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Diagnostic strategies for C-reactive protein

Harriëtte Riese*[1], Tanja GM Vrijkotte[1], Piet Meijer[2], Cees Kluft[2] and Eco JC de Geus[1]

Address: [1]Department of Biological Psychology, Vrije Universiteit, Amsterdam, The Netherlands and [2]Gaubius Laboratory, TNO-PG, Leiden, The Netherlands

E-mail: Harriëtte Riese* - h.riese@psy.vu.nl; Tanja GM Vrijkotte - tgm.vrijkotte@psy.vu.nl; Piet Meijer - p.meijer@pg.tno.nl; Cees Kluft - kluft@euronet.nl; Eco JC de Geus - eco@psy.vu.nl

*Corresponding author

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Abstract

Background: Serum C-reactive protein (CRP) has been identified in prospective epidemiological research as an independent risk marker for cardiovascular disease. In this paper, short-term biological variation of CRP is documented and a strategy to test the reliability of a single CRP sample is proposed.

Methods: Data were obtained from three groups of healthy volunteers: men, no oral contraceptives (OC-)using women and OC-using women. Blood samples were obtained 3 times in men and twice in women during a workweek.

Results and discussion: CRP values were highest in the OC-using women, followed by the men, and lowest in the no OC-using women. Averaged over the three groups the within-subject coefficients of variation (CVi) was 49.24% for CRP, and 29.90% for lnCRP. Using the repeated measures, individual samples were identified that reflected a 'suspicious' unreliable high value, i.e. a value that was more than 2 standard deviations higher than the lowest value obtained from the same subject. In an *a posteriori* analysis, three strategies to identify these suspicious high CRP values were then tested. In terms of maximizing detection of suspicious values and minimizing unnecessary resampling, best results were obtained for the most pragmatic criterion of using an absolute level, stratified for gender, and OC-use, to decide whether a second sample should be obtained.

Conclusion: A single high CRP value must be followed by re-sampling when it is above 1.75 mg/l for men, above 1.00 mg/l for no OC-using women, and above 2.00 mg/l for OC-using women.

Background

Prospective epidemiological research has identified C-reactive protein (CRP), for both men and women, as an independent predictor for cardiovascular diseases (CVD) [1,2]. Clinical utility of CRP values as diagnostic for CVD risk awaits a validated classification scheme to convert the CRP blood value of a single individual into a risk probability. Three major problems impede development of such a scheme. First, CRP is highly sensitive to injury and recent inflammatory events not necessarily related to the chronic atherosclerotic inflammation. CRP levels can increase to as much as 1–1000 fold from baseline concentrations with bacterial infection, trauma, surgery, and other inflammatory events, declining to baseline level in

12–14 days [3]. Second, there is a large impact of oral contraceptives (OC) use and gender on CRP values, that may be unrelated to CVD risk. Women using OC have higher CRP values [4] but are not at more risk for CVD than men [5]. Third, even within infection-free subjects, stratified for gender and OC-use, large intra-individual variability in CRP may be found, as hinted at by our previous results on acute phase proteins [6]. As long as interindividual variability is large, this need not present a problem, but documentation of intra- and interindividual variability of CRP, to our knowledge, is largely limited to two recent publications [7,8].

A first step in the research presented in this paper was to collect data on intra-individual variability -or its reverse: test-retest reliability – in gender and OC-use specific subgroups, with no reports of recent infection. As previously reported, we repeatedly assessed levels of fasting insulin, high density lipoprotein cholesterol (HDL-C), low density lopoprotein cholesterol (LDL-C), triglycerides (TG), fibrinogen, tissue-type plasminogen activator (t-Pa) activity, t-PA antigen, plasminogen activator inhibitor-1 (PAI1) antigen within a single work week in three groups of healthy subjects separately: men, no OC-using women and OC-using women [6,9]. CRP has now additionally been analysed in these samples. A variance components method was used to evaluate the different sources of variance of (ln)CRP: analytical, intra-individual and inter-individual. Intraweek test-retest reliability was computed over three and five days. As a second step we tested three different criteria that could be used to determine the necessity of repeated sampling after a high CRP value was found in an individual. Using the repeated measures, individual samples were identified that reflected a "suspicious" unreliable high value, i.e. a value that was more than 2 standard deviations (SD) higher than the lowest CRP value obtained from the same subject. We then attempted to detect this suspicious value in an *a posteriori* analysis in which all samples were regarded to come from a (typical epidemiological) study design with only one blood sample available per subject. Three different criteria were used: (1) The first criterion was to label all values above an arbitrary absolute threshold level as an unreliable sample. (2) Second, we used an individual's predicted CRP value as a criterion. The individual CRP values were predicted from known correlates like gender, BMI, smoking behaviour and alcohol consumption [4,10]. (3) Finally, we used a similar regression procedure to obtain a predicted CRP value, this time adding fibrinogen and the full spectrum of metabolic syndrome X risk indicators (insulin, HDL-C, LDL-C, TG, t-PA antigen, t-PA activity, and PAI-1 antigen) obtained in the same blood sample as additional predictors. Recent studies suggest that CRP should be included in the insulin resistance (IR) syndrome cluster, since both elevated CRP levels and IR have been found to be markers of the presence of infections, and inflammation in the vessel wall with atherosclerosis [11,12]. We reasoned that future clinical CVD risk assessment may increasingly measure various parameters of the metabolic syndrome in a single blood sample. Thus, we explored the possibility that multivariate measures could help decide on the reliability of an observed CRP value as an alternative for the classic multiple sampling procedure. In all instances, criteria for resampling were evaluated separately for men, no OC-using women and OC-using women.

Methods

Subjects

In the present study, 92 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 ± 8.0 years, range 22–55 years; BMI 23.9 ± 4.0) volunteered to participate in cardiovascular risk assessment. Subjects were included only if healthy, non-pregnant, not receiving treatment or taking medication for hypertension, 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. One of the female subjects had CRP levels > 10 mg/l on both occasions (15.94 and 16.26, respectively). She reported having used diet pills before both blood withdrawal days and was therefore excluded from the final analyses. 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 most 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 11:00 p.m. 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 minutes rest. In the male population blood was drawn on Monday, Wednesday and Friday, at the beginning of a workday, between 8:00 and 9:30 a.m. In the nurses, blood was drawn solely at daytime shifts, between 7:00 and 7:30 a.m. Nurses had to work at least three successive daytime shifts and the first measurement day had to be preceded by at least two 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 two days later, (to make sampling comparable to the Wednesday measurement in the male population) 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 [13,14]. 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), citrate (5 ml), EDTA (3 ml), NaF (2 ml). All vacutainers were mixed directly after sampling by inverting them 5 times. Fasting insulin (pmol/l) was determined with an immunoradiometricassay kit (Medgenix Diagnostics Fleurus, Belgium) from blood taken out of the serum-vacutainer. Blood had to clot for 60 minutes at room temperature. Serum was separated by centrifugation at 2,000 × g for 20 minutes 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 total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) the serum of the clot-activator vacutainer was used. Blood was allowed to clot for 30–120 minutes at room temperature. Serum was separated by centrifugation at 2,000 × g for 20 minutes at 4°C. Lipid determinations were performed at the same day using the Vitros 250 Clinical Chemistry analyser (Johnson & Johnson, Rochester, USA) with Vitros clinical chemistry slides for TC and TG. HDL-C was determined in serum after a precipitation step with HDL-C percipitant (Boehringer Mannheim, Mannheim, Germany). LDL-C was calculated according to the formula of Friedewald [15]. All lipid values are given in mmol/l. Stabilyte blood was drawn for the determination of tissue-type plasminogen activator activity (tPA-Act). Citrated blood was withdrawn for determination of CRP, fibrinogen, tissue-type plasminogen activator antigen (tPA-Ag) and plasminogen activator inhibitor-1 antigen (PAI-1-Ag). Immediately after withdrawal, the vacutainers were put in melting ice and centrifuged within 60 minutes (2.000 × g, 20 minutes at 4°C). Aliquots of plasma were snap-frozen immediately using solid carbon dioxide and stored at -80°C. tPA-Act was measured using the biofunctional immunosorbent assay Chromolize™tPA (Biopool, Umeå, Sweden). Results were expressed in IU/ml. Fibrinogen was determined using the STA coagulation analyser (STAG-O, Asnières, France) and the STA Fibrinogen kit (Boehringer Mannheim, Germany). The results are expressed in g/l. tPA antigen was measured using the enzyme immunoassay Imulyse™tPA (Biopool, Umeå, Sweden). PAI-1-Ag was measured using enzyme immunoassay Innotest PAI-1 (Innogenetics, Zwijndrecht, Belgium). Results of tPA-Ag and PAI-Ag are expressed in ng/ml. C-reactive Protein (CRP) was measured using a sensitive enzyme immunoassay. Briefly, polystyrene microtiterplates were coated with a polyclonal antibody against human CRP (DAKO A/S, Glostrup, Denmark). Test samples were incubated in a 1000 times dilution for 2 hrs at room temperature. After a washing procedure, detecting antibody solution was added (peroxidase labelled polyclonal antibodies against human CRP, DAKO A/S, Glostrup, Denmark). After a 1 hr incubation at room temperature, the microtiterplate was washed and substrate solution was added. After a 15 minutes incubation period at room temperature the reaction with substrate was stopped with the addition of sulphuric acid. The colour development was recorded spectrophotometrically and the CRP content of the test sample was calculated from the calibration curve. Standard CRP serum (Dade behring, Marburg, Germany) was used for calibration, which was calibrated against the international standard for CRP (WHO-code 85/506). Results of CRP are expressed in mg/l. The reference range for healthy individuals of the method used is 2.5 – 97.5% content interval: 0.07 – 5.25 mg/l. The intra-assay coefficient of variation at different points within the reference range were 2.4% for CRP concentrations < 0.5 mg/l, 1.8% for CRP concentrations between 0.5–2.5 mg/l, 1.8% for CRP concentrations between 2.5–5.5 mg/l, and 3.1% for CRP concentrations higher than 5.5 mg/l. The intra-assay and inter-assay coefficient of variation were less than: 5.0 and 7.0 % for fasting insulin, 4.0 and 6.0 % for TC, 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 tPA-Ag, 7.5 and 10.0 % for tPA-Act, 10.0 and 10.0 % for PAI-1-Ag, 2.0 and 10.0 % for CRP. For each of the blood variables the samples were analyzed in the same batch. Moreover, the blood samples, drawn from the same subject on repeated blood withdrawal occasions, were analyzed simultaneously on the same plate. No sample had been stored for more than seven months.

Behavioural risk factors

Smoking behaviour was assessed as number of cigarettes smoked a day. Alcohol consumption was assessed as number of glasses consumed per week in 7 categories which were: less than 1 glass (1), 1–2 (2), 3–5 (3), 6–10 (4), 11–20 (5), 21–40 (6), and more than 40 glasses a week (7). Oral contraceptive use was assessed on the first blood withdrawal session and nurses were categorised as non users (0) or users (1).

Statistical analyses

Analyses of intra-week effects and group differences were performed in a repeated-measurement General Linear Modelling (GLM, SPSSWin 9.0) procedure using the data obtained from the first two withdrawal days of all subjects. The dependent variable in the GLM model was CRP.

We used a natural logarithm transformation of CRP (lnCRP) to meet the demands of normal frequency distribution. Men, no 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 [16].

Test-retest reliability was assessed by 1) computing Pearson correlation coefficients and 2) by a method described in Fraser, and Harris [17] and Marckmann et al. [18] 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 coefficient of variation (CV)). Based on the criteria of Cotlove, Harris, & Williams [19] repeated in Fraser et al. [17] 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).

Using the repeated measures, individual samples were identified that reflected a "suspect" high value, i.e. a value that was more than 1.96 standard deviations (SD) higher than the lowest value obtained from the same subject. The $\sqrt{Si}$, calculated as described above, was used as the group specific SD in this procedure. A posteriori all samples were then treated as coming from a study design with only one single blood sample for all subjects, i.e. a typical epidemiological study. Three strategies were then used to see whether the "suspicious" high CRP values could be detected in such an epidemiological setting. This means that for the 75 no OC-using women who gave blood twice all 150 CRP values were used as if they were a single sample that needs to be diagnosed as reliable, and for the 92 men all 276 CRP values. The first (1) criterion was to label all values above an arbitrary absolute threshold level as suspect. Second (2), we used an individual's predicted CRP value as a criterion. The individual CRP values were predicted from known correlates like gender, BMI, smoking behaviour (in cigarettes a day) and alcohol consumption (in categories indicating glasses consumed a week). We used lnCRP because regression analysis demands normal frequency distribution. Subsequently for all values an expected lnCRP value was calculated. This individual lnCRP value was back-transformed to a simple non-transformed CRP value. The difference between the actually measured CRP level and expected CRP was computed and a threshold level above which a difference between these two values cannot be considered reliable anymore was computed. Third (3), we used a similar regression procedure as described above to obtain a predicted CRP value, this time including fibrinogen and the full spectrum of metabolic syndrome X risk indicators (insulin, HDL-C, LDL-C, TG, fibrinogen, t-PA antigen, t-PA activity, and PAI-1 antigen), as obtained in the same blood sample as well.

Results

Data was checked with regard to frequency distribution. Like CRP, insulin, TG, fibrinogen, t-PA antigen, t-PA activity, PAI-1 antigen were transformed to normal distribution by natural logarithmic transformation and age and BMI by ^{10}log logarithmic transformation before entering into statistical analysis, unless otherwise indicated. For readability, means and ranges of the variables are given in Table 1, for the 3 groups separately.

Table 1: Age, BMI and risk indicators for men, no-oral contraceptives (OC) using women and oral contraceptives using women for each blood withdrawal day separately (means and ranges are given)

	Men (n = 92)			No OC-using women (n = 75)		OC-using women (n = 56)	
Age (years)	45.22 (35–54)			37.1 (23 – 55)		30.0 (22 – 49)	
BMI (kg/m^2)	25.17 (18.70 – 34.11)			24.4 (18.3 – 40.6)		23.2 (18.2 – 35.6)	
Smoking (#/day)	3.28 (0 – 35)			3.41 (0 – 25)		3.68 (0 – 25)	
Alcohol use (cat.)	3.64 (1 – 6)			2.28 (0 – 6)		2.25 (0 – 6)	
Blood values	**day 1**	**day 3**	**day 5**	**day 1**	**day 3**	**day 1**	**day 3**
Mean CRP (mg/l)	1.22	1.12	1.19	0.83	0.80	2.04	1.86
Range CRP (mg/l)	0.04–4.88	0.02–6.53	0.07–7.98	0.05–3.00	0.05–3.78	0.16–5.58	0.14–4.60

(Note: BMI = body mass index; CRP = C-reactive protein)

Table 2: Test-retest correlations for men, no-oral contraceptives (OC) using women and oral contraceptives using women (all p < .001)

	Men (n = 92)			No OC-Using Women (n = 75)	OC-using Women (n = 57)
Risk Factors	**day 1-day 3**	**Day 3-day 5**	**day 1-day 5**	**day 1-day 3**	**day 1-day 3**
CRP	.78	.69	.55	.76	.75
LnCRP#	.73	.76	.60	.82	.80

(Note; CRP = C-reactive protein; # = transformed to normal distribution before entered into the analyses)

Intra week changes and group differences in absolute levels
Values on CRP obtained at day 1 and day 3 were tested for group differences across men, no OC-using women and OC-using women. CRP levels did not differ over the days. On average CRP values differed between the groups ($F(2,230) = 23.19$, $p < 0.001$). Pairwise comparison in this first multivariate group test showed that OC-using women had higher CRP values than men ($p < 001$) and no OC-using women ($p < 0.001$), and that men had higher CRP values than no OC-using women ($p = 0.025$). Including age and BMI as covariates in this analysis did neither change this group effect (F(2,219 = 36.54, $p < 0.001$) nor revealed interaction effects between group and age or group and BMI. Pairwise comparison in this second multivariate group test showed that OC-using women had higher CRP values than men ($p < .001$) and no OC-using women ($p < 0.001$), and that men had only slightly higher CRP values than no OC-using women ($p = 0.096$). In the latter analysis, higher CRP levels were found in subjects with higher BMI's ($F(1,219) = 45.21$, $p < 0.001$), but no effects of age.

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 for (ln)CRP are given in table 2. All correlations were highly significant ($p < 001$). Higher correlations were found for lnCRP than for non-transformed CRP values. For lnCRP the 3-day test-retest correlation was 0.73 for men, 0.80 for OC-using women and 0.82 for no OC-using women. In men, the additional 5-day test-retest correlation was 0.60.

As is shown in Table 3, lnCRP correlated highly with BMI and fibrinogen in all three groups and with t-PA activity and PAI-1 antigen in men and no OC-using women, but less with lipids and insulin. Correlation of lnCRP to age was absent or weak in all groups.

Following Fraser et al. [17] we computed the components of variance due to between subject variance, within-subject variance (excluding analytical variance) and analytical variance (see Table 4) for CRP and lnCRP. Variation estimates were made on both scales for comparative purposes. To check on the analytical precision of (ln)CRP, we additionally report the intra-assay coefficient of variation (CV) that is minimally required to reliably detect intra-week within-subject variance ($CV_{analytical} \leq 0.5 * CV_{within-subject}$)[19]. In all three groups the analytical precision of (ln)CRP is excellent. However, the within subject coefficients of variation (CVi) varied between 33.39–67.17 (49.24 on average) for CRP, and between 19.89–39.69 (29.90 on average) for lnCRP, which is substantially higher than for e.g. HDL-C and LDL-C [6]. Note that although the absolute values of the different variance components are higher in the untransformed CRP values (as expected, and previously reported by Ockene et al., [8]), but that the ratio of the three variance components (Sg, Si, and Sa), is very similar for CRP and lnCRP.

Strategies to assess individual CRP levels reliably
Using the repeated measures, individual samples were identified that reflected a "suspicious" unreliable value, i.e. a value that was more than 2 standard deviations (SD) higher than the lowest value obtained from the same subject. In figure 1,2,3 graphical representations of the so-defined suspicious values in the three groups are given.

Absolute values
When all suspicious deviants have to be identified by an absolute criterion, the absolute value has to be different in the three groups (see table 5). The decision to make sure all suspicious deviants are identified, implies that many subjects need to be invited for resampling; 14 (=15 %) in men, 34 (=24 %) in no OC-using women, and 40 (=38 %) in OC-using women. In order to find a criterion with a more favourable trade-off a second strategy using a regression approach was explored.

Regression analyses
For the second strategy multiple (stepwise) regression analyses were used. As is shown in table 6, age, BMI,

Table 3: Correlations between blood levels of C-reactive protein, and body mass index, age, and parameters of the insulin resistance syndrome. Results are given for men, no-oral contraceptives (OC) using women and oral contraceptives using women, separately (significant correlations are given in bold face; * p < .05; ** p < .01).

C-reactive protein#

	Men			No OC-using women		OC-using women	
	day 1	day 3	day 5	day 1	day 3	day 1	day3
Age#	-.10	-.11	-.12	.20	**.29****	-.10	-.04
BMI#	.16	**.24***	**.28****	**.52****	**.55****	**.49****	**.51****
insulin#	.06	.10	**.27****	.20	.19	.25	.21
TC	.13	.08	.15	.11	.02	.19	.16
LDL-C	**.23***	.05	.14	.21	.13	.10	.08
HDL-C	**-.26***	-.13	-.21	**-.31****	**-.22****	.05	.10
TG#	.10	**.21***	**.28***	**.26***	.10	**.29***	.22
Fibrinogen#	**.62****	**.66****	**.70****	**.54****	**.56****	**.56****	**.56****
t-PA antigen#	.14	.14	**.27***	**.35****	.33	.08	.18
t-PA activity#	**-.21***	**-.26***	**-.38****	**-.36****	**-.32****	.16	-.14
PAI-1 antigen#	.20	**.28****	**.41****	**.42****	**.39****	-.04	.24

(Note: BMI = body mass index; HDL-C = high density lipoprotein cholesterol; LDL-C = low density lipoprotein cholesterol; PAI-1 = plasminogen activator inhibitor-1;t-PA= tissue-type plasminogen activator; TC = total cholesterol; TG = triglycerides; # = transformed to normal distribution before entered into the analyses).

Table 4: Estimates of between subject (BS), within subject (WS) and analytical (A) variation (percentages of total variance in brackets), and coefficients of variation (CV) of CRP for men (n_{ind} = 92, n_{obs} = 552), non-oral contraceptives (OC) using women (n_{ind} = 75, n_{obs} = 300) and oral contraceptives using women (n_{ind} = 56, n_{obs} = 224), separately.

	Between subject		Within subject		Analytical		
	Variance (%)	CV_{BS}	Variance (%)	CV_{WS}	Variance (%)	CV_A	$^1/_2CV_{WS}$
Men							
CRP	1.17 (65.3)	92.20	0.62 (34.6)	67.16	0.00055 (0.03)	2.00	33.58
lnCRP	0.15 (68.8)	57.98	0.068 (31.2)	39.69	0.00017 (0.08)	1.98	19.85
No OC-using women							
CRP	0.47 (75.8)	84.51	0.15 (24.2)	47.17	0.00026 (0.04)	2.00	23.59
lnCRP	0.11 (82.0)	65.32	0.024 (17.9)	30.13	0.00011 (0.08)	2.02	15.07
OC-using women							
CRP	1.23 (74.5)	56.92	0.42 (25.4)	33.39	0.0015 (0.09)	1.99	16.70
lnCRP	0.15 (79.2)	43.49	0.039 (20.6)	19.89	0.00038 (0.20)	1.98	9.95

(Note: CRP = C-reactive protein; lnCRP = natural logarithmic transformed C-reactive protein; n_{ind} = number of individuals; n_{obs} = number of observations)

smoking behaviour and alcohol consumption were used to predict CRP level. However, the amount of explained variance was to low to further explore this strategy.

Next multiple (stepwise) regression analyses were performed wherein besides age, BMI, smoking behaviour and alcohol consumption, also the full spectrum of metabolic syndrome X risk indicators (insulin, HDL-C, LDL-C, TG, fibrinogen, t-PA antigen, t-PA activity, and PAI-1 antigen), were used to predict CRP level. As is shown in table 7, the amount of explained variance varied between 45–51% in the three groups which was considered high enough to continue with this third strategy.

Using the regression weights, as given in table 7, predicted CRP values for all blood samples were calculated. Then the actually measured (or observed) CRP value was subtracted from this predicted value and used as a criterion to decide whether a suspicious unreliable deviant is correctly indicated as unreliable. As is shown in table 8, the criterion necessary to detect all suspicious unreliable deviants differed between men, no OC-using women and OC-using women. The number of subjects that are indicated as having an unreliable CRP sample and need to be invited for resampling is; 29 (=11 %) in men, 42 (=30 %) in no OC-using women and 46 (=43 %) in OC-using women. These numbers, compared to the numbers obtained when

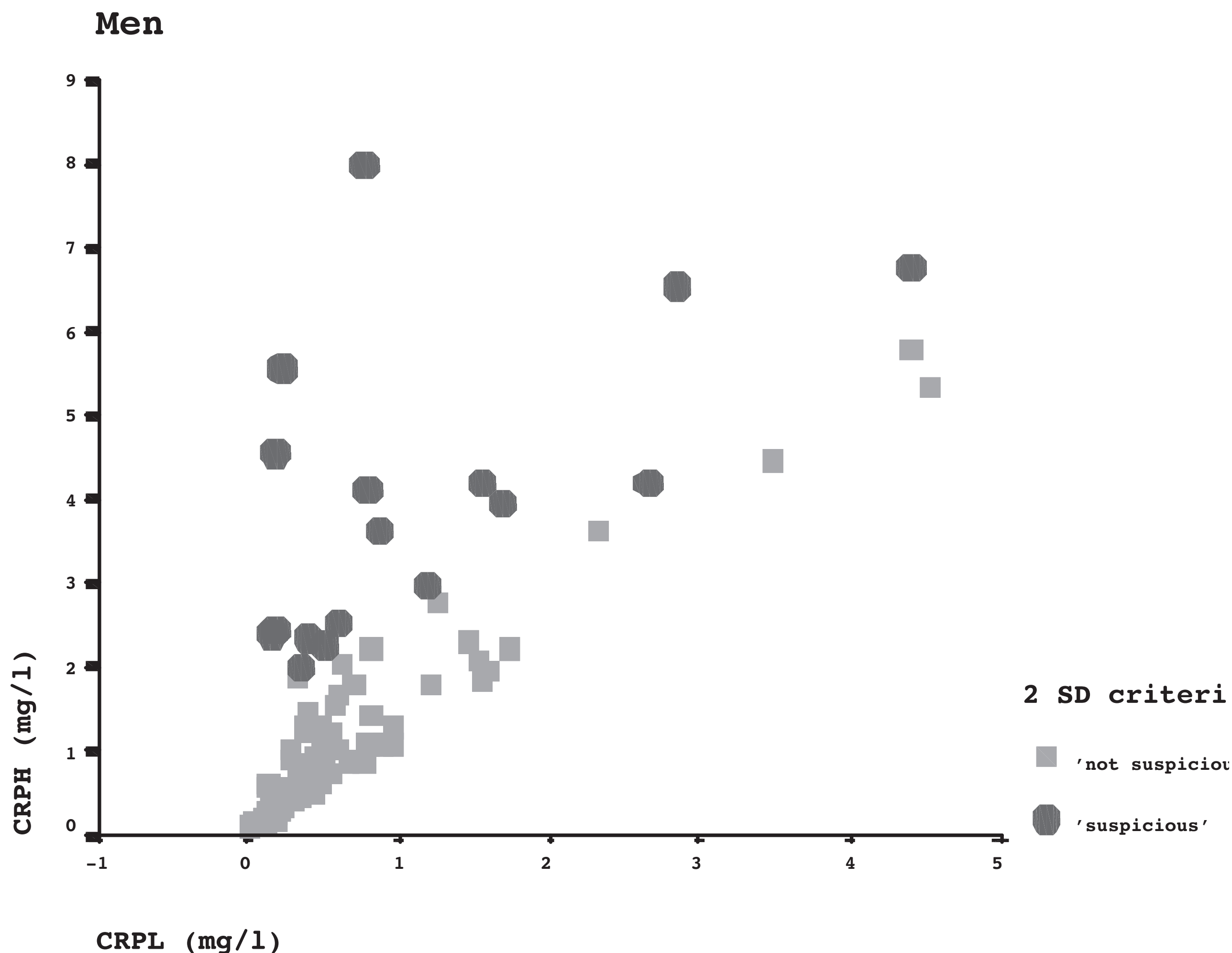

Figure 1
Graphical representation of the identification of the suspicious and not suspicious CRP values in the men (see text for more information).

Table 5: Calculated sensitivity and specificity of detecting unreliable CRP values using an absolute criterion. Results are given for men, no-oral contraceptives (OC) using women, and oral contraceptives using women, separately.

	Men		**No OC-using women**		**OC-using women**	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
2.75 mg/l	0.65	0.93	0.60	0.99	0.67	0.78
2.50 mg/l	0.71	0.93	0.60	0.98	0.83	0.74
2.25 mg/l	0.88	0.90	0.70	0.97	0.67	0.78
2.00 mg/l	0.94	0.88	0.70	0.96	**1.00**	**0.62**
1.75 mg/l	**1.00**	**0.85**	0.80	0.93	1.00	0.52
1.50 mg/l	1.00	0.80	0.90	0.89	1.00	0.45
1.00 mg/l	1.00	0.69	**1.00**	**0.76**	1.00	0.29

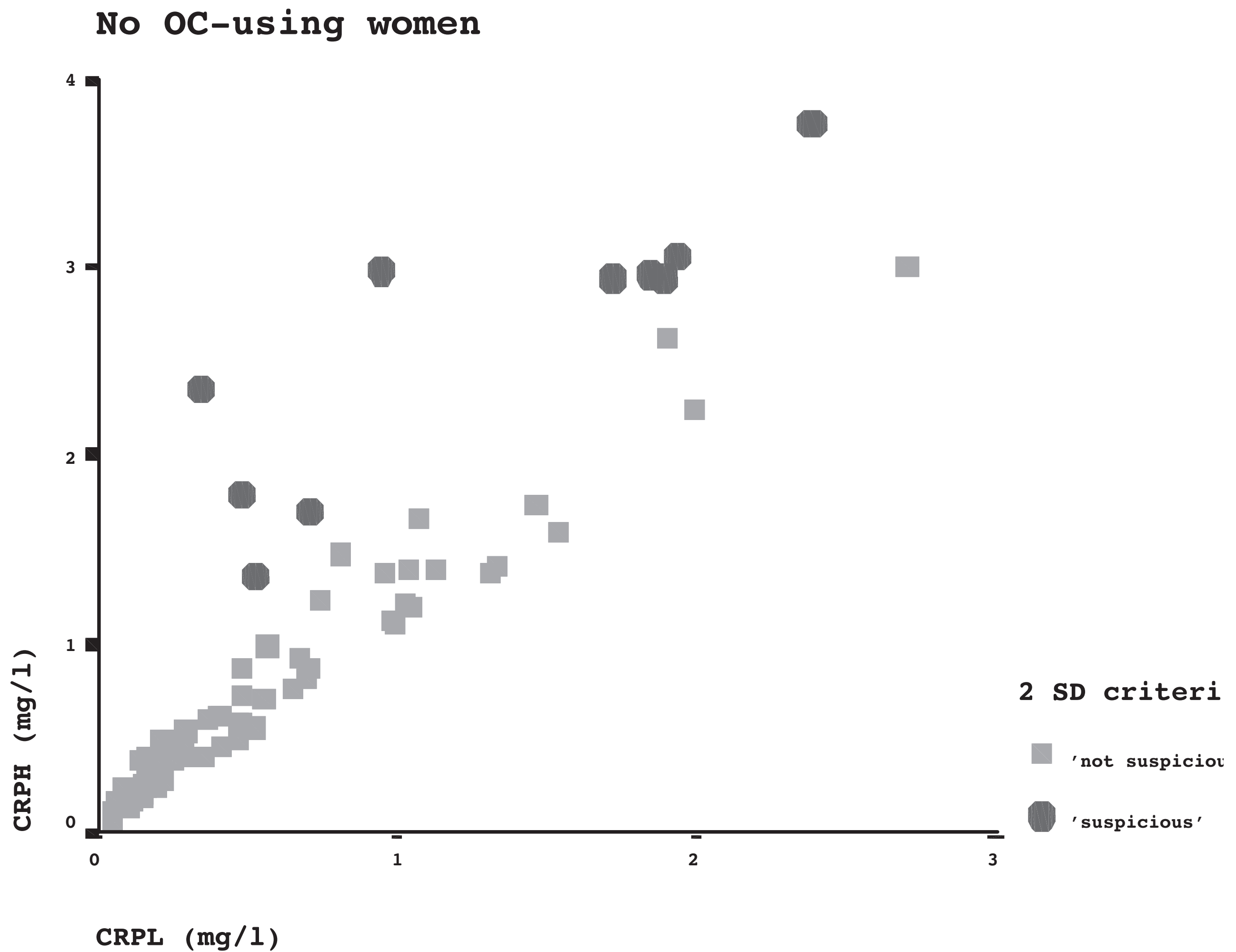

Figure 2
Graphical representation of the identification of the suspicious and not suspicious CRP values in the no oral contraceptives (OC)-using women (see text for more information).

using an absolute criterion are smaller in men, but higher in both groups of women.

Discussion

C-reactive protein has been identified in prospective epidemiological research as an independent risk factor for CVD. Using this acute phase protein in risk prediction in individuals is still under debate. In this paper test-retest reliability of CRP and its log-transformed was found to be adequate; the relative rankings of the subjects remained fairly constant within one workweek. In addition, studying the variability of (ln)CRP, the $CV_{analytical}$ was less than half of the $CV_{within\ subject}$ indicating that the analytical precision of (ln)CRP is satisfactory as well. On average for the three studied groups (men, no OC-using women, and OC-using women), the $CV_{within\ subject}$ found in this paper is 49.24% for CRP, and 29.90% for lnCRP. This is lower than the value of 63.0% for CRP reported by Clark et al., ([20]; in 29 healthy men and women, aged 20–46 years), and comparable to the value of 42.2%for CRP reported by Macy et al., ([21]; in 143 healthy men and women; aged 18–67 years), and comparable to the average value of 30% reported in a review of Kluft et al. [7] for lg^{10} transformed CRP.

Such large biological intra-individual variance makes it very difficult to decide whether a single blood sample is an individuals 'true' habitual level. Based on our data, we advise against classifying a subject as high using a single CRP sample only, for instance by upper tertiles or quar-

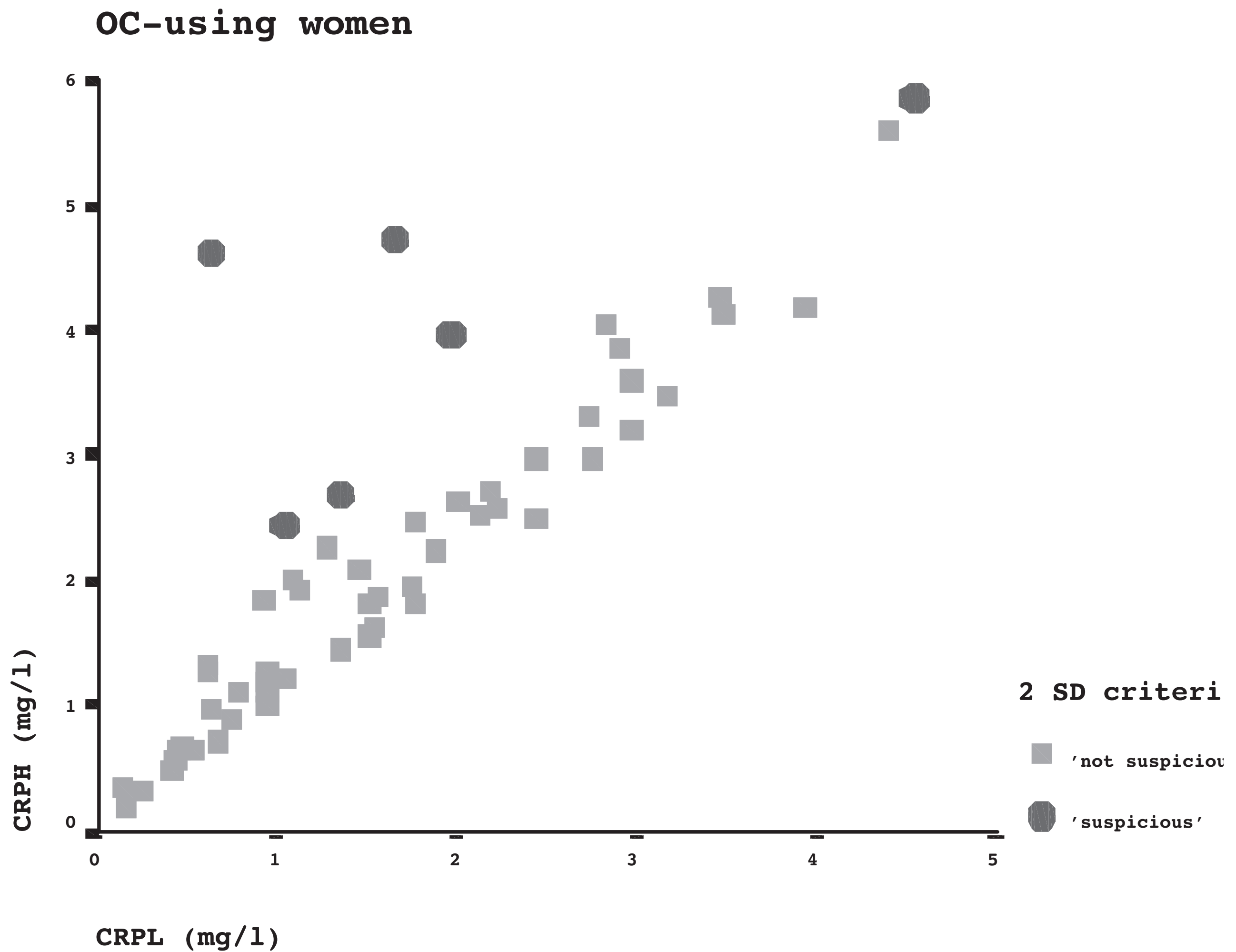

Figure 3
Graphical representation of the identification of the suspicious and not suspicious CRP values oral contraceptives (OC)-using women (see text for more information).

tiles as is common practice for e.g. total cholesterol. Instead multiple sampling of CRP within one individual is necessary. To reduce the number of sampling needed, i.e. for application in epidemiology-scaled research, and after Kluft et al. [7] we aimed to provide a criterion to decide whether a single high sample can be trusted, or whether resampling is necessary.

Results clearly favour an absolute level criterion. Specificity and sensitivity were as good as that of the more elaborate regression approach that is more complicated and expensive since BMI and other metabolic and fibrinolytic risk indicators for CVD need to be assessed. Different criteria for the three groups (men, no OC-using women and OC-using women) are needed to decide whether a single high sample suffices or whether a second sample should be taken. These group specific values above which resampling of CRP is deemed necessary are 1.75 mg/l for men, 1.00 mg/l for no OC-using women, and 2.00 mg/l for OC-using women.

How to proceed when a second sample is indeed required by the criteria above? A pragmatic strategy was suggested by Kluft et al. [7]: after a first blood sample is diagnosed as above the criterion, blood sampling should be repeated no earlier than 2 weeks after the first sample, or later. If the second sample is below the criterion, the first sample must be distrusted and the value of the second sample is more likely to reflect the 'true' value. If the second sample is again above the criterion, the subject should be regard-

Table 6: Multiple regression analyses (stepwise); predicting lnCRP levels (lowest value obtained from 2 or 3 blood withdrawal days) entering body mass index (BMI), age, smoking behaviour, and alcohol consumption into the regression. Results are given for men, no-oral contraceptives (OC) using women, and oral contraceptives using women, separately.

Variable	β coefficient	p-value	R^2
Men			
Constant	-1.485	0. 057	
Smoking	0.161	0.000	0.211
BMI#	2.646	0.000	0.315
Age#	-1.107	0.003	0.338
No OC-using women			
Constant	-3.912	0.000	
BMI#	2.623	0.000	0.342
Age#	0.467	0.049	0.359
OC-using women			
Constant	-4.085	0.000	
BMI#	3.659	0.000	0.282

(Note; BMI = body mass index; lnCRP = natural logarithmic transformed C-reactive protein; OC = oral contraceptives; # = transformed to normal distribution before entered into the analyses)

Table 7: Multiple regression analyses (stepwise); predicting lnCRP levels (lowest value obtained from 2 or 3 blood withdrawal days) entering body mass index (BMI), age, smoking, alcohol use and the insulin resistance parameters (insulin, total cholesterol, HDL-C, LDL-C triglycerides, t-PA antigen, t-PA activity, PAI-1 antigen) into the regression. Results are given for men, no-oral contraceptives (OC) using women, and oral contraceptives using women, separately.

Variable	β coefficient	p-value	R^2
Men			
Constant	-2.697	0.001	
Fibrinogen#	1.182	0.000	0.325
BMI#	2.306	0.000	0.390
Smoking	0.106	0.000	0.461
Age#	-0.975	0.004	0.478
t-PA antigen#	-0.141	0.004	0.487
t-PA activity#	-0.064	0.016	0.498
LDL-C	0.044	0.031	0.506
No OC-using women			
Constant	-3.613	0.000	
BMI#	1.957	0.000	0.344
Fibrinogen#	0.919	0.000	0.452
PAI-1 antigen#	0.108	0.006	0.473
Insulin#	-0.136	0.035	0.491
OC-using women			
Constant	-4.246	0.000	
Fibrinogen#	1.375	0.000	0.327
BMI#	2.192	0.000	0.411
HDL-C	0.202	0.011	0.445

(Note: BMI = body mass index; HDL-C = high density lipoprotein cholesterol; LDL-C = low density lipoprotein cholesterol; lnCRP = natural logarithmic transformed C-reactive protein; PAI-1 = plasminogen activator inhibitor-1;t-PA= tissue-type plasminogen activator; # = transformed to normal distribution before entered into the analyses).

ed as a patient who potentially suffers from a chronic disease. He or she needs to be regularly bled for CRP level monitoring over a prolonged time, possibly accompanied by in depth medical examination as prompted by the specific anamnesis of the patient [4,7].

A limitation of this study is that the criteria cannot be generalised to the general population. Strict inclusion was applied such that all our subjects were very healthy and free of chronic diseases like hypertension, CVD, or diabetes. Additionally, our subjects were young. Sampling in older subjects with further advanced atherosclerotic inflammation would certainly have resulted in a larger CRP variance. Secondly, resampling in the current study took place within one week. In clinical settings persons ideally are invited for resampling 12–14 days after the first increased level sample was obtained, when CRP level characteristically has returned to baseline after a moderate inflammatory stimulus [3]. The positive side of these limitations is that our criteria are probably too conservative. Multiple repeated sampling after longer intervals in a larger general population could certainly sharpen the proposed criteria such that, in the terminology of this paper, specificity is increased without loss of sensitivity.

Table 8: Sensitivity and specificity of the difference (Δ) between the expected CRP level based on the formula obtained from the regression analyses (regression weights given in table 7) *minus* the measured (observed) CRP level (when a negative difference is found the expected value was lower than the observed valued). Results are given for men, no-oral contraceptives (OC) using women, and oral contraceptives using women, separately

	Men		No OC-using women		OC-using women	
Δ in mg/l	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
-1.75	0.76	0.93	0.40	0.99	0.83	0.91
-1.50	0.82	0.92	0.40	0.98	0.83	0.88
-1.25	**1.00**	**0.89**	0.50	0.97	0.83	0.84
-1.00	1.00	0.85	0.70	0.90	0.83	0.80
-0.75	1.00	0.83	0.80	0.90	0.83	0.75
-0.50	1.00	0.75	0.90	0.82	0.83	0.64
-0.25	1.00	0.64	**1.00**	**0.70**	**1.00**	**0.57**
0.00	1.00	0.40	1.00	0.47	1.00	0.42
0.25	1.00	0.15	1.00	0.18	1.00	0.30

Conclusions

A single high CRP value must be followed by re-sampling when it is above 1.75 mg/l for men, above 1.00 mg/l for no OC-using women, and above 2.00 mg/l for OC-using women. Using additional information from gender, BMI, smoking behavior, alcohol use, insulin, HDL-C, LDL-C, TG, t-PA antigen, t-PA activity, and PAI-1 antigen does not meaningfully improve on the use of absolute CRP levels stratified for gender and OC-use.

Competing Interests

None declared.

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