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Parameters of coagulant and fibrinolytic capacity and activity in postmenopausal women: within-subject variability

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Abstract

We have analyzed the within-subject variability of a battery of parameters of coagulant and fibrinolytic capacity and activity in postmenopausal women. We observed large differences in within-subject variability among the tests and have demonstrated how such data can be used to estimate the number of times a parameter must be measured to produce a statistically adequate sample.

Keywords: Postmenopause; Thrombophilia; Hyperhomocysteinemia; Statistics

Clinical evidence indicates that hormone replacement therapy (HRT) for postmenopausal women increases their risk of venous thromboembolic disease two- to fourfold [1–6]. The risk appears to be concentrated in the first year of HRT, as though the hormones were activating a latent hypercoagulable state [1,2,4,5]. In fact, studies of non-thrombophilic women have revealed biochemical evidence that coagulation and fibrinolysis are both stimulated by HRT [7–13]. This suggests that women with thrombophilic traits might be particularly susceptible to the thrombotic risk of hormone replacement. On the other hand, although their relative risk is increased, most women with these traits do not have thrombosis, even when taking oral contraceptives [14,15]. Withholding hormones from all of these individuals would deny 5–10% of Caucasian women the potential benefits of HRT [16]. Measuring biochemical markers of HRT-induced hypercoagulability in women with these traits, however, might identify women at greatest risk of thrombosis who should use HRT cautiously if at all.

When the response of biochemical markers of coagulation and fibrinolysis has been assessed in women without known thrombophilic traits, cohorts of 20–30 individuals have typically been studied [7–13]. This number of subjects has been adequate to detect statistically significant differences between single measurement of factors tested before and after periods of HRT [7,9–13]. However, because the frequency of specific thrombophilic traits is relatively low in individuals without a history of thrombosis, identifying adequate numbers of subjects for studies of this design would require screening hundreds of women [16]. An alternative design is to make multiple measurements of each study variable in a relatively small number of well-characterized subjects, since changes based upon the mean of multiple measurements are more likely to reflect true changes in the "homeostatic set-point" for that individual [17]. Selecting the appropriate number of measurements requires knowledge of the within-subject variability of the factors to be tested, but studies of the within-subject variability of these factors have included few postmenopausal women [18–25].

We addressed this void by screening healthy postmenopausal women who had no history of thrombosis to identify three cohorts for a longitudinal study: two groups with common thrombophilic traits, heterozygous factor V Leiden (fVL) and mild hyperhomocysteinemia, and an age- and weight-matched control group [16,26]. All three groups had blood drawn each month for a total of four times for measurements of a battery of coagulant and fibrinolytic factors.

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1. Methods

The protocol for this study was approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute, Bethesda, MD. All participants gave written informed consent.

1.1. Study eligibility

Women were eligible for the study if they had been amenorrheic for at least 4 months and met the following criteria: (1) No hormonal therapy within the previous 2 months and no more than a cumulative total of 3 years postmenopausal hormone therapy in the past; (2) No history of venous or arterial thrombosis at any time and no history of diabetes mellitus or malignancy within the previous 10 years; and (3) Within the previous 6 months, no major surgery or even minor surgery involving the hips or lower extremities.

After fasting for at least 8 h, participants had blood drawn for the following routine tests in the Department of Laboratory Medicine of the Clinical Center at the National Institutes of Health, Bethesda, MD: C-reactive protein (CRP), follicle stimulating hormone (FSH), prothrombin time (PT), activated partial thromboplastin time (aPTT), functional fibrinogen, functional antithrombin, functional protein C, functional protein S, plasma homocysteine, and red cell folate. The coagulation tests were performed with an STA Hemostasis System (Diagnostica Stago, Parsippany, NJ). The homocysteine was quantitated with high-pressure liquid chromatography as previously described [27]. Other analytes were measured by routine automated methods. In addition, the women were tested for the genes for fVL, prothrombin 20210A, and thermolabile methylenetetrahydrofolate reductase (t-MTHFR) using standard PCR methods.

Women were excluded from further participation if they had any of the following: FSH ≤ 40 U/l, CRP ≥ 1 mg/dl, red cell folate <155 ng/ml (normal, 155–600 ng/ml), any abnormality in the PT, aPTT, fibrinogen concentration, or blood counts, or functional antithrombin (AT) $<75\%$ (normal range, 75–127%), functional protein C (PC) $<72\%$ (normal, 72–149%), or functional protein S (PS) $<64\%$ (normal, 64–131%), or the gene for prothombin 20210A.

1.2. Longitudinal study of postmenopausal women

Women who were found to be heterozygous for fVL or to have plasma homocysteine >12.4 μM ($\geq$97.5th percentile of the laboratory's normal range) were invited to enter the longitudinal study, if they had none of the exclusion criteria listed above. Women who lacked fVL, had homocysteine ≤ 12.4 μM, and were normal by all other screening tests were the source for age- (within 5 years) and weight- (within 5 kg) matched controls for each of the women with fVL or hyperhomocyteinemia.

Subjects had blood drawn between 8 and 11 AM every 4 weeks for 3 months (a total of four blood draws) following an overnight fast. Aliquots of plasma were frozen (-70 °C). After the samples from a study subject had been collected at all time points, the aliquots were thawed and tested together in single runs for functional tPA and PAI-1 (Chromolize tPA and Chromolize PAI-1, Biopool International, Ventura, CA), antigenic tPA and PAI-1 (TintElize tPA, Biopool; Asserachrom PAI-1, Diagnostica Stago), thrombin–antithrombin complexes (Enzygnost TAT micro, Dade Behring, Diagnostics, San Jose, CA), and plasmin–antiplasmin complexes (Enzygnost PAP micro, Dade Behring), as well as factors II, VII, and VIII activities, and activated protein C resistance (APCR), which were measured with an STA Hemostasis System. Histidine-rich glycoprotein (HRG) in the aliquots was quantitated by a previously described ELISA [28].

1.3. Statistical methods

Statistical analyses were performed using StatView (SAS Institute, Carey, NC). One-way analysis of variance (ANOVA) was used to compare the mean data from the control women, those with fVL, and those with hyperhomocysteinemia. Post hoc analysis was performed with the Games–Howell test. Sources of variation (between-subject and within-subject) in the data of the longitudinal study were segregated by repeated measures analysis of variance (ANOVA). The within-subject coefficients of variation (CVs) were calculated as the means of the CVs for each subject's data. For most assays, the analytic (within-run) component of variance was calculated from the variances of duplicate determinations of 15–20 individual samples [29]. For the coagulation factor and APCR assays, the within-run variances were derived from repeated measurements using a single normal plasma sample.

CVs necessary to achieve a P-value of 0.05 and a power of 0.90 with varying sample sizes were calculated using a rearrangement of the formula for the t-statistic,

$$\mathrm{CV} = 100\sqrt{\frac{N(X_\mathrm{A} - X_\mathrm{B})^2}{(t_{\alpha,df} + t_{\beta,df})^2(X_\mathrm{A}^2 + X_\mathrm{B}^2)}}$$

where X_A and X_B are the means of two independent samples of size N. The t-statistic $t_{\alpha,df}$ is the two-tailed t for $\alpha = 0.05$ with degrees of freedom (df) $= 2N - 2$, and $t_{\beta,df}$ is the one-tailed t for $\beta = 0.10$ and the same df.

2. Results

2.1. Screening

Three hundred subjects were screened for the study. The data were incomplete for 1, leaving 299 evaluable, 75.3% of

Table 1
Summary of ANOVA of data from the longitudinal study[a]

Test	Grand (range of individual means)	Variance (% of total)		Coefficient of variation (%)	
		Between-subject	Within-subject	Within-subject mean (range)	Within-run mean
PAI-Ag	24.0 ng/ml (1.1–97)	91	9	55 (9.7–139)	4.3
PAI-funct	25.1 U/ml (1.8–126)	94	6	49 (9.5–120)	1.9
tPA-Ag	8.3 ng/ml (2.9–14.6)	96	4	11 (0.8–32)	2.4
tPA-funct	0.56 IU/ml (0.1–1.9)	84	16	32 (0–76)	8.5
PAP	470 μg/ml (156–1232)	83	17	17 (3.1–33)	4.2
HRG	224 μg/ml (170–320)	78	22	9.9 (2.2–42)	3.8
TAT	3.0 ng/ml (2.3–4.6)	80	20	11 (1.3–22)	1.9
FII	108% (81–135)	89	11	5.7 (1.3–22)	3.7
FVII	130% (87–205)	95	5	8.5 (2.2–18)	3.3
FVIII	154% (69–290)	81	19	16 (1.5–35)	4.9
APCR	2.6 (1.2–3.8)	87	13	6.7 (1.3–22)	3.3
Hcy	10.2 μM (6.3–24.5)	98	2	7.0 (3.4–16)	3.6

[a] The data are from 34 subjects, except APCR, which was measured in 17 subjects, all lacking FVL.

whom were Caucasian, 17.1% African American, 5.4% Asian, and 2.3% Hispanic. Of these 299, 12 (10 Caucasians, 2 African Americans) or 4.0% were heterozygous for fVL, 26 (25 Caucasians, 1 Hispanic) or 8.7% were homozygous for MTHFR, and 6 (all Caucasian) or 2.0% were heterozygous for prothrombin 20210A. Only 3 (all Caucasians) or 11.5% of the 26 women with homozygous MTHFR had hyperhomocysteinemia. These 3 represented only 13.0% of all the women (15 Caucasians, 8 African Americans) with hyperhomocysteinemia (concentrations between 12.4 and 19.8 μM, mean 14.3 μM, S.D. 2.1 μM).

Forty-five women were excluded from the study either because of an FSH ≤ 40 U/l (40 women) or because of a CRP >1 mg/dl (5 women). Of the 12 women with fVL, 4 were excluded because of a low FSH or protein S or an elevated aPTT. Of the 23 subjects with hyperhomocysteinemia, 11 were exclude because of a low FSH or protein S, an elevated CRP or aPTT, or the presence of fVL.

2.2. Longitudinal study

Thirty-nine women entered the longitudinal phase of the study. Five, all in the hyperhomocysteinemic group, did not complete three blood drawings, one because of a hip fracture, the others because of noncompliance. Their data were omitted from subsequent analysis. Therefore, 34 women had blood drawn on three ($N=1$) or four ($N=33$) occasions over 3 months. Eight of these 34 were homozygous for fVL, and 7 women had mild hyperhomocyteinemia. The remaining 19 women were age- and weight-matched controls. (The number of controls exceeded the sum of the other two groups because all of the drop-outs were among the women with mild hyperhomocysteinemia.)

By ANOVA, none of the test results differed significantly in the women with fVL or those with mild hyperhomocysteinemia from the 19 controls ($P \geq 0.22$) with the exception of APCR ratio, which was lower in the women with fVL ($P=0.0009$). Therefore, with the exception of the APCR ratios, all of the longitudinal data from the postmenopausal women were combined for analysis.

Table 1 summarizes the information about variability in the data from the longitudinal study. Between-subject variance accounted for 78–98% of the total variance. The within-subject CV was greatest for PAI-1 antigen (55%), although it was less (30%) when only higher mean values (>40 ng/ml) were considered. Similarly for functional PAI-1, overall within-subject CV (49%) was greater than the CV of means >20 IU/ml (34%). The opposite was true for HRG: overall within-subject CV (9.9%) was less than the mean CV for values >250 μg/ml (17%). Variability due to technical error (within-run variability) was generally small (CV = 1.9–8.5%) compared with total within-subject variability (CV = 6.7–55%).

Within-subject CV did not vary with the mean values for the analytes ($R=-0.29$ to 0.23) except for antigenic and function PAI-1 and HRG, for which there was a weak correlation of the CV with the mean ($R=-0.40$, -0.44,

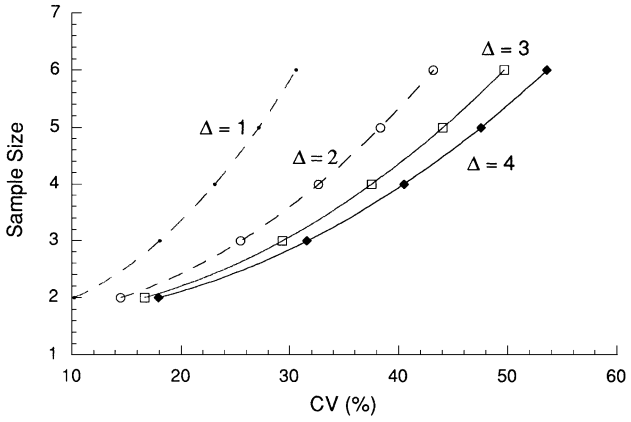

Fig. 1. CV and sample size required to detect twofold ($\Delta=1$), threefold ($\Delta=2$), fourfold ($\Delta=3$), and fivefold ($\Delta=4$) differences in the means of two independent samples calculated for $\alpha=0.05$ and $\beta=0.10$, as described in the text.

and 0.49, respectively). Therefore, to estimate sample sizes necessary to detect hypothetical changes in the means of the samples with an $\alpha = 0.05$ and a $\beta = 0.10$ (power of 0.90), the pooled variances of the samples were calculated using the within-subject CVs of the analytes instead of their average within-subject variance. Sample sizes calculated to detect several fold differences in the means of two samples are shown in Fig. 1.

3. Discussion

The primary goal of this study was to assess the suitability of certain laboratory parameters to detect significant changes in the capacity and activity of the coagulant and fibrinolytic systems in postmenopausal women. The variability in all of the analytes was predominantly due to biologic differences among the subjects (between-subject variance) (Table 1). However, when an individual is being studied over time, the more important component of variability is within-subject. This includes both biologic factors (physiologic plus those related to the venepuncture and sample processing) as well as error intrinsic to the assay method. Since all of the samples from each woman in our study were assayed together in single runs, the relevant analytical variability in the study described here is the within-run error (Table 1). The size of the within-run CV relative to the within-subject CV was generally small, but for HRG, APCR, Hcy, and FII was $\sim$ 50% as large as the total within-subject CV. For these tests, therefore, the precision of the results could be improved and within-subject variability could be reduced by simply testing more replicates in each run.

For other tests, especially the measurements of PAI-1, the within-run CVs were relatively small compared to the within-subject CVs, and more precise estimates of an individual's mean values over time require more numerous blood samples. It is important to point out that the within-subject CVs that we determined are based upon monthly sampling over 3 months and may not necessarily reflect variability over longer or shorter time periods. Nor do they necessarily reflect the biologic variability of other patient populations. From the data published, however, it appears that the CVs we observed are generally representative. In 26 healthy individuals (16 women, 4 of whom were postmenopausal, and 10 men), for example, tested every 3 weeks for 6 months, the within-subject CV for PAI-1 antigen (47%), PAI-1 activity (30%), tPA antigen (15%), PAP (20%), and TAT (25%) were similar to the CVs we found [25]. In another study, 14 subjects (8 women, 6 men) were tested every other month for 3 years, and the within-subject CVs for factors II, VII, and VIII were 7.7–16% [18].

Because of the relatively large within-subject CVs of some of these analytes, single measurements are not helpful in detecting changes even several fold in magnitude. This limitation can be overcome by studying a sufficiently large number of individuals. However, when sufficient numbers are not practical or possible, an alternative approach is to make serial measurements in each subject. Fig. 1 shows that to be relatively certain (i.e., $\alpha = 0.05$, power = 0.90) of measuring a true doubling or halving ($\Delta = 1$) of an analyte with a within-subject CV of $\sim$ 30% requires at least six measurements in each sample. Therefore, factors such as PAI-1 must be measured many times, or the investigator must be willing to detect only larger changes (e.g., $\Delta = 3$ or 4). In contrast, the other analytes generally require only two or three measurements to achieve such reliability.

The information we have reported will be valuable in designing protocols to detect changes of these analytes related to HRT in relatively small numbers of healthy postmenopausal women with specific traits associated with thrombophilia. The outcome of the screening phase of this study demonstrated the difficulty in accruing such subjects. From a total of 300 women screened, we identified only 8 with FVL and 12 with hyperhomocysteinemia who did not have an exclusion criterion, and only 7 of the women with hyperhomocysteinemia completed the 3-month longitudinal phase of the study.

If sufficient pre- and post-HRT measurements are made, significant changes should be detectable even with small cohorts of subjects.

An unavoidable limitation of a small cohort, however, is that it may not faithfully reflect the diversity of the larger population, regardless of how precisely the homeostatic set points of the individuals are defined by serial testing. The effect of fVL on the response to HRT, for example, may depend on other variables, many unknown, that could only be appreciated by studying a large population of women with this genetic trait. Therefore, small cohorts of well characterized subjects may offer limited insights into the effect of a single trait, but this possibility should not preclude appropriately designed studies of such women.

Another problem that could be addressed by serial testing is the evaluation of women who give a history suggesting they might be at an increased risk of thrombosis but who lack the genetic and metabolic traits known to be associated with thrombophila. Serial testing of such women before and during HRT might identify individuals whose basal level of coagulability has been increased by the hormones. The data we have reported about the within-subject variability of candidate analytes will aid in designing these longitudinal studies.

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