, PML-tHCy, creatinine, serum folate, vitamin B12 and vitamin B6 (pyridoxal-5-phosphate, PLP).

Results. In 147 subjects (M/F = 82/65, age range: 14-94 years), FtHCy was significantly higher in men than in women. In men, age and folate levels explained 20.5% and 19.0% of FtHCy variance, respectively. In women, age and vitamin B12 accounted for 22.6% and 17.8% of FtHCy variance, respectively. PML-tHCy was similar in men and women. PML-tHCy was negatively correlated with folate in both sexes, and with vitamin B12 and age in women only. Folate accounted for 20% of the variance of PML-tHCy in men, while in women vitamin B12 and PLP explained 40% and 20% of variance of PML-tHCy, respectively. The reference values of FtHCy and PML-tHCy were: 19.63 and 40.18 μmol/L, respectively, for men under 45 years, 14.26 and 28.31 μmol/L, respectively, for men above 45 years, 28.38 and 36.48 μmol/L for women under 45 years, and 22.49 and 44.06 μmol/L for women above 45 years.

Interpretation and Conclusions. Age, gender and vitamin status influence both FtHCy and PML-tHCy in normal subjects. Reference values should be calculated according to age and sex.
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Key words: homocysteine, folate, vitamin B12, vitamin B6, risk factor, cardiovascular disease.

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Design and Methods

Subjects and study criteria
We investigated healthy volunteers from among the staff working at S. Orsola-Malpighi Hospital of Bologna, Italy, and from among 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 less than 14 years, known thyroid, liver or kidney disease, diabetes or glucose intolerance, lipid disorder, gout, obesity (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, and use of alcohol.

Materials
L-methionine for human oral use, was purchased from ACEF (Piacenza, Italy). All other chemicals were of analytical reagent grade. Ethylenediaminetetracetic dipotassium salt (EDTA 2K+) was obtained from Carlo Erba (Milan, Italy).

Commercial chemiluminescence assays for vitamin B12 and folate were obtained from Chiron Diagnostics, East Walpole, MA, USA.

Investigational procedure
Subjects were asked to fast from midnight. At 8.00 a.m. blood samples for measurement of tHCy and PLP in plasma were collected into tubes containing 4.4 mmol/L EDTA 2K+ and immediately placed on ice in the dark. Platelet-poor plasma (PPP) was then separated within 1 hour by centrifugation at 3000 × g for 20 min at 4°C and multiple 0.8 mL aliquots were stored at –80°C. Blood samples for measurements of folate, creatinine and vitamin B12 in serum were collected into empty glass tubes. The ML test was carried out in a subgroup of 97 subjects. 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 protein-free dry cookies). Blood sampling for post-load tHCy was performed two hours after methionine administration.

Validation of the abbreviated oral methionine loading test
Among the ML test subjects, 39 volunteered for a pilot study aimed at validating a 2-hour ML test and comparing it with the 4-hour test. In these subjects blood samples were 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 Sako19 modified by Sassi et al. (personal communication). The plasma PLP concentration was measured by HPLC according to Sassi et al.20 The concentrations of HCy and PLP were both calculated from the peak-area by applying the method of external standard. Serum folate and vitamin B12 were determined using a commercial automated chemiluminescence assay system ACS-100 (Chiron Diagnostics, East Walpole, MA, USA). Serum creatinine was determined by a commercial automated assay (Roche Diagnostics; Indianapolis, IN, USA). After the ML, tHCy was expressed as the following: 1) absolute increment of FtHCy (PML-tHCy); 2) absolute difference between PML and FtHCy (ΔtHCy); 3) percentage difference over FtHCy, (%ΔtHCy: [(PML-FtHCy) × 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 and Spearman’s correlation coefficients were calculated to analyze the relation between 2- and 4-hour PM-L-tHCy.

Statistical analysis of tHCy, BMI, age and vitamin concentrations was performed using log-transformed data. Group means were compared by the t-test. Correlations between variables are reported as Pearson’s or Spearman’s coefficients. Separate stepwise multivariate regression analyses were performed for men and women. A two-sided 5% level of significance was considered significant for all statistical tests; exact probability values are reported down to p<0.01. Reference values were expressed as 0.975 fractile of the reference distribution with 90% confidence intervals as indicated by the International Federation of Clinical Chemistry.21 Data were analyzed using the Statistical Package SOLO (BMDP, Statistical Software Los Angeles, CA, USA).

Results
Characteristics of subjects and biological determinants of fasting total homocysteine
We evaluated 147 apparently healthy subjects (M/F = 82/65, age range: 14-94 years, median: M/F = 27.3/28.4 years). The biological characteris-
tics, FtHCy and plasma vitamin concentrations for the whole group of study participants and separately by sex are shown in Table 1. BMI and creatinine were significantly higher in men than in 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 was the reason for the missing values. At baseline, FtHCy 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.

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-hour tHCy concentration accounted for 88% of the variability in the 4-hour tHCy concentration.

The correlation between ∆tHCy at 2 hours (2-hour value, t=2, minus the fasting value, t=0) and ∆tHCy at 4 hours (4-hour value, t=4, minus the fasting value, t=0) for the same subjects (n=39) were: Pearson’s r =0.93 and Spearman’s r= 0.89 (data not shown).

On the basis of these results, for further calculations we considered the 2-hour results in all 97 subjects who underwent the ML test.

Results of the post-methionine load test

In the 97 subjects who underwent the 2-hour ML test, PML-tHCy and ∆tHCy were not significantly different according to gender (Table 2). Only the difference expressed in percent of baseline value (%∆tHCy) was significantly higher in women than in men.

Distribution of fasting total homocysteine and post-load plasma total homocysteine and vitamins

The frequency distributions of FtHCy and PML-tHCy are shown in Figure 2. The concentrations of both FtHCy (panel A) and PML-tHCy (panel B) covered a similarly wide range in men and women with a skewed distribution to the right. The frequency distribution was significantly different between men and women only for FtHCy (p<0.05).

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

Table 1. Characteristics of healthy subjects.

<table>
<thead>
<tr>
<th></th>
<th>All (14-94 y) n=147</th>
<th>Men (14-91 y) n=82</th>
<th>Women (14-95 y) n=65</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong></td>
<td>27.50</td>
<td>28.40</td>
<td>27.30</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>27.34</td>
<td>25.26</td>
<td>22.66</td>
</tr>
<tr>
<td><strong>Creatinine (mg/dL)</strong></td>
<td>0.80</td>
<td>0.86</td>
<td>0.70</td>
</tr>
<tr>
<td><strong>FtHCy (mmol/L)</strong></td>
<td>8.60</td>
<td>9.43</td>
<td>7.80</td>
</tr>
<tr>
<td><strong>PLP (nmol/L)</strong></td>
<td>29.80</td>
<td>30.40</td>
<td>28.80</td>
</tr>
<tr>
<td><strong>Folate (ng/mL)</strong></td>
<td>5.30</td>
<td>5.05</td>
<td>5.05</td>
</tr>
<tr>
<td><strong>Folate (nmol/L)</strong></td>
<td>12.00</td>
<td>11.44</td>
<td>12.46</td>
</tr>
<tr>
<td><strong>Vitamin B12 (gg/mL)</strong></td>
<td>395.00</td>
<td>399.00</td>
<td>394.50</td>
</tr>
<tr>
<td><strong>Vitamin B12 (pmol/L)</strong></td>
<td>291.43</td>
<td>294.38</td>
<td>291.06</td>
</tr>
</tbody>
</table>

**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; °significantly different from men p<0.01; §significantly different from men p<0.01; #values of 83 subjects: 46 men and 37 women.
Correlates of fasting total homocysteine
Correlation coefficients between log \( \text{FtHCy} \) and other measured traits are shown in Table 3. Significant positive correlations were found between \( \text{FtHCy} \) and age both in the whole group and separately in men and in women. \( \text{FtHCy} \) 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 \( \text{tHCy} \) concentrations (data not shown).

Age was negatively correlated with PLP, vitamin B12 and folate levels in the whole group (data not shown). Following gender separation, the correlations were confirmed in both sexes, 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 (data not shown).

Correlates of total homocysteine after methionine load
Correlation coefficients between log \( \text{PML-tHCy} \) and log \( \Delta \text{tHCy} \) and other measured traits are shown in Table 4. Age positively correlated with \( \text{PML-tHCy} \) and \( \Delta \text{tHCy} \) in women only. No significant correlations were found for BMI and creatinine. A significant negative correlation of \( \text{PML-tHCy} \) and \( \Delta \text{tHCy} \) with folate, in both sexes. A significant negative correlation of both \( \text{PML-tHCy} \) and \( \Delta \text{tHCy} \) with vitamin B12 and PLP was observed only in women. \%\( \Delta \text{tHCy} \) was correlated with age in men only (data not shown). The correlation between \( \text{FtHCy} \) and \( \text{PML-tHCy} \) loading in all subjects was also calculated (data not shown). Significant correlations were observed between \( \text{FtHCy} \) and \( \text{PML-tHCy} \) (\( r=0.79; \ p<0.0001 \)) and between \( \text{FtHCy} \) and \( \Delta \text{tHCy} \) (\( r=0.405; \ p<0.0001 \)).

Multiple regression analysis for fasting and post-load methionine levels
Parameters with significant correlation with \( \text{FtHCy} \) were entered as independent variables into a backward multiple stepwise regression analysis. Table 5 presents the results, separately in men and women. Age was the major determinant of \( \text{FtHCy} \) levels in both sexes and explained 20.5% and 22.6% of \( \text{FtHCy} \) variance in men and women, respectively. The second major determinant was different according to gender: folate accounted for 19.0% of \( \text{FtHCy} \) variance in men only, while vitamin B12 accounted for 17.8% of \( \text{FtHCy} \) variance in women only. The influence of creatinine (5.3% and 7.7%), like that of the PLP contribution, was small and non-significant in both sexes.

A similar procedure was used for absolute post-
Biological determinants of plasma homocysteine

Table 6 presents the stepwise variable selection in men and women. Age had no determinant influence on post-load values (PML-tHCy) in either sex. In men, folate levels explained 19.9% of PML-tHCy variance, with no significant effect of PLP, creatinine or vitamin B12. In women vitamin B12 explained a great portion of the PML-tHCy variance (41.2%), followed by PLP (19.0%) and creatinine (14.6%), while a non-significant effect was found for folate. Similar results were obtained for δtHCy (data not shown). However, for %δtHCy, age explained the major percentage of variance in both sexes (data not shown).

Reference values of fasting and post-load homocysteine

Reference values for tHCy on the basis of sex and age were calculated as the 0.975 fractile of the reference distribution with 90% confidence intervals21 as indicated in Table 7.

Discussion

The aim of our study was to evaluate the biological determinants of fasting and post-methionine load tHCy concentrations in a cohort of healthy subjects in the area of Bologna, Italy.

Our results confirm the influence of age and sex on fasting tHCy concentrations observed by other investigators in other European populations.17,18,22 Age was correlated negatively with vitamins. However, the increase in tHCy observed with increasing age is attributed not only to lower vitamin levels, but also to the reduced efficiency of HCy metabolic pathways and to the decline in renal function, a critical factor for HCy metabolism.6,11,12,13,23 Estrogen decrease after menopause may also be relevant in elderly women.18,24 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 the presence in women of a smaller muscle mass as indicated by lower creatinine levels, limited creatine phosphate synthesis, and different hormonal status with a lowering effect of estrogens on homocysteine levels. Estrogens seem to have an up-regulatory effect on the hepatic enzyme betaine:homocysteine methyltransferase, another pathway in HCy remethylation, which can decrease FtHCy in women.

In our multiple regression analysis, after age, the second major determinant of FtHCy variance was folate in men (but not in women) and vitamin B12 in women (but not in men). This gender-related difference in the influence of vitamins on FtHCy has been observed by other authors, as it was not correlated with BMI either in the whole group or separately in men and women. It is likely that differing body composition (greater proportion of body weight as fat in women) could account for this feature.

We found a significant correlation of PML-tHCy and ∆tHCy with age only in women at the univariate analysis. This finding could be attributed to the effects of age-related differences in estrogen in women. Several investigators have drawn attention to the need to consider age and gender differences in the response to the ML test. However, in our stepwise regression analysis there

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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Men (n=42)</th>
<th>Women (n=65)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage explained</td>
<td>p</td>
</tr>
<tr>
<td>Log10 age</td>
<td>20.5</td>
<td>0.0007</td>
</tr>
<tr>
<td>Log10 creatinine</td>
<td>5.3</td>
<td>0.0006</td>
</tr>
<tr>
<td>Log10 folate</td>
<td>1.5</td>
<td>0.32</td>
</tr>
<tr>
<td>Log10 folic acid</td>
<td>19.0</td>
<td>0.0011</td>
</tr>
<tr>
<td>Log10 vitamin B12</td>
<td>1.0</td>
<td>0.42</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Men (n=46)</th>
<th>Women (n=41)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage explained</td>
<td>p</td>
</tr>
<tr>
<td>Log10 age</td>
<td>1.5</td>
<td>0.54</td>
</tr>
<tr>
<td>Log10 creatinine</td>
<td>2.3</td>
<td>0.44</td>
</tr>
<tr>
<td>Log10 folate</td>
<td>19.9</td>
<td>0.0300</td>
</tr>
<tr>
<td>Log10 vitamin B12</td>
<td>0.8</td>
<td>0.64</td>
</tr>
<tr>
<td>Log10 PLP</td>
<td>3.6</td>
<td>0.28</td>
</tr>
</tbody>
</table>

*Model included: age, creatinine, PLP, folate and vitamin B12.
was no significant influence whatsoever of age on post-load Hcy values in either sex.

With regard to associations of vitamin status and post-methionine load tHCy levels, our data show that folate levels are determinant for post-load values in males, while vitamin B12 and PLP levels are the main determinants in females. It was suggested that the transsulfuration pathway could be less efficient and more dependent on vitamin B6 and vitamin B12 in females. Moreover, it has also been surmised that the vitamin B12-dependent remethylation pathway, activated by the transient post-load increment in tHCy, could be more important in females than in males.

Our PLP values are lower than those reported in other studies. This can be attributed to the differences between our HPLC method and other commonly used methods, such as radioenzymatic assays, both in pre-analytic and analytic variables.

So far the majority of authors have provided FTHCy and post-ML-tHCy reference values without considering the biological determinants of tHCy. Our observations indicate that differences in age and gender should be taken into account in establishing reference values of fasting and post-load homocysteine levels. Recently some investigators have also recommended that tHCy reference values be established in populations with apparently adequate vitamin status. However, the adequacy of vitamin status might be difficult to determine as it varies widely across different populations, due to different nutritional habits and lifestyles.

Regarding the vitamin status, our data emphasize the predominant influence, on the fasting and post-load homocysteine levels, of folate in males and vitamin B12 and, to some extent, vitamin B6 in females. The differential influence of vitamins according to gender may have practical implications in the diagnosis and treatment of increased levels of fasting and post-load tHCy in subjects with vascular disease. Isolated increased FTHCy levels may warrant the determination of folates, vitamin B12 and creatinine in both men and women, while an isolated increase in PML-tHCy may warrant the determination of folate alone in women and creatinine, vitamin B12 and PLP in women. High fasting tHCy levels are usually treated with folates alone in the presence of adequate vitamin B12 levels. In the case of an isolated increase in PML-tHCy, treatment with folates may be more relevant in men, while women may need treatment with PLP and vitamin B12.

Contributions and Acknowledgments
SS designed the study, was responsible for the HPLC methods and HPLC analysis, data management and wrote the paper. BC designed the study, was responsible for data management and revised the manuscript. GP and CL recruited the participants and supervised the laboratory data analysis. GG was responsible for the HPLC methods and HPLC analysis. SM was responsible for statistical analysis. SC, senior investigator, revised the manuscript and gave final approval for its submission. We are grateful to SS and AC for helping in the recruitment of normal and normal pediatric subjects.

Disclosures
Conflict of interest: none.
Redundant publications: no substantial overlapping with previous papers.

References


29. Vicente Vicente, Deputy Editor

This manuscript was peer-reviewed by two external referees and by Professor Vicente Vicente, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Prof. Vicente Vicente and the Editors. Manuscript received January 15, 2002; accepted June 10, 2002.

What is already known on this topic
Elevated homocysteine plasma levels have been associated with an increased risk of atherosclerosis and thrombosis. Fasting homocysteine concentration seems to be associated to age, gender and vitamin status.

What this study adds
This study evaluates the determinants of fasting and post-methionine load total plasma homocysteine with regard to age, sex, creatinine, folate and vitamin B12.

Potential implications for clinical practice
Age, gender and vitamin status influence fasting total plasma homocysteine levels and two-hour post-methionine load. Reference values should be calculated according to age and sex.

Vicente Vicente, Deputy Editor