Effect of Time Between Measurements on Within-Subject Variability for Total Cholesterol and High-Density Lipoprotein Cholesterol in Women

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A single blood cholesterol measurement may not accurately reflect an individual's true mean concentration. If duplicate blood samples are taken, what number of days between sampling gives the best chance of detecting the maximum within-subject variation? In this study, we analyzed 20 serial blood samples obtained from each of 13 healthy, menstruating women over 35 days. Variability was calculated as the semivariogram, which gives the average squared difference between replicate samples taken over a range of sampling intervals. Data were available for a complete set of intervals from 1 to 26 days. Variability in total cholesterol (TC) increased as the interval between sampling increased from 1 to 12 days. With high-density lipoprotein cholesterol (HDL-C), variability increased from 1- to 7-day intervals. In practice, our results suggest that, irrespective of the time of menstruation, the minimal interval for collecting a second blood sample for TC and HDL-C assays is ~2 weeks.

Indexing Terms: variation, source of statistics/monthly cycle/semivariogram/time-series analysis/heart disease/biological variation

A single blood measurement seldom coincides with the true mean concentrations of total cholesterol (TC) and lipoproteins (LPs) in blood (1). \(^2\) Because of biological variation (CV\(_b\)) and analytical variation (CV\(_a\)), there is considerable within-subject variability of plasma lipid concentration even when the blood is collected under the same conditions and the lipids are analyzed in the same laboratory (2). Lifestyle factors such as diet, exercise, change of body weight, drugs, and season contribute to the CV\(_b\), which accounts for a major part of the total variability (CV\(_T\)) (3-7). CV\(_a\) arises from the lack of desired precision (reproducibility of measurements) and accuracy (deviation from true value) of the assay; its contribution to CV\(_T\) is potentially small (5-7).

Although the CV\(_b\) of TC has been recognized since 1928 (8), many dietary and epidemiological studies have not taken this into account and still rely on a single blood sample for estimating subjects' average concentrations of TC and LP. In some population screening programs, people have been classified as moderate or high-risk individuals on the basis of a single cholesterol determination (9); investigators in the Whitehall study used a single measure for TC determination (10); and, in some intervention studies, the efficacy of treatment by lipid-lowering drugs or diet has been tested by comparison with only one pretreatment value (11, 12). Genetic studies on the association between plasma lipids or LPs and coronary heart disease have also frequently been based on a single determination of lipids (13-15).

Repeated blood sampling is advocated to improve the reliability of an estimate for TC or LP concentration (1). Great heterogeneity in the sampling number and sampling interval is reported in the literature, with blood samples generally being obtained at intervals ranging from 1 day to several weeks (16-32). As a guide to management of patients with TC ≥5.2 mmol/L, the National Cholesterol Education Program recommended that an average of two test results be taken, between 1 and 8 weeks apart (33); however, the exact interval was not specified.

Most previous studies have calculated variability with the CV. In a review of 15 papers (2, 7, 34-46), we found that the CV for plasma TC (unweighted) averaged 6.3% (range 4.1-9.4%). Although the CV is widely used to describe variability, it does not address the ideal interval between successive blood measurements necessary to sample the maximum CV\(_b\). A metaanalysis of CV\(_b\) in serum lipid concentrations by Smith et al. (46) showed that the sampling interval was a significant source of variability, although interpretation of the interval information was difficult because of confounding between the interval and the number of subjects in the study. The effect of sampling interval on variability is a dimension that has been otherwise neglected. Too short an interval may lead to underestimation of the within-person CV\(_b\), whereas intervals of greater than several weeks may compromise patients' compliance and allow long-term seasonal changes to exert an influence (45, 47).

Given the insufficient information about optimal intervals for sampling, we set out to calculate the CV\(_b\) of TC and high-density lipoprotein cholesterol (HDL-C) as a function of time. We wanted to determine the minimal number of days between repeat cholesterol measurements that would give the greatest difference in variability. We used data from 13 women who had blood taken four times a week over 5 weeks for our time-series analysis. Because concentrations of TC and LP in women may fluctuate with the menstrual cycle (48), we recruited women of reproductive age and sampled their blood at known points in the menstrual cycle. This is seldom possible when a random sample is taken into a study. However, knowledge about the fluctuations of

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2 Nonstandard abbreviations: TC, total cholesterol; LP, lipoprotein; HDL-C, high-density lipoprotein cholesterol; CV\(_b\), biological variation; CV\(_a\), analytical variation; and CV\(_T\), total variability.

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these variables over time will aid in blood sampling with an appropriate interval between repeat analyses.

Subjects and Methods

Subjects

Female volunteers (n = 15, ages 19–37 years) participated in the study. The primary results have been reported elsewhere (49). Subjects were recruited through poster advertisements at the University of Sydney. They were apparently healthy, not taking any regular medication (including oral contraceptives) in the preceding 2 months, and not on a weight-reducing diet or other special diet. Their menstrual cycles had been regular over the preceding 6 months, with a mean cycle length of 28.9 days (range 23–34); normal ovulation was confirmed by hormone measurements. One subject did not complete the study, and another subject, as seen from her hormone profile, did not ovulate (48). Data from the remaining 13 subjects were used for statistical analysis.

Experimental Design

Subjects were participating in a prospective study on variations in plasma amino acids, LPs, and reported food intake during the menstrual cycle. The study extended over 5 weeks, during which time fasting blood samples were taken every Monday, Wednesday, Friday, and Saturday between 0730 and 0900, giving a total of 20 blood samples per subject. The subjects remained seated for at least 15 min before blood was taken by a qualified nurse, who used several different sites in the arm to allow healing of veins before withdrawal of samples. An interview was conducted to determine food and drink consumed the previous evening, and to check compliance with overnight fasting. Subjects traveled to the study site by various means, including walking, cycling, and driving by car, motorbike, or bus. Subjects were asked to use the same means of transport each morning; otherwise, no restriction was placed on morning exercise. Dates of menstruation were obtained during and at the end of the study period, and subjects were weighed weekly. This protocol had the approval of University of Sydney Medical Ethics Review Committee and all subjects gave their informed written consent.

Analytical Methods

Blood samples were collected into tubes containing EDTA or lithium heparin and centrifuged immediately (1200g, 15 min) to minimize osmotic shifts between erythrocytes and plasma (4, 5). Plasma was stored at −80°C until analysis. Because of the short supply of EDTA plasma for two subjects, respective aliquots of EDTA and heparin plasma were pooled for the lipoprotein analyses in these subjects (48). Before deciding to pool the samples, we conducted tests to determine whether the type of anticoagulant, EDTA or heparin, used in sample collection influenced the LP concentrations. No significant differences were found in the mean ± SEM concentration between EDTA and heparin plasma, respectively: TC, 5.66 ± 0.03 vs 5.60 ± 0.05 mmol/L; HDL-C, 0.98 ± 0.02 vs 0.99 ± 0.01 mmol/L (n = 24 each). TC was measured enzymatically (CHOD-PAP Monotest; Boehringer Mannheim, Mannheim, Germany) with a centrifugal autoanalyzer (Cobas Fara; Roche Diagnostica, Basel, Switzerland). HDL-C was measured by the method of Warnick et al. (49). For each analysis, samples from one subject were run within the same assay. Between-assay CVs for TC and HDL-C, estimated from control samples run in each assay, were 2.7% (n = 24) and 3.0% (n = 22), respectively. Within-assay CVs for TC and HDL-C were 1.8% (n = 18) and 1.5% (n = 20), respectively.

Statistical Analysis

The mean CVi for TC and HDL-C (n = 13) was calculated, and, using the estimated values for CVi, and between-assay CVsn, we calculated CVb according to the following formula (46):

\[ (CV_b)^2 = (CV_i)^2 - (CV_n)^2 \]

To study the interdependence of TC or HDL-C values for each subject over time, we calculated the semivariogram, γ(d) = \( \sum (x_i - x_{i+2})^2 / 2n \), where d denotes the time interval between any two observations, \( x \) denotes the measured values (in our case TC or HDL-C), t denotes one position in time, and n is the number of sample pairs (50). The semivariogram is an estimate of the average squared difference between any two samples at a specified interval apart. As the time interval between observations increases, the correlation decreases to the point where eventually the values are effectively independent. Therefore, as the value of d increases, the semivariogram increases to a plateau that corresponds to the full variance of x. One aim of this study is to estimate the smallest value of d for which \( \gamma(d) \) can be regarded as having reached the plateau. This value of d will correspond to the smallest intersampling interval necessary to obtain effectively uncorrelated samples from a given subject, that is, the smallest time interval needed to obtain the greatest variation between repeat samples. Semivariograms for TC and HDL-C were calculated for each subject for intervals of 1 to 26 days, and the mean semivariogram value was obtained for each interval. The total number of sample pairs used to calculate the mean semivariogram for each interval was obtained by multiplying the number of pairs available for that interval over the 5 weeks by the number of subjects (n = 13). We then converted the semivariograms for TC and HDL-C to their corresponding CVs, according to the method of Rotterdam et al. (45), in which the square root of the semivariogram was divided by the mean concentration of TC or HDL-C and expressed as a percentage. The mean CVs of the semivariograms were plotted as a function of sampling interval from 1 to 26 days; to observe the underlying trends in variability more clearly, we smoothed the data by the technique of moving averages (order 3) in which three successive data points are progressively averaged (51). Although the study lasted 35 days, calculation of the
semivariograms beyond intervals of 26 days was unreliable, there being too few sample pairs available per interval.

Results

Mean ± SEM concentrations of TC and HDL-C were 4.47 ± 0.85 mmol/l and 1.30 ± 0.24 mmol/l, respectively (n = 13). CVs, CVs, and calculated CVs, are given in Table 1. The CVs for TC and HDL-C (8.3% and 8.0%, respectively) were comparable with values reported in previous studies (2, 7, 34–46). For both TC and HDL-C, ~75% of the observed variability was biological.

The numerical values for the semivariograms are listed in Table 2. Between 26 and 208 sample pairs were used to calculate each value. The CVs of the semivariograms for TC and HDL-C from 1- to 26-day sampling intervals are shown in Fig. 1, with corresponding smoothed data in Fig. 2. The CVs for TC rose from 5% to a maximum of 10% as the distance between blood sampling increased from 1 to 12 days, and remained relatively constant up to 20-day intervals; there was a nonsignificant fall in CV between the 21- and 26-day intervals (Figs. 1A and 2A). The CVs for HDL-C rose from 5% to 8% as sampling intervals increased from 1 to 7 days; there was a nonsignificant second increase between 22- and 26-day intervals (Figs. 1B and 2B).

Discussion

In this study, data for calculation of a semivariogram were available for consecutive intervals from 1 to 26 days. Variability in TC concentrations rose from 5% to 10% as the interval between sampling increased from 1 to 12 days. A similar pattern was observed with HDL-C, with the variability increasing from 5% to 8% over sampling intervals of 1 to 7 days. At longer intervals of up to 26 days, the sample pairs became effectively independent, and variability remained relatively constant.

These results demonstrated that a minimum sampling interval of ~12 days was required to obtain the widest variation between repeat samples. On the basis of these findings, we suggest that the best estimates of the true mean concentrations for plasma TC and HDL-C can be

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**Table 1. Mean variabilities of determinations for TC and HDL-C.**

<table>
<thead>
<tr>
<th>Interval (days)</th>
<th>TC CVa</th>
<th>HDL-C CVa</th>
<th>CVb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.3</td>
<td>8.0</td>
<td>2.7</td>
</tr>
<tr>
<td>2</td>
<td>2.7</td>
<td>3.0</td>
<td>7.8</td>
</tr>
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</table>

CVa was estimated from control samples run in each assay; CVb was calculated according to the formula (49): CVb = CVa² - CVa².

**Table 2. Mean semivariograms for TC and HDL-C (n = 13).**

<table>
<thead>
<tr>
<th>Interval between sampling (days)</th>
<th>TC CV</th>
<th>HDL-C CV</th>
<th>No. of observation pairs per intervala</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.037</td>
<td>0.004</td>
<td>65</td>
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<tr>
<td>2</td>
<td>0.059</td>
<td>0.006</td>
<td>182</td>
</tr>
<tr>
<td>3</td>
<td>0.076</td>
<td>0.006</td>
<td>119</td>
</tr>
<tr>
<td>4</td>
<td>0.108</td>
<td>0.010</td>
<td>115</td>
</tr>
<tr>
<td>5</td>
<td>0.118</td>
<td>0.009</td>
<td>169</td>
</tr>
<tr>
<td>6</td>
<td>0.110</td>
<td>0.012</td>
<td>52</td>
</tr>
<tr>
<td>7</td>
<td>0.229</td>
<td>0.012</td>
<td>208</td>
</tr>
<tr>
<td>8</td>
<td>0.138</td>
<td>0.011</td>
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</tr>
<tr>
<td>9</td>
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<td>0.011</td>
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<td>15</td>
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<td>23</td>
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<td>0.019</td>
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<tr>
<td>26</td>
<td>0.156</td>
<td>0.021</td>
<td>52</td>
</tr>
</tbody>
</table>

**Fig. 1.** Mean within-subject CVs (n = 13) of semivariograms for TC (A) and HDL-C (B) as a function of time between repeat blood measurements. Vertical bars represent the SEM.
obtained by taking repeat blood samples ~2 weeks apart.

To our knowledge, only two studies have examined the within-person variability in TC and HDL-C as a function of the sampling time interval. In the first of these, Harris (52) collected data from 29 male students and calculated the variance of the differences in TC between successive observations at intervals of 1, 7, and 30 days. He examined only these three intervals and did not calculate semivariograms. There was a linear increase in variance from the 1-day to the 30-day interval. The only previous paper reporting variability in terms of the semivariograms for plasma TC and HDL-C was by Rotterdam et al. (45). Data were derived from 22 men and 19 women participating in four different dietary trials that lasted 2–4 weeks. Variability was plotted as a function of sampling interval from 1 to 18 days, although data were available for only six intervening intervals: 3, 4, 7, 10, 11, and 14 days. These authors found an increase in variability as the interval increased from 0 (simultaneous duplicates) to 4 days; they also noted a small, nonsignificant second increase between the 10- and 14-day intervals. The maximum variability of ~5% reported by Rotterdam et al. (45) for intervals of 10 to 14 days was less than the corresponding value of ~10% we observed in our study. Different lifestyle conditions might explain the discrepancy in the two data sets; subjects in their study were participating in metabolic experiments in which diet was strictly controlled, whereas our subjects were free-living.

Concentrations of lipids and lipoproteins can undergo fluctuations with the menstrual cycle. In our previous analysis (48) of the current data, we reported that the maximum increase in TC and HDL-C occurred between the time of menstruation and ovulation, ~14 days apart. Compared with TC, the increase in HDL-C at ovulation was more prolonged and the concentrations remained increased until the following premenstrual phase. These hormonally determined changes would be expected to exert a significant effect on the current analysis and could be reflected in the maximum variability we observed at sampling intervals of ~2 weeks. Furthermore, because calculation of the semivariogram for a particular interval includes all possible combinations of sample pairs, a 2-week interval would be expected to maximize the within-subject variation, irrespective of the day of the menstrual cycle. We also suggest, in light of our results, that the second increase in variability at 10- to 14-day intervals reported by Rotterdam et al. (45) may have been due to their use of female subjects.

Our data for within-person variability represents both CVa and CVn (we did not separate these components). Possible sources of CVa include changes in diet or lifestyle factors such as alcohol consumption and exercise, but also fluctuations in ovarian hormones (4–6, 48). In the longer term, seasonal changes could also exert an effect, although our 5-week study was too short for these to be an important influence. With regard to CVn, our subjects were representative of the normal population of menstruating women: They were free-living, not following any special dietary regimen, and, as confirmed by hormonal measurements (48), had typical ovulatory cycles with a mean (range) cycle length of 29 (23–33) days. Therefore, the variability in blood lipid concentrations we observed is probably typical of the normal variability in this group. CVn can contribute significantly to the CVa, particularly in the measurement of HDL-C, where the analysis involves a precipitation procedure (43, 46, 53–55). In view of this potential source of error, some authors have suggested that a single estimate of HDL-C measured in an individual may not be useful in the primary assessment of risk for heart disease (54). However, even after accounting for factors known to affect plasma TC and HDL-C concentrations, a considerable proportion of the variability for TC and HDL-C remains unexplained (3). Characterization of the usual variations in plasma TC and HDL-C, as conducted in this study, will enable the use of duplicate blood samples to come nearer to reflecting a subject’s true mean values.

We believe this study is the first to examine the within-subject variability in blood samples from young women over a 5-week period. In our previous analysis of the current data examining fluctuations of TC and HDL-C during the menstrual cycle, we observed peaks and troughs occurring ~2 weeks apart (48). By applying time-series analysis of variability to these same data (reported here), we also find the maximum difference at ~2-week intervals. Given the mutual support of these two sets of results, we propose that the best estimates of

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 2. Smoothed CVs of semivariograms for TC (A) and HDL-C (B) as a function of time between repeat blood measurements.
the true mean for concentrations of plasma cholesterol and HDL-C in young women can be obtained by taking samples 2 weeks apart.

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References

41. Costongs GMPJ, Jansen PCW, Bas BM, Hermans J, Van