samples bearing mutations must be correct. In the case of X-linked diseases, this requires the equimolar mixing of PCR products between a wild-type DNA sample and the sample to be tested. The type of mutations searched for must be detectable by D-HPLC. D-HPLC is more sensitive in the screening of point mutations, which is the case of most FIX mutations (1). In any case, once the D-HPLC conditions have been defined, a prospective study of a novel population sample should be performed to confirm the detection rate of the D-HPLC procedure.

The D-HPLC scanning procedure described here is fast because all of the DNA fragments can be amplified under the same conditions, the post-PCR phase is automated, and each D-HPLC run requires only 6 min. The protocol for D-HPLC scanning of the entire FIX gene is completed in <5 h. Finally, the D-HPLC method is cost-effective: we calculated that it costs approximately US $25 to scan the whole FIX gene, excluding instrument and personnel costs. Direct sequencing has a 100% sensitivity, and rapid protocols have been set up to analyze all FIX gene fragments under the same PCR conditions (6) or in single multiplex PCR amplifications (5). Direct FIX gene sequencing has been shown to be efficient in various ethnic-geographic groups (5–8). However, direct sequencing is more expensive than D-HPLC. On the other hand, scanning procedures have a sensitivity of ~75–90% (10), and in most studies D-HPLC was more sensitive than other scanning procedures, as recently demonstrated for the factor VIII gene (11) and for several other disease genes (12, 13). Furthermore, denaturing gradient gel electrophoresis and single-strand conformation polymorphism analysis are more difficult to automate (9).

In conclusion, D-HPLC scanning of the FIX gene with the procedure described here is suitable for the routine diagnosis of HB and for carrier diagnosis if the proband is not available. Using this procedure, we have confirmed the heterogeneity of FIX gene mutations in HB patients from Southern Italy.

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References

Fasting vs Nonfasting Plasma Homocysteine Concentrations for Diagnosis of Hyperhomocysteinemia, M. Rebecca Fokkema,1,2 Marleen F. Gilissen,1 Jasper J. van Doormaal,2 Marcel Volmer,1 Ido P. Kema,1 and Frits A.J. Muskiet1 (Departments of 1 Pathology and Laboratory Medicine and 2 Internal Medicine, University Hospital Groningen, NL-9700 RB Groningen, The Netherlands; * address correspondence to this author at: Pathology and Laboratory Medicine, University Hospital Groningen, CMC-V, Room Y1.165, PO Box 30.001, NL-9700 RB Groningen, The Netherlands; fax 31-50-3612290, e-mail m.r.fokkema@path.azg.nl)

Hyperhomocysteinemia, a risk factor for cardiovascular and thrombotic disease, pregnancy complications, and cognitive disorders, is defined as a fasting plasma total Hcy (tHcy) above a chosen cutoff value (1, 2). Reducing both the analytical and biological variation may add to the diagnostic value of any test. tHcy analytical variation (CVa) is method-dependent and ranges from 2.7% to 4.9% for fluorescence polarization immunoassays and from 2.5% to 14% for HPLC assays (3). The reported biological variations for tHcy under fasting or otherwise standardized conditions include 20–34% interindividual variation (CVi) and 7–11% (with a single extreme of 15–17%) intraindividual variation (CVi) (4–9). Because of the short-term influence of meals, e.g., protein, on CVi (10), it is generally recommended that blood samples be collected under fasting or otherwise meal-standardized conditions. The influence of protein intake on between-day CVi is, however, unknown. The necessity for fasting has
been questioned because fasting and nonfasting tHcy concentrations and reference values, as well as fasting and postprandial short-term CV_{i} and CV_{g} values, are similar (11, 12).

We investigated whether tHcy concentrations and variations under nonfasting conditions differ from those under fasting conditions. For this we calculated tHcy biological variation from samples taken at different clock times during a single day and during 3 weeks.

We studied 16 apparently healthy individuals [8 men and 8 women; median (range) age, 44 (25–58) years]. Our study design is illustrated in Fig. 1A. Six blood samples were taken from all participants on Monday of week 1. A standardized breakfast (0815 in the morning) and lunch (1215) and a dinner (1730–1800) of unrestricted composition and quantity were provided during the day. Participants documented food and beverage intake on Monday and the preceding Sunday and fasted overnight from 2200 on Sunday. Participants were subsequently divided into two groups. Group 1 [five men and five women; age, 45 (25–58) years] took 5 mg of folic acid, 1 mg of cyanocobalamin, and 50 mg of pyridoxine daily at breakfast for 3 weeks (last intake on Sunday of week 4). On Monday of week 4, they repeated the protocol of Monday of week 1. Group 2 [three men and three women; age, 40 (25–57) years] had additional blood samples taken on eight occasions during weeks 1–3. They had no specific dietary restrictions apart from those on Monday of week 1. The study protocol was approved by the medical ethics committee of our hospital and was in agreement with local ethical standards and the Helsinki Declaration of 1975, as revised in 1996.

The compositions of the documented and standardized meals were calculated with the software package Becel (Hartog Union and Van den Bergh). Whole-blood vitamin B_{6} and vitamin B_{12} were determined by HPLC, serum folate and vitamin B_{12} by immunofluorometric methods (Autodelfia; Wallac Oy), and serum creatinine by a MEGA (Merck Darmstadt). tHcy was determined within 1 month of storage at –20 °C with a fluorescence polarization immunoassay (IMx; Abbott Laboratories) that measures tHcy, i.e., the sum of reduced and oxidized forms. tHcy samples from each individual were analyzed in a single run to minimize the contribution of CV_{a} to total variation. The accuracy and precision of the tHcy method and the total, within-run, and between-run CV_{b} values were calculated according to NCCLS procedures (13), using calibrators (l-homocystine in human serum, from the IMx reagent set) at three concentrations and duplicate pool samples with a mean concentration of 15.3 μmol/L.

<table>
<thead>
<tr>
<th>A</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mon</td>
<td>Wed</td>
<td>Fri</td>
<td>Mon</td>
</tr>
<tr>
<td>Group 1 (n=10)</td>
<td>0800 1000 1200 1400 1600 1830</td>
<td>0800 1000 1200 1400 1600 1830</td>
<td>0800 1000 1200 1400 1600 1830</td>
<td>0800 1000 1200 1400 1600 1830</td>
</tr>
<tr>
<td>Group 2 (n=6)</td>
<td>0800 1000 1200 1400 1600 1830</td>
<td>0800 1000 1200 1400 1600 1830</td>
<td>0800 1000 1200 1400 1600 1830</td>
<td>0800 1000 1200 1400 1600 1830</td>
</tr>
</tbody>
</table>

Fig. 1. Study design (A) and time courses of tHcy during a single day before and after 3 weeks of vitamin supplementation (B). (A), the numbers indicate clock times for groups 1 and 2 separately. We provided a standardized breakfast (0815) and lunch (1215) and a nonstandardized dinner (1730–1800) on Monday of weeks 1 and 4. Samples collected at 0800 were taken after a 10-h fast, and samples collected at 1200 were taken before lunch. Group 1 took folic acid (5 mg/day), cyanocobalamin (1 mg/day), and pyridoxine (50 mg/day) supplements daily during weeks 1–3. The six samples taken on Monday of week 1 (groups 1 + 2) and Monday of week 4 (group 1) were used for calculation of within-day biological variation before and after vitamin supplementation, respectively. The nine underlined samples from group 2 were used for calculation of between-day biological variation. (B), time course of tHcy concentrations on Monday of week 1 (before vitamin supplementation) and Monday of week 4 (after vitamin supplementation). The filled and open circles represent mean tHcy concentrations (error bars, 95% reference interval). Before supplementation, tHcy decreased from 0800 to 1000 (P < 0.0001), did not change from 1000 to 1400, and increased from 1000 to 1830 (P = 0.002) and from 1400 to 1830 (P = 0.002). After supplementation, tHcy decreased from 0800 to 1000 (P = 0.024) and increased from 1000 to 1830 (P = 0.13). tHcy day-means decreased on vitamin supplementation (P < 0.0001).
Between-day biological variation was calculated based on the nine underlined data points shown in Fig. 1A for group 2. We did not use the ideal calculation method, i.e., nested ANOVA (14), because costs prevented us from measuring samples in duplicate. The between-day CV\textsubscript{m} (using the formula: SD $\times$ 100/mean tHcy) was calculated for each individual. After correction for within-run CV\textsubscript{a}, [using the formula: CV\textsubscript{a} = CV\textsubscript{a(measured)}$^2$ - CV\textsubscript{a(set)}$^2$], these calculations yielded a median, mean, and range for the between-day CV\textsubscript{m}. The median, mean, and range for between-day CV\textsubscript{a} were calculated in a similar manner, using the three tHcy samples collected at those time points. The between-day CV\textsubscript{a} was calculated from the individual 3-week mean tHcy values, using their overall means and SD after correction for total CV\textsubscript{a} (tCV\textsubscript{a}). An individual’s mean provided an indication of that person’s homeostatic set-point during the 3 weeks of the study. Between-day CV\textsubscript{a} for samples collected at 0800, 1200, and 1400 were calculated in a similar manner, using the three tHcy samples collected at those time points. The within-day CV\textsubscript{a} was calculated from the individual 3-week mean tHcy values, using their overall means and SD after correction for total CV\textsubscript{a} (tCV\textsubscript{a}). An individual’s mean provided an indication of that person’s homeostatic set-point during the 3 weeks of the study. Between-day CV\textsubscript{a} for samples collected at 0800, 1200, and 1400 were calculated in a similar manner, using the three tHcy samples collected at those time points, after correction for within-run CV\textsubscript{a}.

Table 1. Analytical and biological variation of plasma tHcy in the present study and as reported by others.

<table>
<thead>
<tr>
<th>Samples\textsuperscript{a}</th>
<th>Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n\textsubscript{1}</td>
</tr>
<tr>
<td>Analytical variation (CV\textsubscript{a})</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
</tr>
<tr>
<td>Within-run</td>
<td>6</td>
</tr>
<tr>
<td>Between-run</td>
<td>6</td>
</tr>
<tr>
<td>Between-day biological variation</td>
<td>6</td>
</tr>
<tr>
<td>Homeostatic\textsuperscript{d}</td>
<td>6</td>
</tr>
<tr>
<td>0800</td>
<td>6</td>
</tr>
<tr>
<td>1200</td>
<td>6</td>
</tr>
<tr>
<td>1400</td>
<td>6</td>
</tr>
<tr>
<td>Fasting or otherwise standardized as reported by others \textsuperscript{f}</td>
<td>(one extreme value, 15–17)</td>
</tr>
<tr>
<td>Before supplementation\textsuperscript{g}</td>
<td>16</td>
</tr>
<tr>
<td>After supplementation</td>
<td>10</td>
</tr>
</tbody>
</table>

\textsuperscript{a} n\textsubscript{1}, number of participants; n\textsubscript{2}, number of samples.

\textsuperscript{b} CV\textsubscript{b} reported as the median (mean) [range] and is corrected for within-run CV\textsubscript{a}.

\textsuperscript{c} CV\textsubscript{c} is corrected for tCV\textsubscript{a}.

\textsuperscript{d} Homeostatic indicates that the basis for calculation of CV\textsubscript{a} was an individual’s mean tHcy, as derived from the nine samples from group 2 collected at different clock times (0800, 1200, and 1400) throughout the study period.

\textsuperscript{e} ND, nondetectable; indicates that the observed variation (combined within-day CV\textsubscript{a} and CV\textsubscript{b}) was lower than CV\textsubscript{a}.

\textsuperscript{f} Corresponding data for group 1 (n = 10) only are as follows: CV\textsubscript{a} = 5.5% (7.4%) [0.9–18%], CV\textsubscript{b} = 20%; RCV = 16.7%; R = 0.92.
before supplementation. tHcy decreased from 0800 to 1000 in the morning ($P < 0.0001$), did not change between 1000 and 1400, and increased from 1000 to 1830 ($P = 0.002$) and from 1400 to 1830 ($P = 0.002$). Day-mean tHcy inversely correlated with day-mean whole-blood vitamin B$_2$. Within-subject changes were also significant ($P = 0.037$) after supplementation: tHcy decreased from 0800 to 1000 ($P = 0.024$) and increased from 1000 to 1830 ($P = 0.013$). Day-mean tHcy decreased ($P < 0.0001$), and although nonsignificant, within-day CV$_g$ and R seemed to decrease and RCV seemed to increase on vitamin supplementation. The within-day CV$_i$ did not change (Table 1).

When data before and after supplementation were combined ($n = 26$), the day-mean tHcy inversely correlated with the day-means of serum folate, whole-blood vitamin B$_6$ and whole-blood vitamin B$_2$, but not with the day-means of serum vitamin B$_12$. Relative tHcy changes from 0800 to 1200 ($r = -0.406; P = 0.039$) were significantly related to protein intake during the preceding evening. The between-day CV$_i$ inversely correlated with the 3-week mean vitamin B$_6$ concentration.

The similarity between our biological variation coefficients and the standardized biological variations published by other investigators (Table 1) suggests that it is not necessary to limit tHcy analyses to the fasting state. This is substantiated by the similarities of our time-specific CV$_i$ and CV$_g$ values. Fasting may also not be necessary for tHcy sampling during follow-up: our RCV was comparable (Table 1) to RCVs reported in studies that collected samples in the fasting state or under otherwise standardized conditions (4–7).

The within-day tHcy fluctuations observed in the present study, i.e., a decrease in tHcy after breakfast and an increase 2 h after lunch, are consistent with the findings of others (10, 16, 17). Our results confirm that CV$_g$ is particularly dependent on vitamin status (1, 18), based on the relationship between tHcy and vitamin status. The within-day CV$_i$ particularly depended on protein intake, as derived from the relationship between protein intake during the evening before sampling and the tHcy decrease from 0800 to 1200, which, together with the tHcy increase 2 h after lunch (i.e., at 1400), is consistent with amino acid uptake kinetics (10). Furthermore, our data suggest that both the CV$_g$ and the between-day CV$_i$ depend on protein intake to some extent because these seemed lowest at 1400 (i.e., after a 10-h fast and a subsequent 6-h period of low protein intake) and highest at 0800 (i.e., after a 10-h overnight fast). Sampling in the fasting state may thus be the least preferred, whereas long periods of low protein intake are likely to have the largest impact on reducing biological variation. Our study had insufficient power, however, to substantiate this difference and was also not designed to visualize the impact of protein intake standardization in detail. It remains questionable, however, whether a further reduction of biological variation is worth pursuing, notably when it requires the institution of patient-inconvenient protocols with probable poor compliance.

We conclude that a 10-h fast is not necessary and is probably a less-preferred metabolic condition for the standardization of diagnosis of hyperhomocysteinemia. Standardization of protein intake during the preceding evening might lower biological variation, but its influence is as yet unknown.

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References