Variability in the measurement of C-reactive protein in healthy subjects: implications for reference intervals and epidemiological applications

Elizabeth M. Macy,1 Timothy E. Hayes,1 and Russell P. Tracy1,2*

We developed a reproducible ELISA for C-reactive protein (CRP), calibrated with WHO Reference Material, for which intra- and interassay CVs were 3.0% and 6.0%, respectively. Analytical recovery was 97.9%. The distribution of CRP in a healthy blood donor population (n = 143) was nongaussian, with 2.5th, 50th, and 97.5th percentile values of 0.08, 0.64, and 3.11 mg/L, respectively. There was no sex-related difference, and the association with age was weak. In a study of variability [by the method of Fraser and Harris (Crit Rev Clin Lab Sci 1989;27:409–37)], the analytical variability was 5.2%; the within-subject variability, CVI, was 42.2%; and the between-subject variability, CVG, was 92.5%. The critical difference for sequential values significant at \( P < 0.05 \) (i.e., the smallest percentage change unlikely to be due to analytical variability or CVI) was calculated as 118%, and the index of individuality, CVI/CVG, was 0.46. This suggests that CRP, like many clinical chemistry analytes, has limited usefulness in detecting early disease-associated changes when used in conjunction with a healthy reference interval. From a molecular epidemiological standpoint, the usefulness of CRP in longitudinal studies is suggested by the small index of individuality and by observations that (a) short-term fluctuations were infrequent, (b) all data stayed within the reference interval, and (c) relative rankings of the subjects over 6 months only moderately deteriorated.

INDEXING TERMS: inflammation • cardiovascular disease • variation, source of • index of individuality • critical difference • molecular epidemiology

We purchased biotinyl-\( \epsilon \)-aminocaproic acid-\( N \)-hydroxysuccinimide ester (Biotin-X-NHS), human CRP, and anti-CRP rabbit IgG from Calbiochem-Novabiochem, La Jolla, CA. We obtained 5 mol/L sulfuric acid, from Fisher Scientific, Fair Lawn, NJ; citric acid (anhydrous powder), polyoxyethylene (20) sorbitan monolaurate (Tween 20),

Materials and Methods

REAGENTS
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3 Nonstandard abbreviations: CRP, C-reactive protein; BSA, bovine serum albumin; DMF, \( N \),\( N \)-dimethylformamide; ABC, avidin–biotin complex; PPACK, \( d \)-phenylalanyl-prolyl-arginyl-chloromethyl ketone; PBS, phosphate-buffered saline; CVD, cardiovascular disease; CVI, analytical CV; CVG, within-subject CV; and CVG, between-subject CV.
and Nunc Maxisorp (immuno-) microtiter plates from J. T. Baker/VWR Scientific, Rochester, NY; bovine serum albumin (RIA-grade Fraction V; BSA); NaHCO₃; Na₂CO₃; NaCl, Na₂HPO₄, N,N-dimethylformamide (DMF), EDTA (tetrasodium salt), H₂O₂ (300 g/L solution), and o-phenylenediamine from Sigma Chemical Co., St. Louis, MO; and peroxidase-labeled avidin–biotin complex (ABC) from Vector Labs., Burlingame, CA. All other reagents were of the highest grade available.

CRP was biotinylated as follows: human CRP, previously dialyzed in 100 mmol/L NaHCO₃ adjusted to pH 8.5 with 5 mol/L HCl, was combined with activated biotin at the rate of 100 μL of 44 mmol/L biotin in dry DMF per milliliter of 100 mmol/L NaHCO₃ containing 10 mg of the protein. The biotin had been activated by combining biotin and DMF in a glass container, 2.0 mg of Biotin-X-NHS per 100 μL of dry DMF. The biotin/DMF, 4.0 μL, was added to 400 μg of previously dialyzed CRP and reacted with gentle agitation for 4 h at room temperature. The resulting biotinylated CRP was dialyzed overnight in phosphate-buffered saline (PBS; 40 mmol/L Na₂HPO₄ and 130 mmol/L NaCl, adjusted to pH 7.4 with 5 mol/L HCl) and then stored at −20 °C in 500 g/L glycerol solution.

Purity of the human CRP and the biotinylated product was assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol, according to Laemmli.[12] Both CRP and biotinylated CRP produced a single molecular mass band corresponding to CRP subunits (Mr 23 037)[13].

**ASSAY PROTOCOL**

Coating antibody (rabbit IgG to human CRP) was diluted to 2.75 mg/L in sodium carbonate buffer (16 mmol/L Na₂CO₃, 34 mmol/L NaHCO₃, adjusted to pH 9.5 with 5 mol/L HCl) and pipetted, 200 μL per well, into a Nunc Maxisorp microtiter plate. The plate was covered with tape and incubated for 18 h at 4 °C. Unoccupied binding sites on the plate were then blocked for 2 h with 250 μL per well of PBS plus 20 g/L BSA, pH 7.4. Plasma samples, controls, calibrators, and biotinylated CRP were diluted in diluent buffer: PBS plus 1.0 g/L Tween 20, 20 g/L BSA, and 5 mmol/L EDTA, pH 7.4. Plasma samples were assayed at 10-fold dilution. We used a nine-point calibration curve beginning at 10 mg/L CRP and diluted threefold serially. Biotinylated CRP, stored at −70 °C in aliquots adequate for use with one assay plate, was determined to work in this assay at a dilution of 54 000-fold. After washing the wells twice with PBS plus 1.0 g/L Tween 20, pH 7.4, we loaded the plates with 100 μL per well of diluted samples, controls, and calibrators and then rapidly added 100 μL per well of diluted biotinylated CRP. The plate was agitated briefly, covered with tape, and incubated overnight at 4 °C.

Freshly prepared horseradish peroxidase-conjugated ABC (ABC reagent) was prepared by adding 1 drop of reagent A and 1 drop of reagent B per 21 mL of PBS-Tween 20 (PBS + 1.0 g/L Tween 20), pH 7.4, and then incubating for 60 min at room temperature. After washing the assay plates four times with PBS-Tween 20, we added 200 μL of ABC reagent per well and incubated the assay plate for 90 min. We repeated the washing procedure and added to each well 200 μL of substrate solution: freshly prepared o-phenylenediamine diluted to 0.2 g/L in citrate buffer (0.1 mol/L citric acid, 0.2 mol/L Na₂HPO₄, adjusted to pH 5.0 with 25 mol/L NaOH) and activated by addition of 0.039 g/L H₂O₂. After incubating the plate for 30 min at room temperature, the dark, we stopped the enzymatic reaction with 4 mol/L H₂SO₄, 50 μL per well. The color formed was read at 490 nm.

For assay standardization, we used purified human CRP that was calibrated against the 1st International CRP Standard (85–506; established in 1986 by the WHO Expert Committee on Biological Standardization, and available from the National Institute for Biological Standards and Control, Herts EN6 3QG, UK).

**BLOOD SAMPLES**

Blood samples were collected from seated volunteers with use of a free-flowing blood-draw and a maximum 2-min tourniquet application. The blood was immediately stored on ice and centrifuged within 60 min for at least 30 000 g-min. Plasma was then stored at −70 °C. Samples were also obtained from the American Red Cross under the same conditions except that volunteers were given the option to lie prone for the blood draw. All procedures were approved by the University of Vermont Committees on Human Research.

For a study of CRP in 143 healthy individuals, blood samples were collected in a citrate–phosphate–dextrose–adenine erythrocytes preservative solution from Baxter-Senwall (Roundlake, IL). For assay validation and variability studies, we used blood samples drawn into serum tubes, citrate-containing blood-collection tubes (0.129 mol/L of citrate/citric acid, one-tenth total volume), and EDTA (K₃)-containing tubes, all from Becton Dickinson (San Jose, CA). We also used SCAT-1 tubes, which contain 200 000 kIU/L aprotinin, 25 μmol/L N-phenylalanyl-prolyl-arginyl-chloromethyl ketone (PPACK), and 4.5 mmol/L EDTA, and SCAT-2 tubes, which contain 25 μmol/L PPACK and 11 mmol/L citrate/citric acid (both from Hematological Technologies, Essex Junction, VT). We have used these tubes frequently for studies of coagulation factors, where thrombin activity must be minimized.[14]

**POPULATION STUDIES**

Citrated plasma samples from 143 blood donors, ages 18 to 67 (69 women and 74 men), were acquired from the Vermont–New Hampshire Red Cross Center, Burlington, VT. For comparison of CRP concentrations and erythrocyte sedimentation rates, we used citrated plasma samples from the Clinical Coagulation Laboratory of Fletcher Allen Health Care.
For a study of CRP variability, 26 individuals (10 male, 16 female) recruited from the university community came into the NIH General Clinical Research Center at Fletcher Allen Health Care for a single fasting phlebotomy in the early morning, at 3-week intervals, for a total of eight visits. Each subject was questioned briefly as to state of health. Blood was collected into one citrate tube and one SCAT-1 tube. In addition, four individuals recruited from our laboratory provided morning blood samples twice a week for 8 weeks; a light breakfast was recommended if the fasting state could not be maintained. These subjects’ blood was collected in SCAT-1 tubes and processed as described above.

**Other Assays**

In addition to the ELISA, we also determined CRP with the Magiwell CRP kit (United Biotech, Mountain View, CA), according to manufacturer’s directions. Erythrocyte sedimentation rate was determined in the Clinical Coagulation Laboratory. Cholesterol was measured with an Ektachem E-700 dry-film analyzer (Johnson & Johnson Clinical Diagnostics, Rochester, NY).

**Statistical Analyses**

Calibration curves were generated by using the four-parameter fitting algorithm of Titercalc data reduction software (Hewlett-Packard, Sunnyvale, CA). CVs based on control pool values were calculated as (SD/mean) × 100%. Pearson correlation coefficients were generated by using the linear regression subroutine of GBSTAT statistical software (Dynamic Microsystems, Silver Spring, MD) and were compared with one another as described by Snedecor and Cochran [15]. Where mean values were compared, we used Student’s t-test (paired samples, two-tailed). Nonparametric statistical tests were used for data that were not gaussian in distribution.

The 0.95 nonparametric interfractile interval for a study of reference values for CRP was calculated with SPSS statistical software (Marija J. Norusis/SPSS Inc., Chicago, IL). Analysis of the biovariability data was done as described by Fraser and Harris [16]. Three CVs were calculated, representing analytical, within-subject, and between-subject variation (CV_A, CV_P, and CV_G, respectively). We also calculated the index of individuality (CV_I/CV_G); the index of heterogeneity, i.e., the ratio of CV_I to the theoretical CV ([2/(n – 1)]^{1/2}, where n = average number of time points); and the critical difference, i.e., the percentage difference in value that is significant at P ≤0.05: 2.77 × (CV_A^2 + CV_P^2)^{1/2}. Before these calculations, we applied three levels of outlier tests to remove outlier points (as described in [16]). Four points were identified and removed from the total of 200 points.

**Results**

Response curves for human CRP serially diluted threefold in buffer vs dilutions of a citrated plasma pool were compared visually and were judged to be parallel throughout the optimum measurement range. As a formal estimate of the detection limit of the assay, the mean ± 3 SD absorbance of the “zero calibrator” (n = 33 in a single run) corresponded to 0.007 mg/L. The CV of the 0.004 mg/L CRP calibrator was 23.7% (n = 14) and the CV of the 0.014 mg/L calibrator was 9.4%. Therefore, we estimate that, for samples diluted 10-fold, CRP in the range of 0.08 to 9.0 mg/L (corresponding approximately to the middle 85% of the absorbance range for the calibration curve) could be measured consistently with reasonable accuracy.

In the interest of minimizing interplate variability, we prepared multiple aliquots of coating antibody, assay calibrator, and biotinylated CRP, each sufficient for one plate, and stored them at –70 °C. Human CRP, 1.9 mg/L, was added to 25 citrated, reference plasma samples. The average analytical recovery (±SD) was 97.9% ± 22.1% (range 46.7–128.8%); 20 of the 25 samples showed recoveries between 75% and 125%. In several experiments we have consistently observed a small number of samples with relatively poor recovery but as yet have no explanation for this.

Intraassay variability was assessed by assaying a single citrated plasma pool 40 times; the mean value was 0.80 mg/L and the CV was 3.0%. Interassay variability was determined by assaying three plasma controls (including one lyophilized lot) once each per plate for 25 plates divided into four assay runs. The mean values of the controls were 1.05, 2.52, and 2.08 mg/L, and the interassay CVs were 5.7%, 6.8%, and 5.5%, respectively. Diurnal changes (0845 vs 1545 h) in CRP were evaluated by assaying citrated plasma from nine healthy subjects; however, the median values for samples collected at each time were the same, 0.93 mg/L CRP, indicating no significant diurnal variation (P = 1.00; Wilcoxon matched pairs signed-rank test).

Reference values for CRP were determined by the methods described by Solberg [17, 18] and recognizing the work of the IFCC Expert Panel on the Theory of Reference Values. For citrated plasma samples collected from 143 healthy individuals, the median CRP concentration was 0.64 mg/L and the 0.95 interfractile interval was 0.08–3.11 mg/L. The median for the 74 men was 0.64 mg/L (range 0.02–3.25 mg/L) and for the 69 women was 0.63 mg/L (range 0.07-3.24 mg/L), indicating no major gender difference. Stratification of data into subgroups, according to the method of Harris and Boyd [19], on the basis of gender was thus not required. The distribution of CRP values for this healthy group (Fig. 1) was nongaussian but was reasonably well normalized by log_{10} transformation. The Pearson correlation coefficient (r) between log_{10}-transformed CRP values and age was 0.20 (P ≤0.02), indicating a relatively weak, but significant, positive age association for CRP in healthy middle-aged persons.

To compare sample types we have used in various epidemiological studies, we obtained serum, SCAT-1
plasma, SCAT-2 plasma, EDTA plasma, and citrate plasma samples from six individuals. Median values were: serum, 0.65 mg/L; SCAT-1, 0.61 mg/L; SCAT-2, 0.65 mg/L; citrate, 0.58 mg/L; and EDTA, 0.70 mg/L. These values were significantly different (Friedman’s test, \( P < 0.002 \)), primarily because of the values from the citrate tubes (\( P < 0.028 \), Wilcoxon matched pairs signed-ranks test: serum vs citrate plasma). We thought it likely that values were lower in sodium citrate tubes because of the 9:1 dilution of sample by the liquid sodium citrate. This was confirmed by linear regression analysis, where: citrate CRP = 0.84 × serum CRP − 0.02 mg/L (\( r = 0.974 \), \( P = 0.003 \), \( n = 6 \)).

In a freeze/thaw experiment, we thawed once, twice, three, and four times samples of citrated plasma, SCAT-1 plasma, serum, SCAT-2 plasma, and EDTA plasma from at least five individuals, and then assayed for CRP with the ELISA. No significant differences in mean values were found for any individual or any anticoagulant type, indicating that CRP is stable to multiple freeze/thaw cycles.

We compared our CRP ELISA with other assays for acute-phase reaction. Between CRP and the erythrocyte sedimentation rate in 14 patients’ serum samples obtained over 24 h from our clinical hematology laboratory, \( r = 0.68 \). For CRP ELISA (\( y \)) vs CRP Magiwell (\( x \)), linear regression of results for plasma samples from 27 healthy individuals showed excellent correlation (\( r = 0.94 \), \( P <0.0001 \)), although the values from our assay were systematically lower than those reported by the commercial method: \( y = 0.62 \) (SE 0.05) \( x + 0.41 \) (SE 0.09) mg/L.

Figure 2 shows the CRP values for 26 individuals from whom samples were obtained every 3 weeks for 8 time points. All values were below the clinical cutoff of 10 mg/L [20], and values for some individuals were more tightly grouped than for others; nonetheless, it is clear that over this 6-month period individuals tended to have relatively stable CRP values. For example, CRP concentrations for subject 10 tended to be high at each blood drawing, whereas those for subject 19 were relatively low.

In a second study, involving a shorter sampling interval, plasma samples were obtained from 4 healthy individuals twice a week for 8 weeks (Fig. 3; one individual only had 6 time points). These data support the first data set, in that two individuals appeared to have relatively low values throughout the study, whereas two others had relatively high values (although still below the generally accepted clinical cutoff of 10 mg/L). Also, these data indicate the presence of short-term fluctuations (i.e., single points that seem high), even though all data stayed below the clinical cutoff.

To determine the consistency of the relationships between the 26 individuals, we correlated the values for the
first blood draw to the values for the second blood draw, the third, and so forth. Table 1 shows that the values from the first blood draw correlated with the values from the later blood draws about as well as (or better than) they did with the values from the earlier blood draws, as judged by the Pearson correlation coefficient and the Spearman rank correlation coefficient. Both statistical methods gave similar results, although the raw data tend to be better correlated than the rankings were. A total of three high CRP values (attributable to respiratory infections) were removed from blood draws 2, 3, and 4. A test of significance showed that, in both Pearson and Spearman analyses, the first correlation coefficient differed from the second, third, and last but not from the other three ($P \leq 0.05$). Regression analysis revealed no significant trend in $r$ values over time for either the Pearson or the Spearman coefficients.

Statistical analysis according to the method of Fraser and Harris [16] was performed to determine the components of variability. After removal of the outlier points, values for analytical, within-subject, and between-subject CVs were computed and are shown in Table 2, along with values from the one other study of CRP variability we found in the literature. For comparison with a commonly measured epidemiological analyte, we also determined cholesterol concentrations in these same samples.

### Table 1. Pearson and Spearman correlations from blood draw 1 to blood draws 2 through 8 for CRP in 26 individuals enrolled in a 6-month longitudinal study.

<table>
<thead>
<tr>
<th>Correlated blood draw</th>
<th>Pearson <em>r</em></th>
<th>Pearson <em>p</em></th>
<th>Spearman <em>r</em></th>
<th>Spearman <em>p</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.955</td>
<td></td>
<td>0.844</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.849</td>
<td>$\leq 0.05$</td>
<td>0.449</td>
<td>$\leq 0.05$</td>
</tr>
<tr>
<td>4</td>
<td>0.604</td>
<td>$\leq 0.05$</td>
<td>0.455</td>
<td>$\leq 0.05$</td>
</tr>
<tr>
<td>5</td>
<td>0.896</td>
<td>NS</td>
<td>0.858</td>
<td>NS</td>
</tr>
<tr>
<td>6</td>
<td>0.914</td>
<td>NS</td>
<td>0.695</td>
<td>NS</td>
</tr>
<tr>
<td>7</td>
<td>0.898</td>
<td>NS</td>
<td>0.684</td>
<td>NS</td>
</tr>
<tr>
<td>8</td>
<td>0.730</td>
<td>$\leq 0.05$</td>
<td>0.520</td>
<td>$\leq 0.05$</td>
</tr>
</tbody>
</table>

*a* The 26 CRP values obtained from the samples of the first blood draw were correlated to the values from the samples of the second blood draw, and so forth.

*b* Significance of the differences between each $r$ value and the first $r$ value. NS, not significant.

### Discussion

As our knowledge of the biochemistry of cytokine-mediated inflammation has increased, interest in CRP as a marker of this process has increased as well. Inflammation has been implicated in cardiovascular disease (CVD) in several ways [21–25], and CRP concentrations have been shown to be related to risk of recurrent ischemic events in patients with unstable angina [6, 9]. We have recently identified CRP as an independent prospective risk factor for CVD in healthy older women [5]. The use of CRP as a variable in longitudinal epidemiological research is increasing, which supports the need for studies of variability.

The immunoassay we developed is easy to perform with commercially available materials and correlates well with a commercial assay. However, these two assays appear to be standardized differently, given that the slope of the regression line comparing the assays was not 1.0. Because we used WHO Reference Material 85–506 for standardization, the source of this difference in absolute values is unclear.

The median value for CRP in this study was 0.64 mg/L, the 0.95 interfractile reference interval 0.08–3.11 mg/L. These values are slightly lower than those of Kindmark [26], which are often quoted as 0.20–6.10 mg/L. However, our results are still consistent with the generally held clinical position that 10.0 mg/L is a cutoff for significant inflammatory disease [20].

The median value for CRP concentrations in the range of 3.0–10.0 mg/L is not well understood at this time. Values in this area, or even values in the higher portion of the healthy reference interval, could represent minimal inflammatory activity in some or all individuals. Chambers et al. have shown that CRP does increase in minimal experimental inflammation but often stays within the reference interval [27]. From an epidemiological standpoint, we are interested in knowing whether values in the higher portion of healthy reference interval, possibly representing slight but important CVD-associated inflammation, are associated with any additional risk for CVD.

Concerning variability from an epidemiological standpoint, our data suggest that over a 6-month period CRP values appear relatively tightly regulated, with some individuals having consistently higher values than others. Although some values ranged upwards for an individual for one measurement (e.g., individual 6, Fig. 2), these changes were transient. The shorter-term study (Fig. 3) supports this consistency. Even with a much shorter sampling interval (every 3–4 days), individuals still appeared to have consistent, and consistently different, CRP values.

### Table 2. Variability analysis for cholesterol (mmol/L) and CRP (mg/L).

<table>
<thead>
<tr>
<th></th>
<th>This study</th>
<th>Other studies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td>CRP</td>
</tr>
<tr>
<td>Mean conc.</td>
<td>5.1</td>
<td>1.7</td>
</tr>
<tr>
<td>CVX, %</td>
<td>1.0</td>
<td>5.2</td>
</tr>
<tr>
<td>CVI, %</td>
<td>9.3</td>
<td>42.2</td>
</tr>
<tr>
<td>CVG, %</td>
<td>19.4</td>
<td>92.5</td>
</tr>
<tr>
<td>Index of individuality</td>
<td>0.48</td>
<td>0.46</td>
</tr>
<tr>
<td>Index of heterogeneity</td>
<td>2.59</td>
<td>2.92</td>
</tr>
<tr>
<td>Critical difference, %</td>
<td>26</td>
<td>118</td>
</tr>
</tbody>
</table>

$^a$ [29].

$^{b}$ [11].
Whether this regulation is under the same cytokine control as occurs in inflammation, or whether some other mechanism is in play, is unknown. Given our recent observations in a population of healthy individuals of a strong correlation between CRP and another protein believed to be regulated to a large extent by interleukin-6, fibrinogen (R. Tracy, unpublished), we believe there is significant coregulation in the absence of overt inflammation and that cytokines are important in regulating the concentrations of these proteins in healthy individuals.

The calculated values for \( CV_A \), \( CV_B \), and \( CV_C \) are similar to those values reported by Clark and Fraser [11], confirming their observation that a CRP measurement value, used in conjunction with a CRP reference interval, is less than optimal for use in diagnosis, especially of early disease. A relatively large \( CV_C \), coupled with a relatively small \( CV_B \) (yielding a low value for the index of individuality), means that many individuals can have early disease-associated changes that do not exceed the reference interval. The calculated “critical difference” (at \( P \leq 0.05 \)) between two serial values for CRP they determined to be 175% [11], whereas we calculated a critical difference of 118%—again supporting the position that relatively large changes in CRP are required before one can feel certain that the change is significant. This difference in critical difference values is due to the smaller within-subject variance in our group compared with theirs, which is reflected in the smaller index of individuality.

Fraser and Harris have reviewed the clinical uses of laboratory tests and the ways in which variability affects the usefulness of a test [16]. However, our interests in molecular epidemiology differ, in some ways, from those of the clinical laboratorian. For example, the characteristics of CRP just mentioned, i.e., small \( CV_B \), large \( CV_C \), can be advantageous for the commonly used epidemiological technique of linear regression. Under some circumstances, an increased “spread” of the data may give this technique more power to detect small, but nonetheless important, correlations with other variables. For comparison purposes, Table 2 includes data we generated for cholesterol values, used in conjunction with a CRP reference interval, particularly important from a molecular epidemiological standpoint. Variability data may be incorporated into multivariate prediction models, as suggested by Liu [28], although this is not yet commonly done in molecular epidemiological research.

We thank Gail Paige of the American Red Cross for assistance in collecting the samples, and Monica Sullivan, Dan Gibson, and Mary Tang of Fletcher Allen Health Care for assistance with the cholesterol measurements. This study was supported by the National Institutes of Health (Bethesda, MD) grant NIH RO1 HL46696 (R.P.T).

References