Kristine S. Alexander, Steven C. Kazmierczak, Caryn K. Snyder, Jon A. Oberdorf and David H. Farrell*

Prognostic utility of biochemical markers of cardiovascular risk: impact of biological variability

Abstract

Background: Although a variety of biochemical markers are used to help predict the risk of cardiovascular disease, the prognostic utility of any marker used as a risk assessment tool is dependent on the long- and short-term biological variability that the marker shows in different individuals.

Methods: We measured total, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol; triglycerides; high-sensitivity C-reactive protein (hsCRP); total fibrinogen; and γ′ fibrinogen in blood samples collected from 15 apparently healthy individuals over the course of 1 year. Repeated measures variation estimates were used to calculate short- and long-term intraclass correlation coefficients (ICC), within- and between-subject coefficients of variation (CV_I and CV_G, respectively), validity coefficients, and indices of individuality for each marker.

Results: HDL cholesterol demonstrated the lowest variability profile, with an ICC of 0.84 and CV_I of 11.1 (95% CI: 8.3, 17.0). hsCRP showed the highest levels of short- and long-term within-subject variability [CV_I (95% CI): 54.8 (32.8, 196.3) and 77.1 (53.3, 141.3), respectively]. Stated differently, it would require five separate measurements of hsCRP, performed on samples collected over multiple days, to provide the risk assessment information provided by a single measurement of HDL cholesterol. γ′ Fibrinogen demonstrated an ICC of 0.79 and CV_I of 14.3 (95% CI: 10.6, 21.9).

Conclusions: hsCRP showed very high biological variability, such that a single measurement of hsCRP lacks sufficient clinical utility to justify routine measurement. The variability profile of γ′ fibrinogen was not markedly different than HDL cholesterol, necessitating only a limited number of measurements to establish an individual's risk of cardiovascular disease.

Keywords: biomarker; cardiovascular disease; inflammation; risk factor; variation.

***Corresponding author: David H. Farrell,** Knight Cardiovascular Institute, Oregon Health and Science University, 3181 S.W. Sam Jackson Park Road, Mail Stop MQ280, Portland, OR 97239–3098, USA, Phone: +1 503 494 8602, Fax: +1 503 222 2306, E-mail: farrelld@ohsu.edu

Kristine S. Alexander: Department of Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR, USA

Steven C. Kazmierczak: Department of Pathology, Oregon Health and Science University, Portland, OR, USA; and DiabetOmics, Inc., Beaverton, OR, USA

Caryn K. Snyder: DiabetOmics, Inc., Beaverton, OR, USA

Jon A. Oberdorf and David H. Farrell: Gamma Therapeutics, Inc., Portland, OR, USA

Introduction

Clinical laboratory tests provide information for a variety of purposes, including diagnosis, monitoring of disease, and risk assessment. With respect to the latter, the prognostic utility of markers of risk assessment is often based on epidemiologic studies involving tens or hundreds of thousands of individuals. Although these large-scale epidemiologic studies enable the identification of markers associated with increased or decreased risk of cardiovascular disease (CVD), they fail to account for the prognostic utility of the marker when it is applied to a single measurement in a single individual. For any marker of risk assessment to have good prognostic utility in an individual subject, the biological variability of the marker must be low enough to enable appropriate risk stratification using as few serially collected blood samples as possible.

The magnitude of variability seen in a biological marker used to diagnose a disease or assess risk is influenced primarily by two parameters: the analytical variation that is observed in the measurement of the analyte, and the within- and between-subject biological variation that the analyte shows over time. For an analyte to have good clinical utility for risk assessment, the variability associated with measurement of the analyte should be relatively small compared with the within- and

between-subject variance. Analytes with large between-subject (inter-individual) variability relative to the other sources of variance are generally considered to be more reliable, and allow better estimation of an association of the marker with disease [1].

Serum high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, total cholesterol, non-HDL cholesterol, triglycerides, fibrinogen, and basal high-sensitivity C-reactive protein (hsCRP) concentrations have been identified as risk markers for CVD in numerous large-scale epidemiological studies [2]. However, these analytes show significant differences in biological variability, which adversely affects the prognostic utility when only a single measurement of the analyte is performed in an individual patient.

In an effort to assess the impact of biological variability of various cardiac risk markers on their prognostic utility when measured in an individual patient, we examined the short- and long-term biological variability of several markers of cardiovascular risk in a cohort of 15 healthy adults. We measured the concentrations of HDL, LDL, total cholesterol, triglycerides, hsCRP, and total fibrinogen over the course of 1 year to determine the biological variability of each of these markers.

In addition to the currently used risk markers described above, we also measured γ′ fibrinogen concentrations. The γ′ isoform of fibrinogen has been proposed as a cardiovascular risk marker independent of total fibrinogen, and is significantly associated with inflammation. This isoform, which represents approximately 10% of an individual's circulating fibrinogen, has a high-affinity thrombin-binding site that may mediate its effect on thrombosis risk. Studies have linked it to coronary artery disease [3], myocardial infarction [4, 5], and ischemic stroke [6]. In this study, we examine the biological variability of this analyte for the first time and compare it with biomarkers currently used for the assessment of CVD risk.

Materials and methods

Study subjects

We recruited 15 apparently healthy adults (nine women and six men) to participate in a 12-month study designed to quantify the magnitude of changes over time in commonly measured risk factors for CVD. Subjects ranged in age from 21 to 54 years at the time of recruitment. Information was collected on each participant's general health and medication use. Individuals taking cholesterol-lowering medications were excluded. The study has been approved by the Institutional Review Committee of the Oregon Health and Science University, and the study subjects gave informed consent for participation in the study. The study complies with the principles laid down in the Declaration of Helsinki, adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, and recently amended at the 59th World Medical Assembly, Seoul, Korea, October 2008.

Blood sample collection

Venous blood samples were collected from each participant between 0700 and 1000 following an overnight fast. Participants were encouraged to reschedule blood collection if they felt ill. Samples were collected on days 1, 3, 5, 7, 14, 21, and 28, and then monthly for 1 year. Thus, a total of 18 samples were collected over 1 year for subjects who completed the entire protocol.

Blood was collected into plain evacuated serum tubes, EDTA tubes, and citrate tubes depending on the analyte to be measured. Blood samples were processed within 45 min of collection, and serum and plasma were aliquoted into 1.5 mL screw-cap tubes for processing and storage. Samples were stored at 4 C for measurements completed within 24 h, and then stored long term at –70 C.

Measurement of cardiac risk factors

Analysis of total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and hsCRP were performed in duplicate using serum samples within 24 h of blood collection at the Oregon Health and Science University clinical chemistry laboratory. Measurement of these analytes was performed using a Beckman Synchron DxC (Beckman Coulter, Inc., Brea, CA, USA) using reagents from the manufacturer and according to the manufacturer's instructions. Non-HDL cholesterol was calculated as total cholesterol minus HDL cholesterol. Long-term imprecision based on analysis of quality control sera shows a %CV of <3.0% for total and HDL cholesterol, and <5% for LDL cholesterol, triglycerides, and hsCRP. Aliquots from each patient were stored at –70 C until measurement of other markers. Measurement of total fibrinogen and γ′ fibrinogen was performed in batch mode using previously frozen citrated plasma after the conclusion of the study, and within a maximum of 16 months after collection. Fibrinogen is known to be stable under these storage conditions [7, 8]. Total fibrinogen was measured according to the clotting method of Clauss using an STA Compact analyzer (Diagnostica Stago, Inc., Parsippany, NJ, USA).

Analysis of γ′ fibrinogen was performed using a microsphere-based bioassay developed at Gamma Therapeutics (Portland, OR, USA). The calibrator for the assay was "Peak 2 fibrinogen" (Enzyme Research Laboratories, South Bend, IN, USA). The total fibrinogen in this fraction was determined by measuring the absorbance at 280 nm. The γ′ fibrinogen content was determined by (1) separating γ′ and γA subunits on SDS-PAGE/Western blot, (2) measuring their respective intensities using a polyclonal anti-γ chain detector antibody coupled to an infrared imaging dye, and (3) using these values to determine the fractional amount of total fibrinogen that is the γ′ vs. γA form, assuming that both γ′ and γA chains randomly incorporate into fibrinogen hexamers and that γ′ chains constitute only a small minority (approx. 3.5%) of the chains.

To perform the assay, a monoclonal antibody (2.G2.H9) specific for the fibrinogen γ′ chain was covalently coupled to Microplex

Microspheres (Luminex Corp., Austin, TX, USA). Approximately 2500 microspheres per well were then preloaded onto 96-well filter plates (MABVN1250; EMD Millipore Corp., Billerica, MA, USA) and excess buffer was removed by vacuum. One hundred-microliter samples of diluted plasma (1:1000 dilution in assay buffer: PBS+0.1% BSA/0.1% Tween/0.05% sodium azide/1 mM EDTA) were then added to each well. Filter plates were incubated for 2 h at room temperature with gentle shaking, and washed three times with assay buffer. Microsphere samples then underwent two sequential 30-min incubations with 100 L of 4 g/mL biotinylated anti-fibrinogen polyclonal antibody (IASHFBGN-GF-BIO; Innovative Research, Novi, MI, USA) followed by 4 g/mL Streptavidin-R-Phycoerythrin (S-866; Life Technologies, Grand Island, NY, USA). The filter plates were then washed five times, the beads were resuspended in 100 L/well of assay buffer, and median R-Phycoerythrin fluorescence/microsphere was determined by flow cell fluorometry using a Luminex 200 system. The γ′ fibrinogen concentrations of plasma samples were determined by comparing their values with those of duplicate calibrators (six 1:2 dilutions of a 2 g/mL solution) run with each plate. Curve fitting of calibrators and back calculation of plasma values were performed using Luminex Xponent software (weighted five-parameter logistic analysis).

Statistical analysis

A thorough descriptive analysis was performed including assessment of analyte distributions and the pattern of analyte variation over time by subject. To account for repeated measures and obtain between-subject (s_G^2) and within-subject, or biological (s_I^2) variance components, a random-effects analysis of variance was fitted for each analyte using maximum likelihood estimation. For this study, it was assumed that there was no systematic within-subject error and that the within-subject variance remained constant at differing analyte concentrations. The within-subject variance estimates included method-dependent analytical error. To account for skewed distributions, hsCRP and triglycerides were log-transformed before these analyses. In addition, all variance components and statistics were calculated to describe both short- and long-term variation. All measures within the first 30 days were included in the short-term analysis, whereas the long-term analysis included all measures obtained from each participant.

To provide multiple methods for comparison across studies, several statistics were computed to describe analyte variation. For comparisons between tests, variance estimates from the repeated measures analyses were presented as within-subject and between-subject CVs (CV_I and CV_G, respectively). The CV_I was calculated as $100\times(s_I^2/\text{mean})$ and the CV_G as $100\times(s_G^2/\text{mean})$. The 95% confidence interval (95% CI) for each CV was computed using exact methods. For analytes with a skewed distribution, CV estimates and 95% CIs were calculated using a lognormal distribution. Variance estimates were also used to compute the intraclass correlation coefficient (ICC), a reliability measure defined as the proportion of the total variance attributed to between-subject variance $[s_G^2/(s_I^2+s_G^2)]$ and the index of individuality (II: CV_I/CV_G). The validity coefficient (VC), representing the difference between a measured value and the true value due to variability, was calculated as $(1/(1+(s_I^2/ks_G^2)))^{1/2}$, with k equal to the number of measurements performed on an individual. Thus, using a set value for the validity coefficient, the minimum number of measurements required to reach this value was determined. Owing to the non-specific nature of hsCRP, analyses were completed with and without measures >9.9 mg/L. This threshold was chosen to mimic the clinical utility of this test in which a hsCRP >9.9 mg/L warrants repeat testing owing to the reflection of an acute phase response rather than actual CVD risk.

To apply the results of this 12-month study to clinical practice where thresholds are used for CVD risk classification, we performed an analysis describing the misclassification of each analyte when used as a single measure. The median value for each analyte was computed for every participant and assumed to be their "true" value. CVD risk thresholds were set at the high end of the normal range for each analyte and applied to each participant's "true" value (Table 1). The number and percent of single measurements across the 12-month study that deviated from this "true" risk classification were tabulated

Table 1 Number and percentage of misclassification of biomarkers as individual tests and as part of a cardiovascular panel in 15 apparently healthy adults over 1 year of follow-up.

Biomarker, units	Normal range classification	Individual biomarker misclassification[a]			Cardiovascular panel misclassification[b]
		Low	Correct	High	
Total cholesterol, mg/dL	<200	28 (11)	216 (85)	10 (4)	36 (17)
LDL cholesterol, mg/dL	<130	21 (8)	218 (86)	15 (6)	34 (16)
HDL cholesterol, mg/dL	≥40	2 (1)	242 (95)	10 (4)	11 (5)
Non-HDL cholesterol, mg/dL	<160	14 (6)	211 (83)	29 (11)	40 (18)
Triglycerides, mg/dL	<150	6 (2)	238 (94)	10 (4)	16 (7)
hsCRP, mg/L	<1.00	16 (7)	197 (80)	32 (13)	47 (21)
Fibrinogen, mg/dL	<391	0 (0)	232 (93)	17 (7)	16 (7)
γ′ Fibrinogen, mg/dL	<30	6 (2)	230 (91)	16 (6)	20 (9)
Full panel					220 (12)

Data are presented as number (%). SI conversion factors: to convert total cholesterol, LDL, HDL, and non-HDL to mmol/L, multiply by 0.0259; triglycerides to mmol/L, multiply by 0.0113; hsCRP to nmol/L, multiply by 9.524. A total of 254 measures are available for each biomarker except hsCRP, fibrinogen, and γ′ fibrinogen (n=245, n=249, and n=252, respectively). [a]The median value for all biomarkers was computed for each participant and used as the "true" measure. Categorization was performed at clinical and reported thresholds for the normal range. [b]Panel misclassification analysis was restricted to days with no missing measures for any individual test (n=238) and includes a total of 1904 measures.

for each analyte. In addition, we determined the total number and percent of measures misclassified when all biomarkers were performed as a multi-analyte cardiovascular panel. The contribution of each analyte to that total is presented.

Statistical analyses were performed using SAS software version 9.3 (SAS Institute, Inc., Cary, NC, USA).

Results

The mean and median age of the study subjects was 39.9 and 39.0 years, respectively. Two participants were unable to complete the protocol; one moved away from the study site and the other was disqualified after beginning treatment with statins for high cholesterol. The mean number of blood samples per subject was 16.9, and the lowest number of samples from any subject was 11.

The assay for γ′ fibrinogen showed good precision. Measurement of quality control material over 20 separate runs showed total %CVs of 8.1%, 5.5%, and 8.5% at γ′ fibrinogen concentrations of 9.0, 26.4, and 53.1 mg/dL, respectively. The limit of detection was <0.1 mg/dL. Long-term storage up to 18 months showed a <10% change in γ′ fibrinogen concentrations when comparing values obtained using fresh vs. frozen samples. Freeze-thaw studies showed a <5% decrease in γ′ fibrinogen concentrations following three freeze-thaw cycles.

The mean, standard deviation, and coefficients of variation for each of the cardiovascular biomarkers are shown in Table 2. Total cholesterol had the lowest long-term within-subject variance, with a CV_I of 7.6 (95% CI: 5.7, 11.7), followed by non-HDL cholesterol [CV_I (95% CI): 9.4 (7.0, 14.3)] and LDL cholesterol [CV_I (95% CI): 10.3 (7.6, 15.7)]. The low within-subject variance for non-HDL cholesterol is not surprising, given the low values for this source of variance seen in both total and HDL cholesterol, from which it is calculated. hsCRP exhibited the highest long-term within-subject variance by far, with a CV_I of

Table 2 Short- and long-term descriptive statistics for CVD markers measured in a cohort of 15 apparently healthy adults over 1 year of follow-up.

Biomarker, units	n	Mean (SD)	CV_I (95% CI)[a]	CV_G (95% CI)[b]
Total cholesterol, mg/dL				
Short-term	89	190 (27.7)	6.4 (4.0, 15.8)	13.0 (9.7, 20.0)
Long-term	254	191 (27.9)	7.6 (5.7, 11.7)	12.1 (8.8, 19.3)
LDL cholesterol, mg/dL				
Short-term	89	126 (27.8)	7.8 (4.8, 19.2)	20.4 (15.0, 31.7)
Long-term	254	126 (28.1)	10.3 (7.6, 15.7)	19.3 (14.0, 31.1)
HDL cholesterol, mg/dL				
Short-term	89	45.5 (13.0)	9.1 (5.7, 22.7)	27.0 (19.8, 42.8)
Long-term	254	47.2 (13.0)	11.1 (8.3, 17.0)	25.2 (18.2, 41.2)
Non-HDL cholesterol, mg/dL				
Short-term	89	144.2 (29.1)	7.3 (4.5, 17.9)	18.6 (13.8, 28.9)
Long-term	254	144 (29.5)	9.4 (7.0, 14.3)	18.4 (13.4, 29.7)
Triglycerides, mg/dL[c]				
Short-term	89	81.9 (38.4)	24.8 (15.3, 65.6)	33.8 (24.4, 55.5)
Long-term	254	81.6 (40.8)	27.5 (20.3, 43.0)	34.0 (24.5, 55.8)
hsCRP, mg/L (all measures)[c]				
Short-term	85	1.12 (2.45)	54.8 (32.8, 196.3)	107.6 (71.4, 240.1)
Long-term	245	1.20 (2.43)	77.1 (53.3, 141.3)	76.2 (53.3, 148.0)
hsCRP, mg/L (measures ≤9.9)[c]				
Short-term	83	1.05 (1.84)	40.4 (24.6, 121.8)	101.1 (67.6, 218.4)
Long-term	239	1.13 (1.84)	58.9 (42.0, 102.0)	77.4 (53.5, 148.9)
Fibrinogen, mg/dL				
Short-term	88	304 (50.7)	8.8 (5.5, 21.9)	14.0 (10.4, 21.5)
Long-term	249	300 (47.7)	10.8 (8.0, 16.6)	11.9 (8.7, 18.9)
γ′ Fibrinogen, mg/dL				
Short-term	89	23.2 (7.1)	13.9 (8.6, 35.0)	27.1 (19.9, 42.9)
Long-term	252	22.9 (6.8)	14.3 (10.6, 21.9)	27.4 (19.7, 45.1)

SI conversion factors: to convert total cholesterol, LDL, HDL, and non-HDL to mmol/L, multiply by 0.0259; triglycerides to mmol/L, multiply by 0.0113; hsCRP to nmol/L, multiply by 9.524. [a]CV_I indicates within-subject variation. [b]CV_G indicates between-subject variation. [c]Owing to skewed distributions, triglycerides and hsCRP were log-transformed for all calculations. For presentation, log-transformed values were transferred back to original units as a geometric mean (SD).

77.1 (95% CI: 53.3, 141.3). This variation remained high after excluding hsCRP measures >9.9 mg/L [CV_I (95% CI): 58.9 (42.0, 102.0)]. The second highest CV_I of 27.5 (95% CI: 20.3, 43.0) was for triglycerides. Between-subject variance showed a similar trend, with the lowest values seen for total cholesterol, and the highest for hsCRP and triglycerides. The overall variance for hsCRP was much higher than for the other analytes, with a standard deviation exceeding the mean value. The short-term CV_I and CV_G estimates derived from repeated measures performed during the first month of follow-up were very similar to the long-term variance estimates. For all but one analyte, the short-term CV_I and CV_G fell within the 95% CI of the long-term estimate. hsCRP was the only analyte where a difference was observed between the short- and long-term within-subject variance [CV_I (95% CI): 40.4 (24.6, 121.8) and 58.9 (42.0, 102.0), respectively].

The proportions of the total variance attributed to each source are presented in Table 3. In this study, the long-term II ranged from 0.44 for HDL cholesterol to 0.99 for hsCRP, and the ICCs were between 0.50 (hsCRP) and 0.84 (HDL cholesterol). The long-term II and ICC for γ′ fibrinogen were 0.52 and 0.79, respectively, whereas these values for total fibrinogen were 0.91 and 0.55, respectively. This reflects the finding that γ′ fibrinogen has a reduced proportion of within-individual variance, which is approximately half that of total fibrinogen. Of note, the II and ICC calculated for total fibrinogen were nearly identical to the II of 0.92 and the ICC of 0.56 found in similar studies [9, 10]. Short-term II and ICC estimates show the greatest amount of variation for triglycerides (0.73 and 0.64, respectively), followed by total fibrinogen (0.63 and 0.72, respectively) and hsCRP (0.51 and 0.75, respectively). Total fibrinogen and hsCRP II and ICC estimates demonstrated less variation within 1 month compared with the full year of follow-up. In contrast, there was little or no difference between these estimates for the other analytes.

To gain insight into the impact of biological variability on the prognostic utility of these markers of risk assessment, the VC for a single measurement of each biomarker was calculated, as well as the number of samples needed for a biomarker with the same VC as HDL cholesterol (0.91), which showed the highest value for this measure (Table 4). For total cholesterol, LDL cholesterol, non-HDL cholesterol, and γ′ fibrinogen, only two measurements were needed to achieve a VC that was similar to HDL. However, for triglycerides, total fibrinogen, and hsCRP, more than four or five measurements were needed to achieve the same VC as that provided by a single measurement of HDL cholesterol.

Table 3 Short- and long-term indices of individuality, intraclass correlation coefficients, and variance components as a percentage of total variance for CVD risk markers in 15 apparently healthy adults over 1 year of follow-up.

Biomarker, units	Index of individuality	ICC	CV_I%[a]	CV_G%[b]
Total cholesterol, mg/dL				
Short-term	0.49	0.81	19.4	80.6
Long-term	0.63	0.72	28.4	71.6
LDL cholesterol, mg/dL				
Short-term	0.38	0.87	12.7	87.3
Long-term	0.53	0.78	22.0	78.0
HDL cholesterol, mg/dL				
Short-term	0.34	0.90	10.3	89.7
Long-term	0.44	0.84	16.3	83.7
Non-HDL cholesterol, mg/dL				
Short-term	0.39	0.87	13.2	86.8
Long-term	0.51	0.80	20.5	79.5
Triglycerides, mg/dL[c]				
Short-term	0.73	0.64	35.6	64.4
Long-term	0.81	0.60	40.1	59.9
hsCRP, mg/L (all measures)[c]				
Short-term	0.51	0.75	25.5	74.5
Long-term	0.99	0.50	49.5	50.5
hsCRP, mg/L (measures ≤9.9)[c]				
Short-term	0.40	0.82	17.7	82.3
Long-term	0.76	0.61	38.8	61.2
Fibrinogen, mg/dL				
Short-term	0.63	0.72	28.5	71.5
Long-term	0.91	0.55	45.4	54.6
γ′ Fibrinogen, mg/dL				
Short-term	0.51	0.79	20.8	79.2
Long-term	0.52	0.79	21.4	78.6

SI conversion factors: to convert total cholesterol, LDL, HDL, and non-HDL to mmol/L, multiply by 0.0259; triglycerides to mmol/L, multiply by 0.0113; hsCRP to nmol/L, multiply by 9.524. [a]CV_I indicates within-subject variation. [b]CV_G indicates between-subject variation. [c]Owing to skewed distributions, triglycerides and hsCRP were log-transformed for all calculations. For presentation, log-transformed values were transferred back to original units as a geometric mean (SD).

Discussion

A large number of biomarkers are used routinely for assessment of risk of CVD. Although the average cost of a single lipid profile is relatively low when compared with the direct and indirect costs of CVD, estimated to be US $503.2 billion in 2010, the cumulative costs of screening can be substantial, especially if biochemical markers of risk assessment require multiple repeated measurements to establish an individual's true baseline [11, 12].

The biological and analytical variability seen in a risk marker is an important factor in determining the prognostic utility of a single measurement; however, this attribute

Table 4 Validity coefficients and the number of repeated measurements needed to reach a validity coefficient of 0.91 in a study with 15 apparently healthy adults over 1 year of follow-up.

Biomarker	Validity coefficient	No. of sample measurements to achieve a VC=0.91[a]
Total cholesterol, mg/dL	0.85	2
LDL cholesterol, mg/dL	0.88	2
HDL cholesterol, mg/dL	0.91	1
Non-HDL cholesterol, mg/dL	0.89	2
Triglycerides, mg/dL[b]	0.77	4
hsCRP, mg/L (all measures)[b]	0.71	5
hsCRP, mg/L (measures ≤9.9)[b]	0.78	4
Fibrinogen, mg/dL	0.74	4
γ′ Fibrinogen, mg/dL	0.89	2

SI conversion factors: to convert total cholesterol, LDL, HDL, and non-HDL to mmol/L, multiply by 0.0259; triglycerides to mmol/L, multiply by 0.0113; hsCRP to nmol/L, multiply by 9.524. [a]The number of measurements is rounded up to the nearest integer. [b]Owing to skewed distributions, triglycerides and hsCRP were log-transformed for all calculations.

tends to be overlooked, with more focus placed on disease-association levels in large epidemiological studies. These studies are extremely valuable in identifying potential biomarkers of risk assessment but do not necessarily provide insight into the reliability of a single measurement performed on an individual patient, an important practical consideration [13].

We measured CVD risk markers in a cohort of 15 apparently healthy individuals over the course of a year to directly compare their within- and between-individual variability. Of the risk markers measured, HDL cholesterol had the best variability profile for a prognostic biomarker, with the majority of its variability due to differences between subjects, and a relatively small variance observed within a single individual. These characteristics gave HDL the highest value for both the intraclass and the validity coefficients.

In contrast, hsCRP measurements demonstrated a high degree of biological variability, both between subjects and within individuals. The within-individual variance of hsCRP was nearly identical to that seen by Clark and Fraser [14], who found a CV_I of 63%, but was higher than that found in other studies, even after exclusion of values >9.9 mg/L [9, 15]. This is probably due in some part to the small size as well as the duration of the study and number of blood samples analyzed. Sakkinen et al. [9] saw a marked increase in the intra-individual CV for hsCRP at 24 weeks of follow-up, compared with 6 weeks, demonstrating the strong effect of study duration on this variance parameter. This finding is consistent with our observation of greater CV_I and II estimates over 1 year of follow-up compared with 1 month for this analyte.

An increasing number of studies have questioned the clinical utility of hsCRP in assessing the risk of CVD where measurement of this marker did not improve the CVD risk estimation when added to other established risk factors [2, 16–20]. This is due in large part to the high intra-individual variability of this analyte. The within-subject standard deviation for hsCRP in our study was 1.2 mg/L. Although others have reported smaller values for this estimate, it is still quite high relative to the cutoff values for risk classification of <1, 1–3, and >3 mg/L, resulting in a considerable chance of misclassification, and making any assessment of a "real" change in an individual's baseline hsCRP level nearly impossible [16]. We found that it would require five separate measurements of hsCRP to achieve the validity of a single measurement of HDL cholesterol in our cohort, and some have suggested that even more repeated measures might be necessary for establishing an individual's true baseline hsCRP value [21].

We also assessed the within- and between-individual variability of the γ′ isoform of fibrinogen. This marker has been gaining attention as an additional CVD risk factor. Recent research has also suggested a connection between γ′ fibrinogen and inflammation [22], with increased concentrations seen in the acute phase of pulmonary embolism and stroke [23], indicating that it may be useful as a marker of inflammatory processes as well. In vitro studies show that γ′ fibrinogen is differentially upregulated from total fibrinogen by the major inflammatory cytokine interleukin-6 [24]. In studies with HepG2 liver cells, interleukin-6 caused a 3.6-fold increase in the γA mRNA, but an 8.3-fold increase in the γ′ mRNA, resulting in a 2.3-fold increase in total fibrinogen within 24 h, and a 3.2-fold increase in γ′ fibrinogen.

In the present study, the variability profile of γ′ fibrinogen was favorable and demonstrated little difference whether assessed over 1 month or 1 year. For these reasons, it may be a more attractive risk marker than hsCRP for clinical risk assessment. The stability of γ′ fibrinogen is likely to be due to its long half-life compared with CRP. Fibrinogen has a half-life of about 88 h [25] compared with the 19 h half-life of CRP [26]. This long half-life may provide a buffering capacity against transient inflammatory spikes compared with hsCRP, which is more sensitive to inflammation than γ′ fibrinogen, but may be less specific for CVD.

In summary, a number of biomarkers for the assessment of the risk of CVD have been identified on the basis of disease association in large-scale epidemiologic

studies. Although these studies are necessary for identification of potential markers, studies are also needed that demonstrate the true prognostic utility of each marker when measured in an individual, and the number of measurements of each marker that are needed to assess individual risk. We found that a single measurement of HDL cholesterol provides the best assessment of CVD risk owing to the smaller intra-individual variability seen in this marker, whereas two separate measurements of total cholesterol, LDL cholesterol, and γ′ fibrinogen would be needed to provide the same level of prognostic information. In contrast, five separate measurements of hsCRP would be needed to assess risk in a single individual.

Conflict of interest statement

Author's conflict of interest disclosure: OHSU and Dr. David Farrell have a significant interest in Gamma Therapeutics, a company that may have a commercial interest in the results of this research and technology. This potential individual and institutional conflict of interest has been reviewed and managed by OHSU.

Research funding: 1 R44HL104885 from the NIH.

Employment or leadership: Dr. David Farrell is founder and Chief Scientific Officer of Gamma Therapeutics.

Honorarium: None declared.

Received November 2, 2012; accepted April 5, 2013

References

1. Block G, Dietrich M, Norkus E, Jensen C, Benowitz NL, Morrow JD, et al. Intraindividual variability of plasma antioxidants, markers of oxidative stress, C-reactive protein, cotinine, and other biomarkers. Epidemiology 2006;17:404–12.
2. Kaptoge S, Di Angelantonio E, Pennells L, Wood AM, White IR, Gao P, et al. C-reactive protein, fibrinogen, and cardiovascular disease prediction. N Engl J Med 2012;367:1310–20.
3. Lovely RS, Falls LA, Al-Mondhiry HA, Chambers CE, Sexton GJ, Ni H, et al. Association of γA/γ′ fibrinogen levels and coronary artery disease. Thromb Haemost 2002;88:26–31.
4. Mannila MN, Lovely RS, Kazmierczak SC, Eriksson P, Samnegård A, Farrell DH, et al. Elevated plasma fibrinogen γ′ concentration is associated with myocardial infarction: effects of variation in fibrinogen genes and environmental factors. J Thromb Haemost 2007;5:766–73.
5. Lovely RS, Yang Q, Massaro JM, Wang J, D'Agostino RB Sr, O'Donnell CJ, et al. Assessment of genetic determinants of the association of γ′ fibrinogen in relation to cardiovascular disease. Arterioscler Thromb Vasc Biol 2011;31:2345–52.
6. Cheung EY, de Willige SU, Vos HL, Leebeek FW, Dippel DW, Bertina RM, et al. Fibrinogen γ′ in ischemic stroke: a case-control study. Stroke 2008;39:1033–5.
7. Alesci S, Borggrefe M, Dempfle CE. Effect of freezing method and storage at –20 C and –70 C on prothrombin time, aPTT and plasma fibrinogen levels. Thromb Res 2009;124:121–6.
8. Woodhams B, Girardot O, Blanco MJ, Colesse G, Gourmelin Y. Stability of coagulation proteins in frozen plasma. Blood Coagul Fibrinolysis 2001;12:229–36.
9. Sakkinen PA, Macy EM, Callas PW, Cornell ES, Hayes TE, Kuller LH, et al. Analytical and biologic variability in measures of hemostasis, fibrinolysis, and inflammation: assessment and implications for epidemiology. Am J Epidemiol 1999;149:261–7.
10. De Bacquer D, De Backer G, Braeckman L, Baele G. Intra-individual variability of fibrinogen levels. J Clin Epidemiol 1997;50:393–9.
11. Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO 3rd, Criqui M, et al.; Centers for Disease Control and Prevention; American Heart Association. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: a statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation 2003;107:499–511.
12. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, et al.; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Executive summary: heart disease and stroke statistics–2010 update: a report from the American Heart Association. Circulation 2010;121:948–54; erratum in: Circulation 2010;121:e259.
13. Hlatky MA, Greenland P, Arnett DK, Ballantyne CM, Criqui MH, Elkind MS, et al.; American Heart Association Expert Panel on Subclinical Atherosclerotic Diseases and Emerging Risk Factors and the Stroke Council. Criteria for evaluation of novel markers of cardiovascular risk: a scientific statement from the American Heart Association. Circulation 2009;119:2408–16; erratum in: Circulation. 2009;119:e606.
14. Clark GH, Fraser CG. Biological variation of acute phase proteins. Ann Clin Biochem 1993;30:373–6.
15. Ockene IS, Matthews CE, Rifai N, Ridker PM, Reed G, Stanek E. Variability and classification accuracy of serial high-sensitivity C-reactive protein measurements in healthy adults. Clin Chem 2001;47:444–50.
16. McCormack JP, Allan GM. Measuring hsCRP-an important part of a comprehensive risk profile or a clinically redundant practice? PLoS Med 2010;7:e1000196.
17. Folsom AR, Chambless LE, Ballantyne CM, Coresh J, Heiss G, Wu KK, et al. An assessment of incremental coronary risk prediction using C-reactive protein and other novel risk markers: the Atherosclerosis Risk in Communities Study. Arch Intern Med 2006;166:1368–73.
18. Shah T, Casas JP, Cooper JA, Tzoulaki I, Sofat R, McCormack V, et al. Critical appraisal of CRP measurement for the prediction of coronary heart disease events: new data and systematic review of 31 prospective cohorts. Int J Epidemiol 2009;38:217–31.
19. Wang TJ, Gona P, Larson MG, Tofler GH, Levy D, Newton-Cheh C, et al. Multiple biomarkers for the prediction of first major cardiovascular events and death. N Engl J Med 2006;355:2631–9.

20. Kavousi M, Elias-Smale S, Rutten JH, Leening MJ, Vliegenthart R, Verwoert GC, et al. Evaluation of newer risk markers for coronary heart disease risk classification: a cohort study. Ann Intern Med 2012;156:438–44.
21. Campbell B, Badrick T, Flatman R, Kanowski D. Limited clinical utility of high-sensitivity plasma C-reactive protein assays. Ann Clin Biochem 2002;39:85–8.
22. Alexander KS, Madden TE, Farrell DH. Association between γ′ fibrinogen levels and inflammation. Thromb Haemost 2011;105:605–9.
23. Cheung EY, Vos HL, Kruip MJ, den Hertog HM, Jukema JW, de Maat MP. Elevated fibrinogen γ′ ratio is associated with cardiovascular diseases and acute phase reaction but not with clinical outcome. Blood 2009;114:4603–4; author reply 4604–5.
24. Rein CM, Anderson NW, Farrell DH. Differential regulation of fibrinogen γ chain splice isoforms by interleukin-6. 2013;131:89–93.
25. Manco-Johnson MJ, Dimichele D, Castaman G, Fremann S, Knaub S, Kalina U, et al.; Fibrinogen Concentrate Study Group. Pharmacokinetics and safety of fibrinogen concentrate. J Thromb Haemost 2009;7:2064–9.
26. Vigushin DM, Pepys MB, Hawkins PN. Metabolic and scintigraphic studies of radioiodinated human C-reactive protein in health and disease. J Clin Invest 1993;91:1351–7.