Optimal Time Interval between Repeated Blood Sampling for Measurements of Total Homocysteine in Healthy Individuals, Annemarie Voortman,1,2 Alida Melse-Boonstra,1,2 Johanna M. Schulz,1 Jan Burema,1 Martijn B. Katan,1,2 and Petra Verhoef1,2*(1 Division of Human Nutrition and Epidemiology, Wageningen University, 6700 EV Wageningen, The Netherlands; 2 Wageningen Centre for Food Sciences, 6703 GW Wageningen, The Netherlands; * address correspondence to this author at: Wageningen Centre for Food Sciences/Division of Human Nutrition and Epidemiology, Wageningen University, PO Box 8129, 6700 EV Wageningen, The Netherlands; fax 31-317-485369, e-mail Petra.Verhoef@Staff.NutEpi.WAU.nl)

Many studies have shown that increased plasma concentrations of total homocysteine (tHcy) are associated with cardiovascular disease (1). Like most biological variables, plasma tHcy fluctuates within an individual around a long-term mean (2, 3). tHcy measurements often are based on a single blood sample. Taking the mean of two or more repeated measurements will reduce the error attributable to within-subject biological fluctuations and reflect more closely a person’s true mean tHcy value within a certain period. However, as the time interval between two repeated measurements becomes shorter, the values become more similar and the error reduction diminishes. In that case, the benefit of taking the mean of two measurements is not fully used. On the other hand, intervals that are too long (several weeks) may compromise the compliance of study participants and allow long-term seasonal changes (4, 5).

Several studies on the weekly and monthly biological variation, as expressed by the CV, in plasma tHcy concentrations have been published (Table 1). From these studies, no conclusions can be drawn about the optimal time interval between two repeated samples to obtain a sufficiently precise estimate of the true tHcy concentration within a predefined period. The optimal time interval is reached when the interval-specific CV does not increase further with increasing time intervals.

The aim of this study was to determine how many days between two samplings within 1 week are needed to reach the maximum biological variation in tHcy. Our findings may be useful in studies involving estimates of an individual’s true mean concentration of tHcy.

The participants in this study were six male and nine female healthy volunteers, ages 19–46 years. Use of medication or dietary supplements known to affect Hcy or folate metabolism were contraindications for participation. We applied strict criteria related to folate metabolism because folate is a strong determinant of Hcy metabolism. All individuals screened were eligible. Volunteers agreed to participate by signing an informed consent form. The Medical Ethical Committee of the Wageningen University approved the study.

On 5 sequential days, 5 mL of venous blood was collected between 0800 and 1000 from fasting (9–11 h) volunteers. Each volunteer’s five visits occurred at the same time in the morning on each day. Participants had their blood collected in the same position, sitting or lying down, throughout the study. Samples were collected in EDTA-containing tubes. Plasma was separated within 30 min and stored at −80°C until analysis.

Plasma tHcy concentrations were measured within 2 weeks after the end of the study on a HPLC with fluorescence detection at our laboratory (6, 7). All samples from one individual were analyzed in duplicate in one analytical run to avoid between-run analytical variation. The within-run analytical CV based on these samples was 4.1%. The between-run CV in this laboratory was known to be 6.0%.

The intercorrelation of tHcy values within individuals over time, i.e., the semivariance, was calculated for intervals of 1, 2, 3, and 4 days. The semivariance, γ(d), is equal to one-half the average squared difference in concentration between any two samples that are d days apart; the sum of the squares is divided by twice the number of pairs available at a specified interval (4). Thus, it is an estimate of the conditional variance of the second measurement, given the outcome of the first one. The formula for this calculation is:

\[ γ(d) = \frac{1}{2n} \sum (x_t - x_{t+d})^2 \]

where d denotes the time interval between any two observations, x denotes the measured values (here tHcy concentration), t denotes one point in time, and n is the number of interval pairs in the summation (4, 5). As the value of d increases, the semivariance gradually increases and will ultimately approach the full within-person variance, i.e., both analytical and biological variation. At this point, the measurements are no longer correlated and the shortest time interval is found for which the maximum biological variation in tHcy is reached (4).

If repeated measurements are more correlated when they are taken at short time intervals than when they are

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**Table 1. Biological variation in plasma tHcy concentrations according to several studies using weekly through monthly sampling strategies.**

<table>
<thead>
<tr>
<th>Design</th>
<th>Participants, n</th>
<th>Biological CV, %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weekly sampling during 5 weeks</td>
<td>24</td>
<td>8.1</td>
<td>(9)</td>
</tr>
<tr>
<td>Weekly sampling during 4 weeks</td>
<td>20</td>
<td>8.3</td>
<td>(10)</td>
</tr>
<tr>
<td>Sampling at 2-month intervals during 1 year</td>
<td>96</td>
<td>8.9</td>
<td>(11)</td>
</tr>
<tr>
<td>Biweekly sampling at four visits</td>
<td>44</td>
<td>9.4</td>
<td>(12)</td>
</tr>
<tr>
<td>Weekly sampling during 4 weeks</td>
<td>20</td>
<td>7.0</td>
<td>(13)</td>
</tr>
</tbody>
</table>
far apart, a model such as the one described in the Appendix can be applied. The within-subject correlation coefficient ($\rho$) between two consecutive measurements, which is called autocorrelation, affects the variance of sums and differences of two consecutive measurements. For uncorrelated measurements ($\rho = 0$), it is a well-known property that the SE becomes smaller by a factor of $1/\sqrt{2} = 0.71$. On the other hand, if the two measurements are completely correlated, i.e., identical, then there is obviously no reduction of variance at all. By plugging in some selected values of $\rho$ and $\sigma^2$ in the model and comparing the resulting curve with the empirical data, we found that values for the autocorrelation in the range of 0.5–0.8 were consistent with the data.

A gradual increase of SD in our data with increasing individual mean values of tHcy measured over 5 consecutive days indicated that the usual assumptions of normality and constant variance were violated. We therefore performed a logarithmic transformation on tHcy. This made it possible to express the variability as a CV. For small to moderate values of the CV, i.e., up to $\sim 20\%$, the (semi)-variance of 100 × ln(tHcy) provides a fair estimate of the squared CV, as can be derived straightforward from the Taylor series: $CV^2 = \frac{\text{Var}[100 \cdot \ln(tHcy)]}{100}$. This is the more natural model to use when within-subject SDs are proportional to the mean value of the variable, as appeared to be the case in our data. All statistical analyses were performed with SAS (Ver. 6.12 for Windows).

Three volunteers were not fasting on the first day but completed the rest of the study. Data from these volunteers were reported as missing on the first day. The plasma tHcy concentrations in the 15 volunteers throughout the 5 days were 4.2–20.9 $\mu$mol/L with a mean of 9.2 (SD, 2.8) $\mu$mol/L.

The relationship between CV and sampling interval is shown in Fig. 1. The values of the semivariance for the different time intervals of 1, 2, 3, and 4 days, based on ln(tHcy) values and expressed as a CV, were 7.9%, 12%, 13%, and 13%, respectively, with corresponding numbers of observation pairs per interval of 57, 42, 27, and 12, respectively. The CV at the time interval of 0 days refers to the analytical variation for duplicates of a single sample, i.e., the within-run analytical CV, which was 4.1% in our study. As Fig. 1 shows, the CV increased monotonically at a decreasing rate when the interval increased from 0 to 4 days. This confirms that measurements on consecutive days were correlated. Beyond the time interval of 2 days, the CV increased very little.

Thus, sampling on consecutive days reduces the benefit of taking the mean of two measurements as a means of improving precision. For uncorrelated measurements, the error would be reduced by a factor of 0.71 when duplicate samples are taken. We estimated that the autocorrelation for consecutive days was 0.6 in this study. Therefore, the error was reduced only by a factor $\sqrt{\frac{1 + \rho^2}{2}} = 0.89$ for a 1-day interval and 0.83 or 0.78 for a 2- or 3-day interval, respectively. For a 4-day interval, the error was reduced by a factor of 0.75 and thus reached the full reduction of a factor of 0.71 that would be achieved if no autocorrelation were present.

This study showed that the CV calculated from the semivariances of two repeated tHcy measurements increases with an increasing sampling interval of 1–4 days. This implicates the presence of autocorrelation of repeated tHcy measurements on short time intervals. The variation hardly increased between intervals of 2–4 days. We therefore suggest that serial blood measurements of tHcy to assess a person’s true mean average should be made at least 2 days apart.

Thirup and Ekelund (8) also examined the day-to-day variation of plasma tHcy on 5 consecutive days, but calculated only one CV instead of interval-specific CVs. They reported a mean within-subject biological CV of 13% (8). We calculated that the within-subject biological variation in our study was 12% at the 4-day interval, which is close to their finding. Studies that used weekly through monthly sampling strategies found lower values for the biological CV for plasma tHcy (Table 1).

We used the method as described by Rotterdam et al. (5) for assessing the optimal time interval in days between repeated cholesterol measurements. Like these authors, we examined the biological variability as a function of time between repeated blood measurements, expressed as a semivariance. An attractive feature of this approach is that it presents different CVs at various time intervals, rather than just one CV.

Because of the presence of autocorrelation, the extent to which the error of the mean can be reduced increases with the time interval between two repeated tHcy measurements. We found that a 4-day interval yields almost the full benefit that can be achieved without the presence of autocorrelation.

In conclusion, this study shows that when the mean of two repeated tHcy determinations is calculated to enhance precision, the measurements should be taken at
least 2 or, even better, 3 or 4 days apart to optimize error reduction.

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References


Appendix

If $x_i$ is a random variate with $E(x_i) = \mu$, $Var(x_i) = \sigma^2$, and $Cov(x_i, x_{i+d}) = \sigma^2 \rho^d$ for $d = 0, 1, 2, \ldots$, then $\rho$ is called the autocorrelation. For this model, the variance of the sum and the difference of two values are:

$$Var(\bar{x}_n + \bar{x}_{n+d}) = \sigma^2 + 2\sigma^2 \rho^d + \sigma^2 = 2\sigma^2(1 + \rho^d)$$
$$Var(\bar{x}_n - \bar{x}_{n+d}) = \sigma^2 - 2\sigma^2 \rho^d + \sigma^2 = 2\sigma^2(1 - \rho^d).$$

Thus it follows that the variance of the average of two measurements is:

$$Var(\bar{x}_n + \bar{x}_{n+d})/2) = 1/4 \cdot 2\sigma^2(1 + \rho^d) = \sigma^2(1 + \rho^d)/2$$

and the semivariance is:

$$1/2\{Var(\bar{x}_n - \bar{x}_{n+d})\} = 1/2 \cdot 2\sigma^2(1 - \rho^d) = \sigma^2(1 - \rho^d)$$

Forty-Eight Hours of Biopsy Culture Improve the Sensitivity of the in Vitro Gliadin Challenge in the Diagnosis of Celiac Disease, Antonio Picarelli,1 Luigi Sabbatella,1 Marco Di Tola,1 Stefania Vettrano,1 Cristina Maffia,1 Cristina Picchi,1 Antonio Mastracchio,2 Paolo Paoluzi,2 and Maria Cristina Anania1 (Departments of 1Clinical Sciences and 2Experimental Medicine, University “La Sapienza”, 155-00161 Rome, Italy; *address correspondence to this author at: Department of Clinical Sciences, Policlinico “ Umberto I ”, University of Rome “La Sapienza”, Viale del Policlinico, 155-00161 Rome, Italy; fax 39-06-49970524, e-mail a.picarelli@flashnet.it)

Celiac disease (CD) is a long-life intolerance to gliadin in genetically susceptible individuals (1–6). Despite contrary views (7), diagnosis is still based on the histologic findings of intestinal mucosal atrophy with crypt hyperplasia in individuals on a gluten-containing diet and a return to normal after a gluten-free diet (GFD). The presence of circulating anti-endomysial antibodies (EMAs) and their disappearance after GFD confirm the diagnosis (8–10). It has recently been shown that EMAs are produced by intestinal mucosa of CD patients. EMAs disappear in treated CD patients but are newly produced after in vitro exposure of intestinal biopsy samples to gliadin (11).

Culture of intestinal mucosa from treated CD patients in the presence of a peptic-tryptic (PT) digest of gliadin for 24 h frequently fails to produce detectable EMAs, but it has high specificity as well as high sensitivity in overt CD (12). Our aim was to increase the sensitivity of the in vitro culture by prolonging the duration to 48 h. We also tested a new culture method in batch to ease testing.

We enrolled 11 untreated, EMA-positive CD patients (5 males and 6 females; mean age, 24.7 years; age range, 17–42 years) and 29 treated CD patients (11 males and 18 females; mean age, 32.0 years; age range, 11–70 years) after at least 12 months of GFD (median length of GFD, 730 days; range, 342–4015 days) and two monthly, consecutive serum EMA tests that were negative. As disease controls we studied 67 patients (20 males and 47 females, mean age, 35.3 years; age range, 20–50 years) with gastrointestinal diseases other than CD (42 with gastroesophageal reflux disease, 8 with ulcer disease, 3 with ulcerative colitis, 3 with Crohn disease, 2 with lymphoma, 1 with small bowel carcinoma, and 8 with Helicobacter pylori infection). All were EMA-negative.

Biopsy samples of duodenal mucosa were obtained from all of these patients for diagnostic purposes. All procedures were in accord with the ethical standards of the responsible institutional committee on human experimentation.