Within-Person Variation of Plasma Homocysteine and Effects of Posture and Tourniquet Application

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Background: Frequently, the result of only a single determination of total homocysteine in plasma (P-Hcy) is used to distinguish between the probability of the presence or absence of risk for vascular disease. A prerequisite for the interpretation of a single P-Hcy test is knowledge of the magnitude of within-person variation and the possible effects of preanalytical variables. However, data on within-person variation are still sparse and inconsistent, and data for the effect on P-Hcy of posture and tourniquet application during venipuncture are not available.

Methods: The within-person variation of P-Hcy and the effects of posture and tourniquet application during venipuncture were studied in 24 healthy subjects. The analytical imprecision of our stable-isotope dilution assay was 3.1%.

Results: The within-person variation (CV) was 8.1%. Daily supplementation with 0.4 mg of folic acid for 2 weeks produced a small but significant decrease in P-Hcy, but there were no significant changes in within-person variation before and after supplementation. After 30 min in the horizontal posture, P-Hcy declined by 6.3%. A 3-min tourniquet application caused a 2.8% increase of P-Hcy.

Conclusions: Our value for within-person variation is consistent with results from studies reported recently in the literature. A 3-min tourniquet application does not add appreciable variation to the measurement of P-Hcy, but the posture of the subject during venipuncture contributes considerably to the within-person variation. We recommend that blood collection when the patient is in a supine position be avoided.

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Over the past decade, there has been an exponential increase in the publication rate of studies on the importance of a slightly increased concentration of total homocysteine in plasma (P-Hcy) as a risk factor for occlusive vascular disease, including atherosclerosis and thrombosis (1). In consequence, clinical demand for the routine determination of P-Hcy has increased dramatically since the mid-1990s. Laboratory data routinely are expressed numerically without comments on their uncertainty. Frequently, the result of only a single P-Hcy test is used to distinguish between the probability of the presence or absence of risk for vascular disease.

In addition to knowledge of analytical and between-person variation, e.g., reference intervals, a prerequisite for the interpretation of a single P-Hcy test is knowledge of the magnitude of within-person variation and the possible effects of preanalytical variables. The importance of this was illustrated by the early and influential report by Wu et al. (2), who found a significant mean difference of <2 μmol/L between proband cases of early coronary artery disease and controls.

In a previous study, we reported data on analytical variation and age- and gender-specific reference intervals (3). However, data on the within-person variation of P-Hcy are still sparse and inconsistent (4–8). Because protein-bound Hcy accounts for 70% or more of the total measured Hcy in plasma from healthy subjects (9, 10), the amount of venous stasis and the position of the subject during venipuncture may affect the measured value. To our knowledge, data for a possible effect of tourniquet application on P-Hcy are not available, and we are aware of only one study (n = 7) on the effects of posture on P-Hcy (11).

The present study was designed to obtain additional data on within-person variation in non-folate-deficient subjects. Because our previous study suggested that one-fourth of the apparently healthy volunteers recruited among the employees of our hospital were not consuming enough folate to keep P-Hcy low (3), we included a folic acid supplementation trial to assess folate status in our reference population. We also examined the magnitude of possible changes introduced by tourniquet application or different postures.

The purpose of this study was to provide further
background information needed to interpret P-Hcy in general and to provide a better basis for accurate assessment of risk factor associations with vascular disease.

**Subjects and Methods**

**Study Design**

During a period of 5 weeks in January and February 1999, 24 apparently healthy volunteers participated in and completed the study. They were recruited among the employees of our department; 12 were women, ages 28–63 years (median, 38 years), and 12 were men, ages 28–63 years (median, 39 years). The volunteers agreed to participate after informed consent, in accordance with the Helsinki Declaration. The study was approved by the local committee of medical ethics.

**Specimens**

Tube 1 was thrown away. Tube 2 contained heparin as an anticoagulant for measurements of albumin and protein. To exclude kidney insufficiency, we measured the creatinine concentration in the first sampling (A1). Tube 3 contained EDTA as an anticoagulant and was used for measurement of the volume fraction of erythrocytes (hematocrit). Tube 4 contained heparin as an anticoagulant with sodium fluoride added to a final concentration of 4 g/L blood, according to the procedure described previously (12), to prevent blood cell production and release of Hcy into the plasma, which would cause an artificial increase in measured concentrations. Plasma was separated by centrifugation within 2 h and stored at −20 °C until determination of Hcy.

**Assays**

P-Hcy was measured by a modification of the assay developed by Stabler et al. (13) and Allen et al. (14). The method is based on the stable-isotope dilution principle with solid-phase extraction of the sample (12). The method was linear over the concentration range examined (0.5–300 μmol/L) (15). Only samples collected on the same day were analyzed in the same assay. Precision was assessed by internal quality-control samples at two concentrations (13.9 and 28.6 μmol/L; n = 60) measured throughout the study. The CVs for both were 5%.

Creatinine and albumin in plasma were measured with a Vitros 950® analyzer (Ortho Clinical Diagnostics), and protein was measured by the biuret method on a Cobas Mira® analyzer (Roche). The ratio between P-Hcy and albumin was calculated as μmol Hcy/L divided by μmol albumin/L, assuming a relative molecular weight of 66 500 for albumin. The volume fraction of erythrocytes (hematocrit) was measured with an H3RTX® (Bayer Technicon).

**Statistical Analysis**

Results are given as mean ± SD, CV, and range. Differences between variances were tested with the F-test. The variance components, as calculated by analysis of variance with nested design (balanced repeated subsampling), were between-person, within-person, and analytical variation. The relationship between variables was assessed using the Pearson correlation coefficient and regression analysis. The reference change, i.e., the minimum detectable (95%) change on two consecutive measurements, was calculated as 2.77(within-person variance + analytical variance)1/2 (16). Unless otherwise stated, a significance value of 0.05 was assumed.

**Results**

The mean creatinine concentration in plasma was 78.3 ± 15.5 μmol/L, and no specimen had a concentration above the upper reference limit (120 μmol/L).

**Analytical Variation**

Analytical variation as determined by duplicate measurement of all samples in the study was 3.1% (Table 1).

**Within-Person Variation Before Folic Acid**

The mean P-Hcy concentration in the two first-morning samples, collected after 5 min rest in a sitting position (A1 and B1), was 8.5 μmol/L. Five women and four men had concentrations above the appropriate reference limits for their age and gender (3) on one or both occasions. The
and in hematocrit were correlated significantly with the decrease in P-Hcy. However, the P-Hcy/albumin ratio of 0.0198 ± 0.0055 did not change with posture (see Fig. 1). Assuming an analytical CV for P-Hcy of 3% and for albumin of 1%, the analytical CV for the ratio will be ~3%. Twenty-three of the 24 participants had changes no larger than 6% of their individual ratios.

EFFECT OF TOURNIQUET APPLICATION

An increase in P-Hcy after a 3-min application of tourniquet (C2 and D2) was observed (Table 3). The increases in P-Hcy, albumin, protein, and hematocrit were all significant, and the increases in albumin and in protein correlated significantly with the increase in P-Hcy. However, the P-Hcy/albumin ratio of 0.0123 ± 0.0032 did not change with the application of tourniquet. All participants had changes no larger than 6% of their individual ratios (Fig. 1).

Discussion

It has been recommended that blood be collected without a tourniquet after 15 min of sitting (17). This procedure is time-consuming and therefore not possible in many clinical settings. We chose 5 min because this time period approximates the time that patients will usually sit before having their blood drawn.

The between-person CV of P-Hcy was 28%, and the concentration range was 4.0–14.4 μmol/L before and 3.8–12.8 μmol/L after supplementation with folic acid. This finding suggests that our study population initially had only subtle or no folate deficiency. The intervals are in the same range reported in an earlier study (3) in which we generated a 0.95 reference interval of 4.5–11.9 μmol/L; using the mean P-Albumin and the analytical SD of 0.69 μmol/L 0.26 μmol/L. This procedure is time-consuming and therefore not possible in many clinical settings. We chose 5 min because this time period approximates the time that patients will usually sit before having their blood drawn.

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The within-person variation was 8.1%. This value is consistent with results from the above-mentioned four studies: 7.0% (20 individuals, ages 21–65 years, from whom samples were drawn weekly for 4 weeks), 9.4% (44
individuals, ages 21–46 years, from whom samples were drawn biweekly at each of four visits per individual), 8.9% (96 individuals, ages 65–74 years, from whom samples were drawn at 2-month intervals over a 1-year period), and 8.3% (20 individuals, ages 31–55 years, from whom samples were drawn at weekly intervals on four occasions), respectively. These concordant results do not confirm previous observations by Santhosh-Kumar et al. (8) who studied 79 individuals, ages 18–65 years, from whom samples were drawn on days 5 and 8 of supplementation with 1 mg of folic acid daily. The authors found a variation of P-Hcy threefold higher for any given individual than that found in our study, reporting that concentrations of Hcy in the second serum sample ranged as low as 36% to as high as 165% of the values on the first serum sample (decreases up to 5 μmol/L and increases up to 7 μmol/L). The discrepant results are puzzling, and the reason is not clear. The imprecision of the method used was remarkably high (8%), but the large variation in Hcy concentrations can only partly be related to analytical imprecision. Other factors could also contribute to the variation, e.g., the sampling procedure using serum instead of plasma (vide infra) or the presence of folate deficiency in the study population.

Fig. 1. Changes in P-Hcy, albumin, and the ratio of P-Hcy to albumin with posture and application of tourniquet. All results are the means of changes measured in two identical experiments performed over an interval of 1 week in 24 subjects. (Left), the decreases in P-Hcy and albumin after 30 min in the supine position. The changes in P-Hcy and albumin were significant, but there was no significant change in the ratio. (Right), the increases in P-Hcy and albumin after a 3-min application of tourniquet. Again, the changes in P-Hcy and albumin were significant, but there was no significant change in the ratio.
It has become increasingly apparent that the blood-sampling procedure may contribute considerably more variation to the measurement of P-Hcy than the total variation in the analytical procedures for the assay. After sampling, the blood cells still produce and release Hcy into the plasma (18, 19), causing an artificial increase of P-Hcy of ~10%/h until centrifugation. Cooling the blood on ice immediately after collection and separating the blood cells from the plasma within 1 h inhibits the release of Hcy by the cells (18). However, this procedure is inconvenient and a source of error, and it will be impossible to implement in many clinical settings. Adding fluoride (12) or acidic citrate (20) to the blood samples controls the problem. For obvious reasons, measurement of Hcy in serum samples is obsolete. We collected blood in tubes with fluoride because standard tubes with sodium fluoride and heparin as an anticoagulant are readily available, and this practical procedure is the daily routine in our laboratory.

Because the analytical variation in this study only was 3.1%, the 6.3% decrease of P-Hcy after 30 min in the horizontal posture indicates that the position of the subject during venipuncture may have a much greater effect on the outcome of the laboratory result than the variation of the assay procedures. Omitting standardization of the blood-sampling procedure with respect to posture by collecting the blood from subjects sitting and lying down at random may increase the intra-individual variation from 8.1% up to ~12%. The observed range of changes (~15.3% to 0.4%) in our 24 subjects is smaller and not consistent with the results reported by Thirup and Ekelund (11), who found that in seven healthy adults, P-Hcy concentrations changed from ~29.9% to ~10.3% after 30 min at supine rest. The reason for the discrepant results is not clear. Because almost all blood collections for the determination of P-Hcy, in our experience, are done on ambulatory subjects or walking inpatients, we recommend that blood collection during supine rest be avoided.

Table 3. Results obtained after 3-min application of tourniquet.

<table>
<thead>
<tr>
<th></th>
<th>P-Hcy, μmol/L</th>
<th>P-Albumin, g/L</th>
<th>P-Protein, g/L</th>
<th>Hematocrit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean change</td>
<td>0.20</td>
<td>1.77</td>
<td>2.98</td>
<td>0.012</td>
</tr>
<tr>
<td>Relative change</td>
<td>2.8%</td>
<td>4.1%</td>
<td>3.9%</td>
<td>2.8%</td>
</tr>
<tr>
<td>SD</td>
<td>0.37</td>
<td>2.25</td>
<td>3.77</td>
<td>0.017</td>
</tr>
<tr>
<td>Relative change</td>
<td>4.3%</td>
<td>3.8%</td>
<td>3.7%</td>
<td>3.2%</td>
</tr>
<tr>
<td>Range</td>
<td>−0.8 to 1.1</td>
<td>−0.6 to 7.11</td>
<td>−2.0 to 11.0</td>
<td>−0.02 to 0.045</td>
</tr>
<tr>
<td>Range of the relative change</td>
<td>−7.4% to 13.6%</td>
<td>−1.4% to 15.9%</td>
<td>−2.6% to 14.7%</td>
<td>−4.5% to 10.6%</td>
</tr>
</tbody>
</table>

* All increases were significant.

In conclusion, our study emphasizes the need for controlled blood collection, particularly when investigating the role of slightly increased P-Hcy as a risk factor for vascular diseases. The factors of tourniquet application and especially posture will contribute significantly to the within-person variation of P-Hcy if not controlled.

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References


