Fibrinolysis & Proteolysis (2001) 15 (1), 9–20
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doi: 10.1054/fipr.2001.0099, available online at http://www.idealibrary.com on IDEAL®

Covariance of metabolic and hemostatic risk indicators in men and women

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Summary *Background and objective*: Multivariate analyses on clusters of metabolic and hemostatic risk indicators implicitly assume good test–retest reliability of these variables, substantial covariance among the various indicators, stability of covariance structure over time, and comparable covariance structure in different subpopulations. The aim of the present study is to investigate these assumptions.

Methods: Repeated samples were taken of fasting insulin, triglycerides (TG), high-density cholesterol (HDL-C), low-density cholesterol (LDL-C), fibrinogen, tissue-type plasminogen activator (t-PA) antigen, t-PA activity, plasminogen activator inhibitor-1 (PAI-1) antigen to address their intra-week reliability and covariance structure. In the same work-week blood was drawn three times from 125 sedentary males (age 45.2 ± 5.3 years) and twice from 132 female nurses (age 33.7 ± 8.0 years).

Results: About half (44.8%) of these women were oral contraceptives (OC) users. Only minor intra-week changes in absolute levels were found. Intra-week test-retest correlations varied between 0.52 (t-PA activity) and 0.94 (HDL-C) with an average value of 0.81. In men, non-OC using women, and OC using women, the covariance matrices of the eight risk indicators were equal at day 1 and day 3, testifying the good stability of covariance structure over time. Differences in covariance structure of all three groups were observed, which remained after correction for BMI and age. In men and non-OC-using women, significant correlation was found on all days between insulin and the other risk indicators with exception of fibrinogen and LDL-C. In OC users, insulin was correlated with TG, LDL-C, and fibrinogen.

Conclusion: The metabolic and hemostatic risk indicators showed good test-retest reliability, and their covariance is stable over time. Multivariate analyses of this cluster should be performed separately for men, non-OC-using women, and OC-using women. © 2001 Harcourt Publishers Ltd

INTRODUCTION

Epidemiological research has shown that blood plasma levels of insulin, high-density cholesterol (HDL-C), low-density cholesterol (LDL-C), triglycerides (TG), fibrinogen and PAI-1 activity are risk indicators for cardio-vascular disease (CVD).[1–7] Although these indicators are considered to represent independent disease processes (e.g. hypertension, atherosclerosis and thrombosis), current thinking emphasizes their clustering in particular individuals. In the insulin resistance syndrome (IRS), high insulin, LDL-C and TG levels and low HDL-C levels co-exist with an unfavourable fibrinolytic profile.[8–12] Due to the additive and possibly synergistic effects of the underlying disease processes, small elevations of multiple metabolic and hemostatic risk indicators may signal a larger CVD disease risk than large deviations in one or few of these indicators. Consequently, recent studies on the effects of lifestyle, social economic class and chronic (work) stress have assessed clusters of hemostatic and metabolic risk indicators to compute the 'multivariate' CVD risk.[13–16]

Validity of a multivariate approach to CVD risk requires that: (1) the test-retest reliability over repeated

Received: 14 August 2000
Accepted after revision: 22 December 2000

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measures of the various risk indicators is sufficiently high, (2) there is substantial covariance between the various risk indicators and (3) the structure of their covariance is reasonably stable over time and conditions. Although some information is available on the reliability and the covariance of metabolic and hemostatic risk of the IRS indicators[17–19] an evaluation of the stability of their covariance structure is lacking entirely. Changes in covariance structure over time could easily emerge due to differential psychosocial, behavioural and infradian (weekly, monthly, seasonally) effects on each of these risk indicators.[20–23] Such effects may compromise the stability of covariance structure, even if the blood-sampling procedures (time of day, dietary restrictions, venipuncture protocol, etc.) and blood analyses (on-site preparation, storage, biochemical assays, etc.) are optimally standardized.

Secondly, the extent to which the covariance structure of the risk indicators differs across (sub-) populations is uncharted. Such group differences in covariance structure could easily exist, for instance, in populations differing in factors known to be associated with the risk indicators like physical activity, experienced (work) stress, gender or oral contraceptive use.[24–27] The latter two, gender and oral contraceptive effects, may selectively affect some of the risk indicators of the IRS,[28,29] and possibly change their covariance to other indicators. Systematic group differences in the covariance of the metabolic and hemostatic risk indicators would imply that between subjects designs should take the differential covariance structure of the groups into account.

The present study repeatedly assessed levels of fasting insulin, LDL-C, HDL-C, TG, fibrinogen, t-PA antigen, t-PA activity and PAI-1 antigen within a single work week in three groups of subjects separately: men, non-oral contraceptive-(OC) using women and OC-using women. Test-retest reliability and covariance structure of the eight target risk indicators were computed for all three groups. Covariance of these multiple indicators is examined with structural equation modelling[30] to test the stability of covariance structure across repeated measures within the groups, and to test for group differences in covariance structure.

MATERIALS AND METHODS

Subjects

In the present study, 125 male clerical workers from a large computer company (mean age 45.2 ± 5.3 years, range 35–55 years; BMI 25.1 ± 2.8) and 132 female nurses from three hospitals (mean age 33.7 ± 8 years, range 22–55 years; BMI 23.9 ± 4.0) volunteered to participate. Subjects were selected from a larger sample to include only healthy non-pregnant subjects who were not receiving treatment or taking medication for hypertension, or known to suffer from cerebrovascular disease, hyperlipidaemia or diabetes mellitus. Also subjects who had used aspirin or other anti-inflammatory or analgesic medication were excluded from the final analyses. 29.6% of the men and 28.1% of the women were smokers, and 44.8% of the women were OC users. All subjects gave written informed consent before entrance to the study. Study and blood-sampling protocol were approved by the Ethics Committee of the Vrije Universiteit, Amsterdam.

Procedure

Because our blood variables are known to respond to a host of confounders (e.g. time of day, shift work, current or previous day meals, alcohol or coffee drinking and physical activity etc.) rigid standardization of blood sampling procedures was enforced to optimize test-retest reliability. Subjects were requested to fast and refrain from use of alcohol, coffee and tea after 23:00 the preceding night and to refrain from high-impact physical activity the preceding day. In both the male and the female population, blood was drawn at the workplace from the arm in a sitting position after at least 15 min rest. In the male population, blood was drawn on Monday, Wednesday and Friday, at the beginning of a workday, between 08:00 and 09:30. In the female population, blood was drawn solely at daytime shifts between 07:00 and 07:30. Female nurses had to work at least three successive daytime shifts, and the first measurement day had to be preceded by at least 2 non-working days (to make sampling comparable to the Monday measurement in the male population). The first blood sample was drawn at the first day of a daytime shift, and the second sample 2 days later, when the nurses were still on a daytime shift. After the first blood sample was drawn, subjects' body weight (to the nearest 100 g) and height (to the nearest cm) were measured in light clothing.

Metabolic risk indicators

Blood withdrawal was according to the Standardized European Concerted Action on Thrombosis (ECAT) assay procedures.[31,32] Blood was drawn by venipuncture of the antecubital vein and sampled in six different vacutainers in the following order; serum with clot-activator (5 ml), serum (3 ml), Stabilyte® (5 ml),[33] citrate (5 ml), EDTA (3 ml). All vacutainers were mixed by moving the vacutainers 5 times 'head over head' immediately after withdrawal. Fasting insulin (pmol/l) was determined with a immunoradiometric assay kit (Medgenix Diagnostics Fleurus, Belgium)[34,35] from blood taken out of the

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serum-vacutainer. Blood had to clot for minimal 60 min at room temperature. Serum was separated by centrifugation at 2000 × g for 20 min at 4°C. Aliquots of serum were stored at −20°C. Values were multiplied by 0.139 to convert fasting insulin into mU/l. For determination of TG and HDL-C the serum of the clot-activator vacutainer was used. Blood was allowed to clot for minimal 30 min and maximal 2 h at room temperature. Serum was separated by centrifugation at 2000 × g for 20 min at 4°C. Lipid determinations were performed at the same day using the Vitros 250 Clinical Chemistry analyzer (Johnson & Johnson, Rochester, USA) with Vitros clinical chemistry slides for TG. HDL-C was determined in serum after a precipitation step with HDL-C precipitant (Boehringer Mannheim, Mannheim, Germany). LDL-C was calculated according to the formula of Friedewald.[36] All results are given in mmol/L. Stabilyte blood was drawn for the determination of t-PA activity. Citrated blood was withdrawn for determination of fibrinogen, t-PA antigen and PAI-1 antigen. Immediately after withdrawal, the vacutainers were put in melting ice and centrifuged within 60 min (2000 × g, 20 min at 4°C). Aliquots of plasma were snap-frozen immediately using solid carbon dioxide and stored at −80°C. t-PA activity was determined using the bio-functional immunosorbent assay Chromolize™tPA (Biopool, Umeå, Sweden).[37] Results were expressed in IU/ml. Fibrinogen was determined using the STA coagulation analyzer (STAG-O, Asnières, France) and the STA Fibrinogen kit (Boehringer Mannheim, Germany).[38] The results are expressed in g/L. t-PA antigen was measured using the enzyme immunoassay Imulyse™tPA (Biopool, Umeå, Sweden).[39] PAI-1 antigen was measured using enzyme immunoassay Innotest PAI-1 (Innogenetics, Zwijndrecht, Belgium).[40] Results for PAI-1 antigen en t-PA antigen are expressed in ng/ml. The intra-assay and the inter-assay coefficient of variation, respectively were less then: 5.0 and 7.0% for fasting insulin, 3.5 and 5.0% for HDL-C, 3.0 and 5.0% for TG, 5.0 and 7.0% for fibrinogen, 10.0 and 12.0% for t-PA antigen, 7.5 and 10.0% for t-PA activity, and 10.0 and 10.0% for PAI-1 antigen. For each of the blood variables all blood samples were analyzed in the same batch. Moreover, the blood samples, drawn from the same participant on repeated blood withdrawal occasions, were analyzed simultaneously on the same plate. No sample had been stored for more than 7 months.

For evaluation of hemoconcentration, hematocrit (Ht), from EDTA blood was used. Immediately after withdrawal per participant 2 capillary tubes (Hawksley & Sons Ltd., Sussex, England) were filled three-quarters with blood, sealed with SEAL-EASE® Tube Sealer (Becton Dickinson, Franklin Lakes, USA) and centrifuged for 6 min in a micro hematocrit centrifuge. A hematocrit-reader was used for determination the Ht values.

Statistical analyses

Analyses of intra-week effects and group differences were performed in a multivariate design using the General Linear Modeling (GLM in SPSS7.5 for Windows) procedure. The dependent variables in the GLM model were fasting insulin, HDL-C, LDL-C, TG, fibrinogen, t-PA antigen, t-PA activity and PAI-1 antigen. Men, non-OC-using women and OC-using women were entered in the model as between subject factor. Age and BMI were entered as covariates in the analyses. The GLM procedure was started with a model specification, which included all main effects and all second-, and third-order interactions. Subsequently, non-significant interactions were removed from the model specification and the GLM procedure was performed again. This step-down procedure ends when an exclusive significant interaction or a main effect is found.[41] Test-retest reliability was assessed in three ways. *First* by computing Pearson correlation coefficients. *Second* by a method described in Fraser et al.[42] and Marckmann et al.[20] using the assay variation in the duplicates and the variation across different measurement days, to obtain estimates of analytical variance (Sa), average within-subject variance (Si) and between subject variance (Sg). Analytical variance was extracted from the variance in the duplicates (as reflected in the intra-assay CV). Based on the criteria of Cotlove et al.,[43] which was repeated in Fraser et al.[42] and adopted by the college of American Pathologists and the World association of societies of pathology, we specify the minimally acceptable CV for each of the assays (the CV of the assay should equal to or less than half of the normal intra-week biological variation also expressed as a CV). *Third* by calculating repeatability coefficients $RC = 2.77 \times (\sqrt{s_w})$, after Bland, and Altman,[44] using the within-subject's variances (s_w) calculated according to the procedure described above.

The idea of a latent syndrome underlying various risk indicators can be easily reformulated in terms of a statistical technique called structural equation modelling (SEM).[30] In SEM, a latent IRS factor can be postulated that has causal pathways to a number of latent factors reflecting the true score of the hemostatic and metabolic risk indicators. These latent factors are based, in turn, on repeated observations of each of the risk indicators (see Fig. 1).

The above approach of repeated measures of multiple risk indicators substantially increases the statistical power of both within subject (e.g. efficacy of medication) and between subject (e.g. case control comparison) designs. Structural equation modelling was performed using LISREL8.[30] First we assessed the stability of the covariance matrices of the risk indicators within each group; this was only done for day 1 and day 3. A model is specified wherein the correlations between all risk

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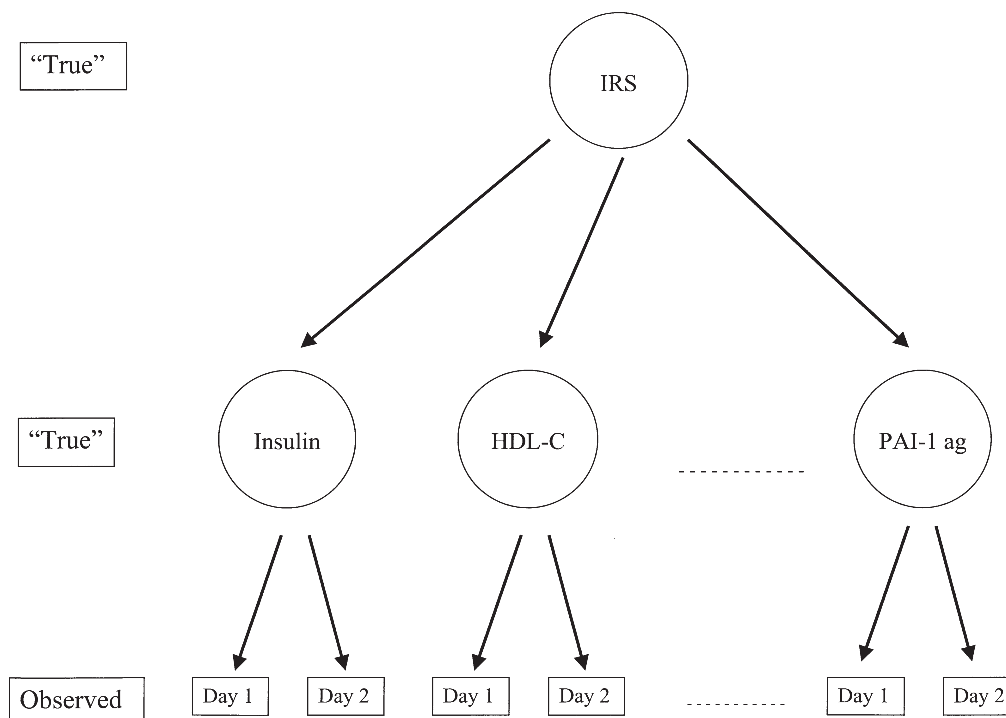

Fig. 1 Schematic representation of the influence of a latent IRS factor on the true score of various risk indicators. The true score of the risk indicators influences the observed measurements over repeated occasions.

indicators and their standard deviations are stated to be equal at both days. This model is fitted to the observed covariance in an iterative maximum likelihood procedure, yielding a chi-square parameter that indicates the goodness of fit of the model. Subsequently, a second model is specified wherein the correlations between all risk indicators are specified to be equal and their standard deviations are unequal. The difference in the chi-square goodness of fit parameters of these two models has itself a chi-square distribution. If the second more parsimonious model does not fit significantly worse than the first full model, the hypothesis that the covariance structure is equal on the 2 days is accepted. Secondly, in the same way comparisons of the covariance matrices between all combinations of the risk indicators were performed for the three groups. As a consequence of different statistical characteristics of the chi-square distribution, chi-square tests were carried out against a significance level of $P<0.01$.

RESULTS

All data were checked with regard to frequency distribution. Fasting insulin, TG, fibrinogen, t-PA antigen, t-PA activity and PAI-1 antigen were transformed to normal distribution by logarithmic transformation before entering in the statistical analysis. For readability, the back-transformed logarithmic mean and range of transformed risk indicators for the groups are given in Table 1.

Intra week changes and group differences in absolute levels

Values on the metabolic and hemostatic risk indicators and Ht, obtained at day 1 and day 3 were tested for group differences across men, non-OC-using women and OC-using women. Age and BMI were included as covariates in this analysis. Two interactions involving group or day were found: group by BMI ($F(18,438)=2.5$, $P=0.001$) and group by day ($F(18,438)=2.3$, $P=0.002$). Inspection of the univariate results showed the group by BMI interaction to be due to fasting insulin ($F(2,226)=3.6$, $P=0.03$), t-PA activity ($F(2,226)=10.1$, $P<0.001$), PAI-1 antigen ($F(2,226)=6.2$, $P=0.003$) and Ht ($F(2,226)=4.4$, $P=0.01$). Parameter estimates indicated that with increasing BMI, fasting insulin increased most in men and least in OC-using women. With increasing BMI, t-PA activity decreased most in men and least in OC-using women. With increasing BMI, PAI-1 antigen levels increased most in men and least in

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Table 1 Age, BMI and risk indicators for men, non-oral-contraceptives-(OC) using women and OC-using women for each blood withdrawal day separately (means and standard deviations, for variables with a skewed distribution the back-transformed logarithmic means and ranges are given)

	Men ($n=125$)			**Non-OC-using women ($n=75$)**		**OC-using women ($n=57$)**	
Age (years)	45.23 ± 5.33			36.71 ± 8.29		30.19 ± 6.21	
BMI (kg/m^2)	24.96 (18.70–34.11)			24.28 (18.29–40.60)		23.00 (18.20–35.62)	
Risk indicators	**day 1**	**day 3**	**day 5**	**day 1**	**day 3**	**day 1**	**day 3**
Insulin (mU/l)	6.63 (2.36–31.83)	6.55 (2.78–32.25)	6.77 (2.50–26.55)	6.65 (2.22–28.08)	6.54 (2.64–20.80)	6.96 (4.17–21.13)	6.95 (3.75–14.73)
TG (mmol/l)*	1.26 (0.49–4.02)	1.36 (0.55–4.28)	1.33 (0.51–4.22)	0.93 (0.48–2.49)	0.89 (0.45–2.05)	1.16 (0.57–3.01)	1.07 (0.57–2.42)
HDL-C (mmol/l)	1.20 ± 0.39	1.19 ± 0.38	1.16 ± 0.35	1.43 ± 0.34	1.40 ± 0.36	1.59 ± 0.37	1.56 ± 0.38
LDL-C (mmol/l)*	3.50 ± 0.81	3.57 ± 0.86	3.52 ± 0.90	2.79 ± 0.67	2.76 ± 0.61	2.69 ± 0.84	2.68 ± 0.80
Fibrinogen (g/l)	2.92 (1.97–5.32)	2.91 (1.71–4.71)	2.91 (1.82–4.58)	2.81 (1.64–4.47)	2.78 (1.63–4.47)	2.85 (1.96–4.28)	2.79 (1.91–4.13)
t-PA activity (IU/ml)*	0.45 (0.01–2.34)	0.47 (0.3–1.5)	0.44 (0.05–1.6)	0.63(0.14–1.96)	0.53 (0.07–1.87)	0.83 (0.25–1.63)	0.73 (0.06–1.60)
t-PA antigen (ng/ml)	9.56 (3.2–20.6)	9.48 (2.2–17.8)	9.36 (3.4–19.6)	5.66 (1.60–17.20)	5.18 (1.40–15.10)	3.81 (1.20–9.20)	3.54 (1.40–8.70)
PAI-1 antigen (ng/ml)	73.45 (11.0–554.0)	66.06 (10.4–446.0)	66.51 (10.0–303.0)	42.03 (8.0–186.8)	41.01 (9.4–210.0)	18.03 (2.8–90.5)	17.43 (4.3–69.90)
Hematocrit (mmol/l)*	43.77 ± 2.50	43.73 ± 2.47	43.80 ± 2.29	39.19 ± 2.30	38.64 ± 1.87	39.32 ± 2.01	38.74 ± 2.24

*: group by day interaction; BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; PAI-1: plasminogen activator inhibitor-1; TG: triglycerides; t-PA: tissue-type plasminogen activator

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non-OC-using women. With increasing BMI, OC users had an increase in Ht level; men and non-OC users had a small decrease in Ht levels. The group by day interaction was due to TG ($F(2,226)=4.9$, $P=0.008$), LDL-C ($F(2,226)=3.7$, $P=0.03$), t-PA activity ($F(2,226)=5.0$, $P=0.008$) and Ht ($F(2,226)=3.8$, $P=0.02$). These risk indicators increased from day 1 to day 3 in men and decreased in both groups of women. Ht did not change in men, but it did decrease in women from day 1 to day 3.

Test-retest reliability

Test-retest correlations between day 1 and day 3 in men and women, and correlations between day 3 and 5, and between day 1 and day 5 in men, varied between 0.52 (t-PA activity in women who used OCs) and 0.94 (LDL-C in men), with a medium value of 0.81. All were highly significant ($P<0.001$). In men, test-retest correlations were slightly lower for day 1–5 compared to day 1–3 as well as day 3–5, except for t-PA antigen. Following Salomaa et al.,[44] we focused on the subjects that were in the highest quartile (lowest quartile for HDL-C and t-PA activity) of each risk indicator distribution on the first blood withdrawal occasion. The proportion of these subjects that were still in the highest quartile on a second withdrawal occasion was computed (for men separate proportions for day 1 and 3, and day 1 and 5 were computed). Table 2 shows these proportions. On average they were 70.1% (range 50.0–94.4%). In men, the highest proportions were found for LDL-C and TG, and the lowest proportions were found for Ht. Compared to OC users, in non-OC users, higher proportions were found for LDL-C, TG and insulin and lower proportions for HDL-C.

Following Fraser et al.[42] we computed the components of variance in each of the risk indicators due to between subject variance, within-subject variance (excluding analytical variance) and analytical variance (see Table 3). We additionally report the intra-assay coefficient of variation (CV) that is minimally required to reliably detect intra-week within-subject variance ($CV_{analytical} \leqslant 0.5 \times CV_{within\text{-}subject}$)[41] HDL-C, LDL-C, fibrinogen, and t-PA antigen did not meet this criterion in all three groups. Using the within-subject's variances (s_w), repeatability coefficients (RC) after Bland and Altman[44] were computed. In general, RCs appear to be higher in men than in women, particularly for insulin, TG, fibrinogen, t-PA antigen and PAI-1 antigen.

Correlation structure of the risk indicators

Table 4 displays the correlation structure of the risk indicators for both sexes. The metabolic and hemostatic risk indicators showed substantial intercorrelations in men (Table 4A) and women (Tables 4B & 4C).

In men and non-OC-using women, significant correlation was found on all days between insulin and the other risk indicators with exception of fibrinogen and LDL-C. In OC users, insulin was systematically associated with TG, LDL-C, and fibrinogen only. Overall, the largest difference in correlation structure was found between men and OC-using women, with the non-OC-using women somewhere in between.

Covariance structure of the risk indicators

Differences in the covariance structure of the metabolic and fibrinolytic risk indicators of the first and second blood withdrawal day were tested using LISREL8.[30] Results are shown in the upper panel of Table 5. Tests of the intra-week stability of the covariance structure were carried out for the three groups separately. In men and

Table 2 Proportion and number of men, non-oral-contraceptives-(OC) using women and OC-using women who were in the highest quartile (lowest quartile for HDL-C and t-PA-act) of the risk indicator distribution on the first blood withdrawal occasion also belonging to the highest quartile on the second blood withdrawal occasion

Risk indicators	Men ($n=102$) day 1–3 %	Men day 1–3 (n)	Men day 1–5 %	Men day 1–5 (n)	Non-OC-using women ($n=75$) day 1–3 %	Non-OC day 1–3 (n)	OC-using women ($n=57$) day 1–3 %	OC day 1–3 (n)
Insulin (mU/l)	73.1	(19)	68.0	(17)	83.3	(15)	61.5	(8)
TG (mmol/l)	76.9	(20)	73.1	(19)	73.7	(14)	57.1	(8)
HDL-C (mmol/l)	69.2	(18)	73.1	(19)	65.0	(13)	92.9	(13)
LDL-C (mmol/l)	84.6	(22)	76.9	(20)	94.4	(17)	64.3	(9)
Fibrinogen (g/l)	65.4	(17)	69.2	(18)	77.8	(14)	78.6	(11)
t-PA activity (IU/ml)	65.4	(17)	70.4	(19)	57.9	(11)	50.0	(7)
t-PA antigen (ng/ml)	69.2	(18)	73.1	(19)	75.0	(15)	78.6	(11)
PAI-1 antigen (ng/ml)	76.9	(20)	61.5	(16)	63.2	(12)	57.1	(8)
Hematocrit (mmol/l)	50.0	(11)	60.0	(15)	73.3	(11)	63.6	(7)

BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; PAI-1: plasminogen activator inhibitor-1; TG: triglycerides; t-PA: tissue-type plasminogen activator.

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both the non-OC-using and OC-using women the covariance matrices were equal for day 1 and day 3 ($\chi^2(8) = 7.72$, $P = 0.46$; $\chi^2(8) = 10.72$, $P = 0.22$; $\chi^2(8) = 7.68$, $P = 0.47$ respectively).

The same procedure was performed in a multi-group LISREL8 analysis to test group differences. Comparisons between men and non-OC users, men and OC users and non-OC users and OC users were performed successively. Results are shown in the lower panel of Table 5. In this analysis the covariance matrices at day 1 and day 3 were specified to be equal within and between the groups. In addition, the standard deviations were equal within and between groups. These models all fitted poorly ($\chi^2(192) = 240.90$, $P = 0.0095$; $\chi^2(192) = 252.50$, $P = 0.002$; $\chi^2(192) = 247.83$, $P = 0.004$ respectively), so no further fitting of more parsimonious models were needed to conclude that the covariance structures between the groups differ. These results indicate that the covariance structure of the metabolic and hemostatic risk indicators, measured at 2 work days, were different in men, non-OC-using women and OC-using women. This remained true after correction for differences in BMI and age in the three groups.

DISCUSSION AND CONCLUSIONS

Previous studies performing multivariate regression and multivariate ANOVA analyses on different clusters of metabolic and hemostatic risk indicators of the insulin resistance syndrome have tacitly assumed that: (1) the univariate test-retest reliability over repeated measures of the various indicators is good to excellent, (2) there is substantial covariance between these risk indicators, and (3) the structure of their covariance is stable over time. This study directly tested these assumptions and found them to be essentially correct. With regard to test-retest reliability, 42.9 to 92.9% of the observed variance in the risk indicators could be attributed to between subject variance with an average value of 81%. Moreover, the proportion of subjects that were in the extreme quartile of the risk indicator distribution on two successive

Table 3 Estimates of between subject (BS), within subject (WS) and analytical (A) variation (percentages in brackets), and coefficients of variation (CV) of the risk indicators for men ($n_{ind} = 102$, $n_{obs} = 612$), non-oral-contraceptives-(OC) using women ($n_{ind} = 75$, $n_{obs} = 300$) and OC-using women ($n_{ind} = 57$, $n_{obs} = 228$), separately. Repeatability coefficients (RC) for the three groups separately are given as well

	Between subject			Within subject			Analytical				
	Variance	(%)	CV_{BS}	Variance	(%)	CV_{WS}	Variance	(%)	CV_A	½CV_{WS}	RC
Men											
insulin	16.71	(79.5)	53.9	4.16	(19.8)	26.9	0.14	(0.7)	5.0	13.5	5.65
TG	0.41	(68.0)	43.4	0.19	(31.7)	29.5	0.0020	(0.3)	3.0	14.8	1.21
HDL-C	0.13	(90.9)	29.9	0.011	(7.9)	8.9	0.0017	(1.2)	3.5	4.5	0.29
LDL-C	0.70	(91.2)	23.5	0.052	(6.8)	6.5	0.015	(2.0)	3.5	3.3	0.63
fibrinogen	0.25	(81.6)	16.9	0.035	(11.3)	6.3	0.022	(7.1)	5.0	3.2	0.52
t-PA activity	0.072	(60.2)	48.3	0.046	(38.3)	37.7	0.0018	(1.5)	7.5	18.9	0.59
t-PA antigen	9.074	(74.8)	29.8	2.028	(16.7)	14.1	1.026	(8.5)	10.0	7.1	3.95
PAI-1 antigen	3935.52	(76.1)	70.0	1152.76	(22.3)	37.9	80.69	(1.6)	10.0	19.0	94.05
Non-OC-using women											
insulin	10.00	(83.5)	43.9	1.85	(15.4)	18.9	0.13	(1.1)	5.0	9.5	3.77
TG	0.095	(68.0)	31.3	0.044	(31.4)	21.7	0.00084	(0.6)	3.0	10.9	0.58
HDL-C	0.11	(88.1)	23.2	0.013	(10.0)	8.1	0.0024	(1.9)	3.5	4.1	0.32
LDL-C	0.36	(84.6)	21.8	0.032	(8.0)	6.5	0.0094	(2.4)	3.5	3.3	0.50
fibrinogen	0.26	(90.0)	17.8	0.0090	(3.1)	3.3	0.020	(6.9)	5.0	1.7	0.26
t-PA activity	0.076	(62.9)	42.1	0.042	(35.0)	31.3	0.0025	(2.1)	7.5	15.7	0.57
t-PA antigen	4.30	(82.4)	35.4	0 .58	(11.1)	13.0	0.34	(6.5)	10.0	6.5	2.11
PAI-1 antigen	954.20	(68.6)	60.9	412.06	(29.6)	40.0	25.66	(1.8)	10.0	20.0	56.23
OC-using women											
insulin	4.91	(64.3)	30.2	2.59	(33.9)	22.0	0.14	(1.8)	5.0	11.0	4.46
TG	0.16	(75.6)	33.5	0.050	(23.8)	18.8	0.0013	(0.6)	3.0	9.4	0.62
HDL-C	0.13	(92.9)	23.0	0.0070	(5.0)	5.4	0.0030	(2.1)	3.5	2.7	0.23
LDL-C	0.64	(92.8)	29.8	0.041	(5.9)	7.6	0.0088	(1.3)	3.5	3.8	0.56
fibrinogen	0.29	(87.8)	18.9	0.019	(5.8)	4.9	0.021	(6.4)	5.0	2.5	0.38
t-PA activity	0.039	(42.8)	23.6	0.048	(52.8)	26.1	0.0040	(4.4)	7.5	13.1	0.61
t-PA antigen	2.56	(82.1)	40.0	0.40	(12.8)	15.7	0.16	(5.1)	10.0	7.9	1.75
PAI-1 antigen	187.52	(85.7)	63.5	124.74	(13.7)	51.8	4.76	(0.6)	10.1	23.5	30.94

BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; n_{ind}: number of individuals; n_{obs}: number of observations; PAI-1: plasminogen activator inhibitor-1; TG: triglycerides; t-PA: tissue-type plasminogen activator.

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Table 4A Correlation structure of the risk indicators in men

	Insulin	TG	HDL-C	LDL-C	Fibrinogen	t-PA act	t-PA ag	PAI-1 ag
Day 1								
insulin	–							
TG	0.34†	–						
HDL-C	−0.41†	−0.39†	–					
LDL-C		0.37†		–				
Fibrinogen				0.30†	–			
t-PA activity	−0.35†	−0.23*	0.35†			–		
t-PA antigen	0.39†	0.39†	−0.43†	0.26*		−0.25†	–	
PAI-1 antigen	0.40†	0.42†	−0.43†	0.21*		−0.76†	0.64†	–
BMI	0.41†	0.27†	−0.36†			−0.54†	0.44†	0.60†
Day 3								
insulin	–							
TG	0.27†	–						
HDL-C	−0.37†	−0.43†	–					
LDL-C		0.27†		–				
fibrinogen				0.28†	–			
t-PA activity	−0.54†	−0.41†	0.32†			–		
t-PA antigen	0.43†	0.39†	−0.34†			−0.44†	–	
PAI-1 antigen	0.56†	0.49†	−0.28†			−0.78†	0.70†	–
BMI	0.54†	0.36†	−0.39†			−0.61†	0.44†	0.61†
Day 5								
insulin	–							
TG	0.35†	–						
HDL-C	−0.38†	−0.36†	–					
LDL-C		0.26†		–				
fibrinogen				0.21*	–			
t-PA activity	−0.57†	−0.40†	0.28†			–		
t-PA antigen	0.45†	0.38†	−0.39†			−0.43†	–	
PAI-1 antigen	0.56†	0.47†	−0.32†			−0.79†	0.76†	–
BMI	0.59†	0.33†	−0.37†			−0.51†	0.40†	0.52†

BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; PAI-1: plasminogen activator inhibitor-1; TG: triglycerides; t-PA: tissue-type plasminogen activator. Non-significant correlations are omitted: *: $P<0.05$; †: $P<0.01$.

Table 4B Correlation structure of the risk indicators in non-oral contraceptives using women

	Insulin	TG	HDL-C	LDL-C	Fibrinogen	t-PA act	t-PA ag	PAI-1 ag
Day 1								
insulin	–							
TG		–						
HDL-C	−0.34†	−0.41†	–					
LDL-C		0.34†		–				
fibrinogen	0.27*			0.28*	–			
t-PA activity	−0.24*			−0.24*	−0.29*	–		
t-PA antigen	0.32†	0.50†	−0.29*		0.28*	−0.36†	–	
PAI-1 antigen	0.38†	0.37†	−0.31†	0.25*	0.30†	−0.75†	0.67†	–
BMI	0.54†	0.26*	−0.25*		0.45†	−0.47†	0.42†	0.51†
Day 3								
insulin	–							
TG	0.27*	–						
HDL-C	−0.27*	−0.31†	–					
LDL-C		0.33†		–				
fibrinogen	0.32†			0.25*	–			
t-PA activity	−0.32*	−0.26*	0.42†		−0.35†	–		
t-PA antigen	0.34†	0.47†	−0.27*	0.23*	0.28*	−0.43†	–	
PAI-1 antigen	0.38†	0.37†	−0.37†	0.23*	0.32†	−0.84†	0.70†	–
BMI	0.40†		−0.28*	0.24*	0.48†	−0.57†	0.37†	0.52†

BMI: body mass index; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; PAI-1: plasminogen activator inhibitor-1; TG: triglycerides; t-PA: tissue-type plasminogen activator. Non-significant correlations are omitted: *: $P<0.05$; †: $P<0.01$.

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Table 4C Correlation structure of the risk indicators in oral contraceptives using women

	Insulin	TG	HDL-C	LDL-C	Fibrinogen	t-PA act	t-PA ag	PAI-1 ag
Day 1								
insulin	–							
TG	0.41†	–						
HDL-C			–					
LDL-C	0.39†	0.30*		–				
fibrinogen	0.30*				–			
t-PA activity						–		
t-PA antigen							–	
PAI-1 antigen						−0.59†	0.65†	–
BMI	0.39†				0.47†			0.37†
Day 3								
insulin	–							
TG	0.38†	–						
HDL-C			–					
LDL-C	0.32*	0.35†	−0.34*	–				
fibrinogen	0.27*	0.34†			–			
t-PA activity	−0.32*			−0.38†		–		
t-PA antigen							–	
PAI-1 antigen	0.33*					−0.61†	0.64†	–
BMI	0.39†				0.49†	−0.28*	0.30*	0.41†

BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; PAI-1: plasminogen activator inhibitor-1; TG: triglycerides; t-PA: tissue-type plasminogen activator. Non-significant correlations are omitted: *: $P<0.05$; †: $P<0.01$).

Table 5 Upper panel: tests for equality of covariance matrices of the metabolic and hemostatic risk indicators on day 1 and 3 for men ($n=104$), non-oral contraceptives using women ($n=75$) and oral contraceptives using women ($n=57$; a non-significant $\Delta\chi^2$-value means that the covariance structures were equal for day 1 and day 3). Lower panels: tests for equality of covariance matrices between the three groups (a significant χ^2-value means that the covariance structures were different between the groups)

Model specification	df	χ^2	*P*-value	Δdf	$\Delta\chi^2$	*P*-value
Day differences						
Men						
1. $R_{1m}=R_{3m}, sd_{1m}\neq sd_{3m}$	56	75.75	0.04			
2. $R_{1m}=R_{3m}, sd_{1m}=sd_{3m}$	64	83.47	0.05			
Model 1 vs. model 2				8	7.72	0.46
Non-OC-using women						
3. $R_{1nooc}=R_{3nooc}, sd_{1nooc}\neq sd_{3nooc}$	56	54.83	0.52			
4. $R_{1nooc}=R_{3nooc}, sd_{1nooc}=sd_{3nooc}$	64	65.55	0.42			
Model 3 vs. model 4				8	10.72	0.22
OC-using women						
5. $R_{1oc}=R_{3oc}, sd_{1oc}\neq sd_{3oc}$	56	63.14	0.24			
6. $R_{1oc}=R_{3oc}, sd_{1oc}=sd_{3oc}$	64	70.82	0.26			
Model 5 vs. model 6				8	7.68	0.47
Group differences						
Men vs. non-OC using women						
7. $R_{1m}=R_{3m}=R_{1nooc}=R_{3nooc}, sd_{1m}=sd_{3m}=sd_{1nooc}=sd_{3nooc}$	192	240.90	0.0095			
Men vs. OC-using women						
8. $R_{1m}=R_{3m}=R_{1oc}=R_{3oc}, sd_{1m}=sd_{3m}=sd_{1oc}=sd_{3oc}$	192	252.50	0.002			
OC-using women vs. non-OC using women						
9. $R_{1oc}=R_{3oc}=R_{1nooc}=R_{3nooc}, sd_{1oc}=sd_{3oc}=sd_{1nooc}=sd_{3nooc}$	192	247.83	0.004			

R: correlation matrix; sd: standard deviation; 1: day 1; 3: day 3; m: men; nooc: women who do not use oral contraceptives; OC: women who use oral contraceptives; df: degrees of freedom; Δ: delta.

withdrawal days was high (70% on average). Other studies have reported comparably short-term reliability coefficients for insulin,[17] lipoproteins,[46] fibrinogen,[47] t-PA antigen[48] and PAI-1 antigen [48,49] in both male and female populations. This study is the first to additionally test for possible changes in the covariance structure of metabolic

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and hemostatic risk indicators across repeated measures. The results show that, at least across a single workweek, the covariance structure between hemostatic and metabolic risk indicators are stable. Taken together, these results suggest that in epidemiological studies blood samples drawn on different days of the week will yield similar results. The huge logistical impact on a study design of having to measure all subjects on exactly the same day of the week is not justified by our results.

In our study: (1) the samples of all subjects were frozen and saved to be analyzed in a single batch, (2) measurement conditions (time of day, shift, protocol for blood handling, transport and storage, etc.) were highly standardized, and (3) experimental protocol minimized confounding factors (diet, smoking, alcohol use, recent exercise etc.). With Fraser et al.,[42] we believe that these conditions should be met in future studies as much as possible. Under these optimal conditions, Fraser et al.[42] specified the minimally acceptable CV of biochemical assays to be equal to or less than half of the average within-subject variation also expressed as a CV. Because of high test-retest reliability, our assays for HDL-C, LDL-C, fibrinogen, and t-PA antigen were not adequate to detect intra-week within-subject variance in all or some of the groups according to this criterion. However, within subject variation in these risk indicators probably increases when measurements are spaced over longer periods of time, due to additional monthly cycles and seasonal effects, although previous studies suggest that such seasonal variation is small in comparison to short-term variation.[19] More importantly, intra-week within subject variation is likely to be higher in patient populations than in the present healthy subjects, because higher mean levels usually coincide with higher variances. Therefore, minimally acceptable CV of the assays in single patient studies in clinical settings is probably larger than reported in Table 3A. A relatively large intra-week within-subject variance was found for TG, t-PA activity and PAI-1 antigen. This means that a single measurement of these risk indicators will be a poor indicator of the 'true' score. In population-based studies, trueness of measurement is usually increased by measuring a variable in more subjects or by assessing the variable a repeated number of times. The former is costly, and the latter is most effective if the correlation between repeated measures is high. The present study demonstrated the validity of a third strategy. Measurement of multiple indicators that are highly correlated can be as informative about the value of a single indicator as many repeated measurements of that single variable. Based on our results, measurement of t-PA activity and t-PA antigen in men (average correlations to PAI-1 antigen is -0.79 and 0.71 respectively) is almost as informative about PAI-1 as measuring PAI-1 three times (average test-retest is 0.80). Logistically, it is much easier to measure three parameters on 2 days, than a single parameter on six different days. More importantly, in the case of the risk indicators of the insulin-resistance syndrome, a multivariate approach is theoretically by far the most attractive. Studies assessing epidemiological risk, and ultimately even clinical studies, want to assess the subject's latent CVD risk. Because of their possible synergistic effects, a combination of high levels of all risk indicators may be more informative about the subjects CVD risk, than a high level in a single indicator. Therefore, a design with a few repeated measures of these multiple correlated risk indicators is an optimal way to assess the multivariate CVD risk conveyed by the insulin resistance syndrome. It has good statistical power, it is easier to implement (measurements on 2 or 3 days within a single week rather than repeated measurements across many weeks) and is theoretically meaningful. Because clear group differences were shown in covariance structure for men, non-OC-using women and OC-using women results of such analyses should be performed separately within each group. In general, when comparing different groups, multivariate analyses of mean, variance *and covariance* of the risk indicators is to be preferred above conventional analyses testing for mean and variance alone.

In conclusion, two measurements within the same week of these multiple correlated metabolic and hemostatic risk indicators showed them to have good test-retest reliability, and a stable covariance structure over time. However, the covariance structure is different for men, non-OC-using women, and OC-using women. Future multivariate model fitting on this metabolic and hemostatic risk cluster should estimate the parameters separately for these three groups.

ACKNOWLEDGEMENTS

Dr C. Dolan and Dr D. L. Knol are thanked for their help and advise on the statistical analyses. This work was supported by grants from the Netherlands Heart Foundation (NHS grant 94.030) and the Netherlands Organization for Scientific Research (NWO grant 904-64-045).

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