

## Hyperhomocysteinemia: could the post-methionine oral loading test sometimes be avoided?

FRANCESCO MARONGIU, LARA FENU, GIULIA PISU, PAOLO CONTU, DORIS BARCELLONA

**Background and Objectives.** Measurement of homocysteinemia, a risk factor for venous and arterial thrombosis, is carried out in patients fasting for 12 hours and after an oral methionine load (PML). The procedure is time-consuming and several of the patients suffer from nausea and malaise. We wondered whether methionine loading could sometimes be avoided by considering fasting homocysteinemia (tHcy) levels.

**Design and Methods.** We evaluated whether fasting tHcy levels were useful to predict PML and  $\Delta$ PML tHcy with acceptable sensitivity and specificity in 381 patients with venous and arterial thrombosis through the generation of receiver operating characteristic curves.

**Results.** Both PML and  $\Delta$ PML tHcy correlated with fasting tHcy values. The cut-off of fasting tHcy value yielding a 100% sensitivity in predicting normal PML and  $\Delta$ PML tHcy was 6.5 and 5.0  $\mu\text{mol/L}$  in females, and 7.1 and 7.2  $\mu\text{mol/L}$  in males. Fasting tHcy values yielding a 95% specificity in predicting a positive PML and tHcy result ranged from 12.5 to 13.1  $\mu\text{mol/L}$  in males and from 10.4 to 10.5  $\mu\text{mol/L}$  in females. A 95% specificity in predicting a positive  $\Delta$ PML tHcy result ranged from 10.8 to 11.6  $\mu\text{mol/L}$  in females and from 15.9 to 17.0  $\mu\text{mol/L}$  in men. Considering PML tHcy, 186 out of 381 patients could have avoided methionine loading while using  $\Delta$ PML tHcy 123 out of 381 could have done so.

**Interpretation and Conclusions.** Nearly 50% of our patients considering PML tHcy, and about 30% considering  $\Delta$ PML tHcy could have been spared the methionine loading test. We propose this model for those who wish to carry out this analysis on their own.

**Key words:** hyperhomocysteinemia, post methionine loading test, arterial thrombosis, venous thrombosis.

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In the past twenty years the interest in the role of hyperhomocysteinemia as a risk factor for both arterial and venous thrombosis has greatly increased.

Plasma levels of homocysteine (tHcy), a sulfhydryl amino acid derived from the conversion of methionine, are affected both by genetic and acquired factors, which on the one hand can mutually interfere but on the other do not help explain its levels completely.<sup>1-3</sup>

The most common genetic defects involved in mild-to-moderate hyperhomocysteinemia are heterozygosity for cystathionine  $\beta$ -synthase or methylenetetrahydrofolate reductase (MTHFR), which are cumulatively present in 0.4 to 1.5% of the general population.<sup>4</sup> Another genetic defect is the thermolabile homozygous mutant of MTHFR (C677T), whose prevalence in normal subjects is between 5 and 20%,<sup>5</sup> and which seems to be responsible for increased levels of homocysteinemia in the presence of low serum folate levels.<sup>2</sup>

Non-genetic factors capable of affecting tHcy levels are age, gender, menopause status,<sup>6</sup> serum levels of B<sub>12</sub>, folic acid,<sup>7</sup> and renal function.<sup>8</sup> Low serum levels of vitamin B<sub>6</sub> may also cause hyperhomocysteinemia, and have been reported as an independent risk factor for venous and arterial thrombosis.<sup>9,10</sup>

The role of hyperhomocysteinemia as a risk factor for arterial thrombosis has been strongly demonstrated in cross-sectional and case-control studies,<sup>11</sup> but there are contrasting data in prospective studies that followed healthy subjects for several years.<sup>12-14</sup> However, positive results in terms of predicting adverse outcomes have been obtained when considering the follow-up of patients with established cardiovascular disease.<sup>15,16</sup> Recent data suggest that mild hyperhomocysteinemia is also involved in the pathogenesis of venous thromboembolic disease.<sup>17</sup> A meta-analysis of 9 studies showed that hyperhomocysteinemia can increase the risk of venous thrombosis by approximately two and half times.<sup>18</sup>

The measurement of tHcy is normally carried out in patients fasting for at least 12 hours, and is repeated after a methionine load (0.1 g/Kg) in a time ranging from two to eight hours. This test was initially undertaken to reveal patients with a heterozygous cystathionine  $\beta$ -synthase defect, but it was later used to detect mild abnormalities of methionine metabolism even in patients with normal fasting tHcy levels.<sup>19</sup> Methionine is normally dissolved in tea or fruit juice to mask its unpleasant taste. Since the procedure is time-consuming and several of the patients attending our

From the Dipartimento di Scienze Mediche Internistiche, University of Cagliari (FM, LF, GP, DB) and Dipartimento di Igiene e di Sanità Pubblica, University of Cagliari (PC), Italy.

Correspondence: Prof. Francesco Marongiu, MD, Policlinico Universitario di Monserrato, Università di Cagliari, SS 554, bivio per Sestu, Monserrato, Cagliari, Italy. E-mail: marongiu@pacs.unica.it

clinic suffered from nausea and experienced malaise, we wondered whether methionine loading could sometimes be avoided by simply considering fasting tHcy levels. The aim of this study was, therefore, to evaluate whether fasting tHcy levels were useful for predicting post-methionine loading (PML) tHcy levels and the increase in PML tHcy levels ( $\Delta$ PML) with acceptable sensitivity and specificity in a large group of patients with venous and arterial thrombosis. Moreover, we studied whether other factors such as age, gender, arterial or venous thrombosis, vitamin B<sub>12</sub>, and folic acid levels could affect the relationship between fasting and both PML and  $\Delta$ PML tHcy levels in the hope of better identifying possible predicting negative or positive tHcy results after methionine loading.

## Design and Methods

### Patients

We studied 381 consecutive patients (193 men, median age 50, range 22–81 years and 188 women, median age 45, range 16–83 years) referred to our Thrombosis Center with an instrumental and/or laboratory documented diagnosis of venous or arterial thrombosis. *Arterial thromboses* (n=158, 107 men and 51 women, median age 48, range 21–78 years) were the following: peripheral thrombosis (n=17), ischemic stroke (n=57), and previous myocardial infarction (n=84). *Venous thromboses* (n=223, 86 men and 137 women, median age 48, range 16–83 years) were the following: deep vein thrombosis (n=147), deep vein thrombosis and pulmonary embolism (n=34), pulmonary embolism (n=27), and superficial venous thrombosis (n=15). A control group of 135 healthy subjects (64 men and 71 women, median age 51, range 17–77 years) were also studied. Healthy controls were selected based on clinical history (negative for drug assumption, cardiovascular, kidney, and liver diseases), and normal routine biochemical and blood tests. From 66 of these control subjects, a blood sample was also taken after methionine loading.

### Methods

Blood was drawn between 7.30 am and 8.30 am after fasting for 12 hours into tubes containing EDTA for tHcy measurement and no anticoagulant for vitamin B<sub>12</sub> or folate assays. Methionine (3.8 g/ m<sup>2</sup> body surface area) was immediately administered orally in 200 mL of tea and a second blood sample was obtained 4 hours later.

In order to measure tHcy, the blood sample was immediately put in an ice bath, and centrifuged at 3000 × g for 20 min at 4°C. Plasma was then frozen at –80°C until assays could be performed. Plasma tHcy assay was carried out by high performance liquid chromatography and fluorimetric detection.<sup>20</sup> The intra-assay coefficient of variation of this test

**Table 1. Median and total ranges of tHcy ( $\mu$ mol/L), folic acid (ng/mL) and B<sub>12</sub> vitamin (pg/mL) measurements in patients with arterial thrombosis (AT), vein thrombosis (VT), and in healthy controls.**

	Patients with AT	Patients with VT	Healthy controls	p*
Fasting tHcy	8.3, 3.5-48.9 <sup>§</sup>	7.6, 3.2-46.6 <sup>§§</sup>	7.5, 3.6-15.8	0.0025
PML tHcy	26.0, 8.2-79.9 <sup>§</sup>	25.1, 10.4-101.3 <sup>§</sup>	22.4, 10.9-35.2	0.0045
$\Delta$ PML tHcy	16.8, 4.6-62.5 <sup>§</sup>	17.2, 2.4-87.2 <sup>§</sup>	14.9, 6.1-27.1	0.021
Folic Acid	5.5, 1.9-32.0 <sup>§§</sup>	5.2, 2.0-38.1 <sup>§§</sup>	5.6, 2.2-28.8	0.74
B <sub>12</sub> Vitamin	407.3, 140.9-2411.7 <sup>§</sup>	375.6, 138.1-2116.6 <sup>§</sup>	444.9, 192.6-2669.5	0.0001

\*Kruskal-Wallis (ANOVA); <sup>§</sup><0.05 versus controls (Dunn's multiple comparison test). <sup>§§</sup>Not different from controls; none of the parameters was significantly different between patients with arterial and venous thrombosis.

is 5.02% in our hands.

Vitamin B<sub>12</sub> and folic acid assays were performed using a radioimmunoassay technique (B<sub>12</sub> Fol- NB, Chiron Diagnostics GmbH, Germany).

### Statistical analysis

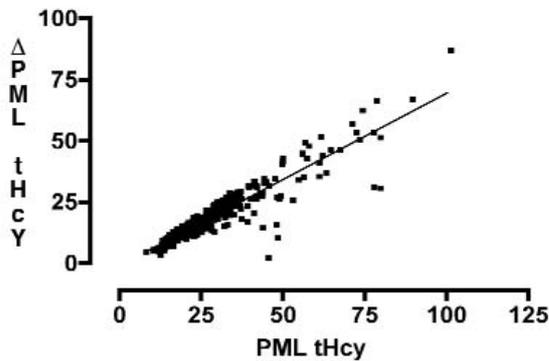
Statistical analysis was performed using the SPSS package (SPSS Inc, Chicago, IL, USA).

Data are expressed as median and range. The Kruskal-Wallis analysis of variance and the Mann-Whitney U test were used for comparisons among the studied groups. Cohen's K test was used to assess the agreement<sup>21</sup> between  $\Delta$ PML and PML tHcy test results. Analysis of covariance was employed to evaluate the influence of other categorical (sex and type of thrombosis) and continuous variables (age, vitamin B<sub>12</sub>, and folic acid levels) on the relationship between fasting and both PML and  $\Delta$ PML tHcy levels. The final model was obtained using stepwise elimination. Linear regressions were then obtained between fasting and both PML and  $\Delta$ PML tHcy levels considering the influence of the co-variates. Sensitivity and specificity of the fasting tHcy values in predicting normal or abnormal PML and  $\Delta$ PML tHcy levels were obtained by generating receiver operating characteristic (ROC) curves.

Moreover we calculated the 95% CI of sensitivity and specificity for different cut-off values as proportion standard errors or Poisson distribution as appropriate.

## Results

Fasting tHcy, PML,  $\Delta$ PML tHcy, vitamin B<sub>12</sub>, and folic acid levels are expressed as median and total



**Figure 1.** Scatter plot and linear regression line between PML tHcy and ΔPML tHcy (mmol/L) described by  $\Delta\text{PML tHcy} = 0.7 \times \text{PML tHcy} - 0.8$  ( $R^2 = 0.85$ ).

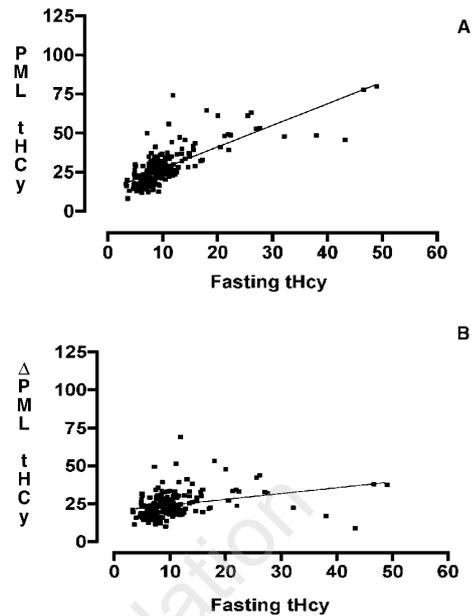
range. Fasting tHcy, PML, ΔPML tHcy and vitamin B<sub>12</sub> levels showed a different behavior in patients and controls while folic acid levels did not (Table 1). We considered as positive all patients with fasting, PML, and ΔPML tHcy levels above the 99<sup>th</sup> percentile of the distribution of control subjects.

Since the distribution of fasting tHcy in controls was significantly affected by gender ( $p < 0.0001$ ), we used two different cut-off values for females (12 μmol/L) and males (15 μmol/L).

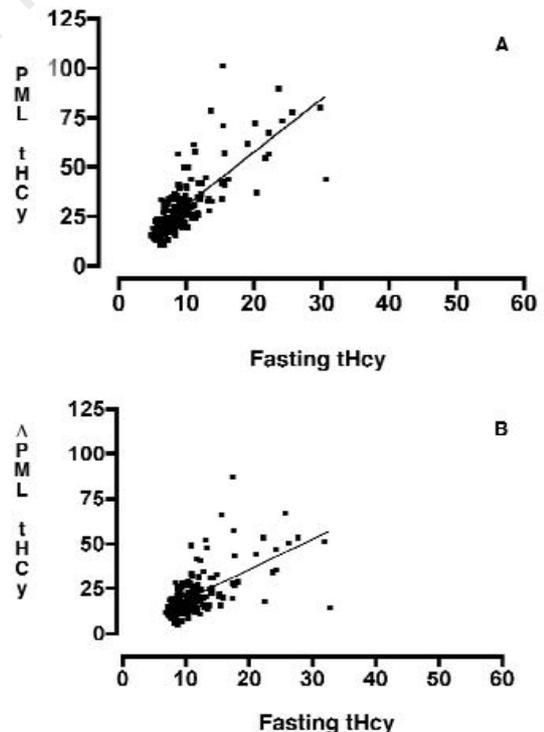
Since the distribution of PML and ΔPML tHcy was not significantly different in females and males, we used the same cut-off for PML (35 μmol/L) and ΔPML tHcy (26 μmol/L).

An increased level of fasting tHcy was found in 44 out of the 381 patients (11.5%), while a normal fasting tHcy level but a positive PML and ΔPML tHcy occurred in, respectively, 38 and 39 out of the 381 patients (10% for both groups).

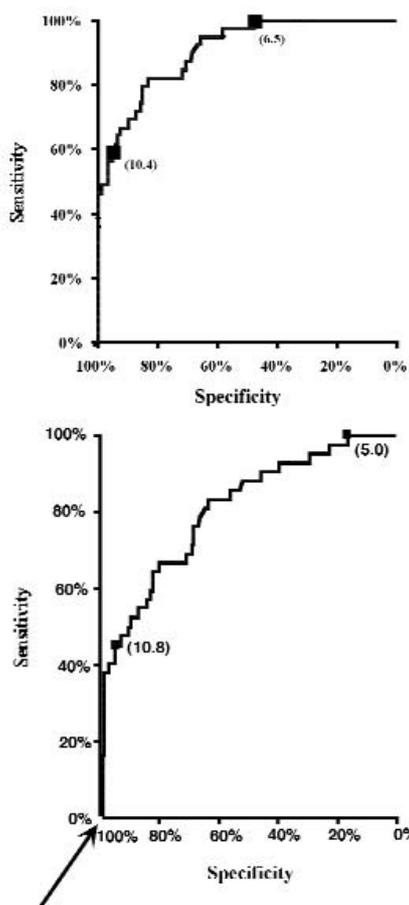
Of the 39 patients who had a ΔPML tHcy positive result, 31 (80.5%) also had a positive PML. This figure was confirmed by Cohen's K test, which gave a substantially good result ( $K = 0.80$ ). The linear regression analysis demonstrated that fasting tHcy explained 49% of the variation in PML tHcy ( $R^2 = 0.49$ ;  $p < 0.0001$ ) and 13% of the variation in ΔPML tHcy ( $R^2 = 0.13$ ;  $p < 0.0001$ ). In spite of the fact that the PML and ΔPML tHcy plasma levels were strongly correlated ( $R^2 = 0.85$ ;  $p < 0.0001$ ) (Figure 1), and Cohen's K test demonstrated a good agreement between the two tests, the next analysis was carried out considering the relationship between fasting tHcy and both PML and ΔPML tHcy levels. The analysis of co-variance (ANCOVA) showed that gender significantly affected the relationship between fasting and both PML and ΔPML tHcy levels (Figures 2, 3). Age, folate levels, B<sub>12</sub> levels, and



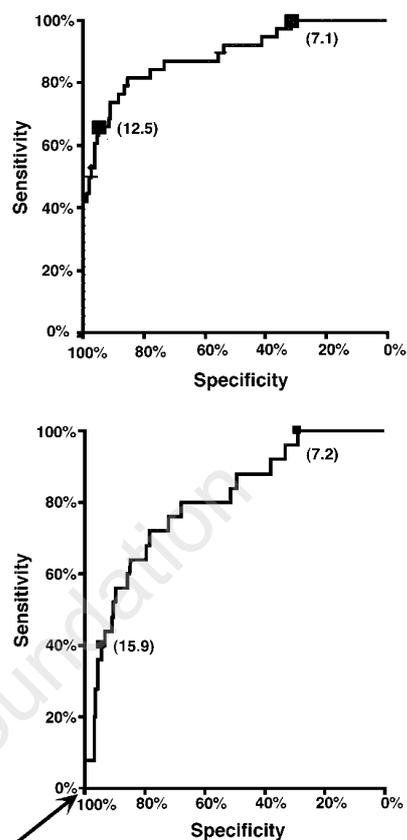
**Figure 3.** Scatter plot and linear regression in men a) between fasting tHcy and PML tHcy (μmol/L) described by  $\text{PML tHcy} = 1.4 \times \text{fasting tHcy} + 13.7$  ( $R^2 = 0.58$ ), b) between fasting tHcy and ΔPML tHcy (μmol/L) described by  $\Delta\text{PML tHcy} = 0.4 \times \text{fasting tHcy} + 13.7$  ( $R^2 = 0.10$ ).



**Figure 2.** Scatter plot and linear regression in women a) between fasting tHcy and PML tHcy (μmol/L) described by  $\text{PML tHcy} = 2.7 \times \text{Fasting tHcy} + 7.4$  ( $R^2 = 0.59$ ), b) between fasting tHcy and ΔPML tHcy (μmol/L) described by  $\Delta\text{PML tHcy} = 2.7 \times \text{Fasting tHcy} + 7.4$  ( $R^2 = 0.36$ ).



**Figure 4.** Receiver operating characteristic (ROC) curve of fasting tHcy levels ( $\mu\text{mol/L}$ ) for prediction of a) PML and b)  $\Delta\text{PML}$  test results in women. Figures in parentheses represent the respective tHcy cut-off levels.



**Figure 5.** Receiver operating characteristic (ROC) curve of fasting tHcy levels ( $\mu\text{mol/L}$ ) for prediction of a) PML and b)  $\Delta\text{PML}$  test results in men. Figures in parentheses represent the respective tHcy cut-off levels.

type of diagnosis (arterial or venous thrombosis) did not affect this relationship. The final adjusted  $R^2$  of the model was 0.59 for PML tHcy and 0.30 for  $\Delta\text{PML}$  tHcy ( $p < 0.0001$ ).

ROC curve analyses were generated to find specific cut-off values of fasting tHcy, yielding high sensitivity in predicting normal PML and  $\Delta\text{PML}$  tHcy, and high specificity in predicting abnormal PML and  $\Delta\text{PML}$  tHcy.

Patients were divided by sex according to the results obtained by analysis of co-variance. The cut-off fasting tHcy value yielding a 100% sensitivity (CI 95%=91%-100%) in predicting a normal PML and  $\Delta\text{PML}$  tHcy value was tHcy=6.5 and 5.0  $\mu\text{mol/L}$ , respectively, in females and tHcy=7.1 and 7.2  $\mu\text{mol/L}$ , respectively, in males. Specificity was 48% (CI 95%=42-54%) and 16% (CI 95%=11-24%) in females for PML and  $\Delta\text{PML}$  tHcy, respectively, whereas it was 32% (CI 95%=24-40%) and 29% (CI 95%=22-39%) in males respectively for PML and  $\Delta\text{PML}$  tHcy, respectively.

On examining fasting tHcy values yielding a 95% specificity (CI 95%=92-98%) in predicting a positive PML tHcy result, these values ranged from 12.5  $\mu\text{mol/L}$  to 13.1  $\mu\text{mol/L}$  in males and from 10.4  $\mu\text{mol/L}$  to 10.5  $\mu\text{mol/L}$  in females. A 95% specificity (CI 95%=91-98%) in predicting a positive  $\Delta\text{PML}$  tHcy result ranged from 10.8 to 11.6  $\mu\text{mol/L}$  in females and from 15.9 to 17.0  $\mu\text{mol/L}$  in men, the latter being beyond the normal cut-off (Figures 4, 5).

Finally we calculated how many patients could have avoided the PML test. Adding up i) patients with fasting tHcy values below, or equal to, the considered cut-off value to predict normal PML tHcy (119/381; 31.2%), ii) patients with fasting tHcy values above, or equal to, the considered cut-off value to predict an abnormal PML tHcy (23/381; 6.0%), and iii) patients with fasting hyperhomocysteinemia (44/381; 11.5%), the result came to 186 out of 381 (48.8%).  $\Delta\text{PML}$  tHcy figures are as follows for the first two classes of patients: i)

72/381; 18.9%; ii) 6/381; 1.6%. The patients who could have been spared the PML test using  $\Delta$ PML tHcy, including those with fasting hyperhomocysteinemia, were 123 out of 381 (32.3%).

## Discussion

Mild hyperhomocysteinemia is considered a risk factor for venous and arterial thrombosis. The mechanisms of how high levels of tHcy may act in inducing vascular damage are still not completely clear. There is some evidence in favor of the role of tHcy as an inducer of oxidant stress, thus affecting NO release from endothelial cells.<sup>22</sup> Other possible mechanisms include activation of factor V,<sup>23</sup> interference with protein C activation and thrombomodulin expression,<sup>24,25</sup> inhibition of tissue plasminogen activator binding,<sup>26</sup> and some others. The laboratory approach to the diagnosis of hyperhomocysteinemia can induce nausea in up to about 20% of the cases.<sup>27</sup> Moreover, a methionine load can acutely impair endothelial function<sup>28,29</sup> and produce protein oxidation as well as lipid peroxidation.<sup>30</sup> The aim of this study was, therefore, to evaluate whether the PML test could be avoided at least in some patients in daily clinical practice, taking into consideration fasting tHcy levels. Our findings indicate that the relationship between fasting tHcy and both PML and  $\Delta$ PML tHcy values is affected only by gender. In particular the regression line is steeper in women, indicating a higher increase in PML and  $\Delta$ PML tHcy results. Since the other co-variables did not affect this kind of relationship, we generated two ROC curves for PML and two for  $\Delta$ PML tHcy based on gender alone. The final result is the simple model presented here which may predict, with high sensitivity, and specificity both normal and high PML and  $\Delta$ PML tHcy values from fasting tHcy. From a practical point of view, male patients will prove negative to the PML test, at least in our Thrombosis Center, if their tHcy value is under the cut-off of 7.1  $\mu\text{mol/L}$  using both PML and  $\Delta$ PML tHcy values, while in female patients we could obtain the same result with a cut-off of 6.5 and 5.0  $\mu\text{mol/L}$  respectively. Contrariwise, a tHcy level of between 12.5 and 13.1  $\mu\text{mol/L}$  in males and between 10.4 and 10.5  $\mu\text{mol/L}$  in females could predict a positive PML test result with high specificity. High specificity  $\Delta$ PML tHcy cut-off values appear to be useful only for females, since cut-off values obtained in men are beyond the fasting tHcy normal cut-off. A limitation of this study may be the relatively small number of healthy controls with a PML test. It is not easy to find a volunteer who will agree to drink an unpleasant substance such as methionine. On the other hand, the values obtained in the PML test were close to those reported in the literature.<sup>31</sup> Again, we chose only those values above the 99th percentile

of a control distribution as positive in order to minimize the risk of having false positive results. This study is not intended to eliminate the PML test from the laboratory approach to diagnosing hyperhomocysteinemia. As a matter of fact the PML test allows detection of a certain number of hyperhomocysteinemic patients not recognized by the measurement of fasting tHcy levels. In our hands this figure rises to about 10% of the patients using both PML and  $\Delta$ PML test results. However we tried to create a model that should allow selection of some patients in whom the PML test may be avoided. In this study nearly 50% of our patients could have been excluded from the procedure: a result that could have a practical impact. The  $\Delta$ PML tHcy results are useful, though not fully interchangeable with PML tHcy values. In fact about 30% of the patients could have been spared a methionine load. Finally we suggest that patients with a fasting tHcy result between the two cut-off values obtained to predict a negative or positive PML (or  $\Delta$ PML) tHcy should be tested with PML. The model presented here can be carried out in any laboratory through the generation of ROC curves to find local sensitive and specific cut-off figures of fasting tHcy levels.

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## Pre-publication Report & Outcomes of Peer Review

### Contributions

FM: designed and supervised the study, he was responsible for the evaluation of the data and wrote the manuscript; LF: performed tHcy, vitamin B12, and folic acid measurements; GP: organized the data and wrote the manuscript along with FM; PC: analyzed and interpreted the data; DB: recruited the patients and prepared the thrombophilia database. She critically revised the manuscript. Primary responsibility for the paper: FM; primary responsibility for all Tables and Figures: GP.

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### Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

### Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper was taken jointly by Professor Cazzola and the Editors. Manuscript received August 13, 2002; accepted December 3, 2002.

In the following paragraphs, the Editor-in-Chief summarizes the peer-review process and its outcomes.

### What is already known on this topic

Elevated plasma homocysteine is a known risk factor for venous and arterial thrombosis. Measurement of homocysteinemia is normally carried out in patients fasting for at least 12 hours and is repeated after an oral methionine load. This procedure allows a greater number of hyperhomocysteinemic subjects to be detected, but it is time-consuming and causes nausea and malaise.

### What this study adds

This study defines useful cut-offs for fasting homocysteinemia that allow methionine in nearly half of the cases. Patients with plasma homocysteine levels below lower cut-offs can reasonably be considered negative to methionine load, while those with values above upper cut-offs are very likely positive.