Determination of the habitual low blood level of C-reactive protein in individuals

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In order to use C-reactive protein (CRP) in risk prediction in individuals, it is necessary to know how to obtain the habitual level of an individual and hence its biological variations: i.e. longitudinal variability within subjects and variability between individuals. This paper provides data on biological variability that is used to propose a strategy for assessing individual low levels of CRP.

The longitudinal variability in individuals (intraindividual variability) is essential to know, but only reported in a very limited way. Additional data were calculated from in-house and requested databases for periods of follow-up from 5 days to 1 year. The intraindividual coefficient of variation (CVi) was found to be rather similar for several groups and periods and on average was ~30%. Reported analytical coefficients of variation of commercial and in-house methods generally are below 6%, which is well below the desired limit of half the CVi.

The distribution of CRP in apparently healthy individuals is wide and skewed with interquartile values ranging between 150-250% of the median and an estimate of the composite coefficient of variation (CVc) of ~120%. The distribution is equally broad in several other groups studied such as type II diabetics and pregnant women.

It is concluded that the coefficient of variation for the determination of CRP in a single blood sample is as high as ~30%, but that this is acceptable for the reliable positioning of individuals within the distribution of CRP in the group with a CVc as high as ~120%.

CRP can show unexpected outliers (increases) which can sometimes be explained by information from a short questionnaire and definitely identified by the analysis of a second blood sample after an interval of ~2 weeks. Similarly, to ascertain high values in a first sample a second blood sample can be analyzed. It should be noted that, in view of the significant intraindividual variability of CRP, the difference between the first and second values may reach 71%.

The large intraindividual variability of CRP approximating 30% renders it difficult to position an individual reliably in smaller categories such as tertiles, quartiles or quintiles of the total distribution. It is suggested that it would be most practical to have a goal of a single decision level or threshold only. Positioning an individual into two groups with equally wide distribution is on the borderline of reliability for one blood sample. Multiple blood samplings are required for smaller categories and higher threshold levels.

The use of a decision limit should further acknowledge the limited interclass stability of around r = ~0.5 for a single blood sample.

The above considerations are summarized in a practical working scheme. This scheme can serve as a basis for further refinement, discussions and development of sampling and decision limits to be selected from medical and economical perspectives and tested in practice.

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Introduction

C-reactive protein (CRP) has been known for a long time as a rapid and strongly reacting acute phase protein[1]. Recent epidemiological studies showing the value of continuously low blood levels of CRP as a prognostic biomarker for cardiovascular disease risk have largely stimulated the interest in this polypeptide[2-6].

Before being able to decide about an adequate strategy for the determination of the habitual blood level of an individual it is necessary to evaluate the analytical and biological variability more closely. This is less important in epidemiological studies since studying large numbers of subjects compensates the imprecision due to these variations.

For analytical specifications we followed the accepted criteria based on biological variation. This was inspired by Tonks[7], specified further by Cotlove et al.[8] and by Fraser and Harris[9] and broadly accepted[10]. The

criteria for the analytical performance of laboratory tests are related to the intraindividual biological variation; for testing individuals they are related to the (composite) biological variation or the reference range[10].

The aim of the present manuscript was to evaluate reported data on analytical, inter- and intraindividual variability. In view of the lack of data on intraindividual variability, these data have been calculated additionally from in-house and requested databases.

This will be the basis for a proposal of a strategy designed to measure the individual level of a chronically low level of CRP.

Variation data

Analytical variation. All methods described for low levels of CRP are immunological methods, mostly enzyme immunoassays and microparticle methods. Recently four commercially available, automated methods for CRP have been evaluated[11]. In table I[11-43], the reported commercial and in-house methods for low CRP levels and the reported analytical variability are listed.

For most methods the intra- and interassay variability is low and, using different techniques, it is possible to measure the CRP values with an analytical interassay coefficient of variation below 6%.

This is well below 15% which is the value derived from the criterion that the analytical coefficient of variation (CVa) should be below half of the intraindividual coefficient of variation (CVi) (which is ~30%)[44].

Interindividual variation. The distribution of CRP concentrations in apparently healthy controls and reported patient groups is skewed. The logarithmically transformed levels have a Gaussian distribution and in epidemiological and clinical studies this transformation is usually applied before further analysis. Otherwise, non-parametric statistics are used. For individual patient care, the use of transformed results is considered impractical and therefore in this manuscript we will discuss the results and consequences for the use of non-transformed data.

The composite coefficient of variation (CVc), including the interindividual variation coefficient (CVg), and the CVi, related to each other via the equation $CVc^2 = CVg^2 + CVi^2$ [8], has been calculated from the reference ranges of various groups of individuals and from reported data. These are shown in table II[14,18,32,45-56]. On the basis of different publications and of different populations we derived that this variability is rather uniform and on average the CVc is ~120%.

Table I. Analytical variability of assays for low levels of C-reactive protein.

Assay type	Availability	Company/author	Intraassay CV%	Interassay CV%	Undefined CV%
Immunonephelometric	Commercial	Dade-Behring	3.7[13]; 1.4[14]; 2.2-3.4[15]; 2.5-3.9[12]	6.4[13]; 1.2[14]; 3.0-5.4[15]; 6.4[12]	< 7.6[11]
Enzyme immunoassay	Commercial	Kordia		5.1-5.3[16]	
Immunochemiluminescent	Commercial	Diagnostic Product Corporation			< 9.8[11]
Enzyme immunoassay	Commercial	Eurogenetics[20]			
Enzyme immunoassay	Commercial	Hemagen Diagnostics[12]			
Immunoenzymometric	Commercial	Medix Diacor		7.9-9.0[19]	
Magiwell	Commercial	United Biotech[18]			
Competitive enzyme immunoassay	In-house	Macy	3.0[18]; 3.0[21]	6.0[18]; 5.5-6.8[13]; 9.9[22]; 6.0[21]; 9[23]; 5.5[24]; 8.9[25]	4.2[26]
Microparticle enzyme immunoassay	In-house	Abbott	3.5-6.3[27]; 2-4[28]; 2.8-11[40]	1.9-6.0[27]; 6.8[28]	< 12[11]
Enzyme immunoassay	In-house	Mendall	8[29]; 9[30]	14[29]; 17[30]	
Enzyme immunoassay	In-house	de Maat	3.8[31]; 2.9[32]	8[33]; 4.7[31]; 7.2[32]	
Enzyme immunoassay	In-house	Gram[34]			
Enzyme immunoassay	In-house	Hak	3.8[35]	4.7[35]	
Enzyme immunoassay	In-house	Mattusch[42]			
Radioimmunoassay	In-house	Claus[43]			
Radioimmunoassay	In-house	Wassunna	5.0[36]	5.4[36]	
Immunoturbidimetric	In-house	Eda		2-5[37]	
Immunoturbidimetric	In-house	Price	1.4-2.4[38]		
Immunoturbidimetric	In-house	Keevil	1.6-14.6[17]	5.3-9.7[17]	
Immunoradiometric	In-house	Hutchinson	4[39]; 1.4-5.6[40]; 1.8-6.2[40]	12[39]; 0.5-8.0[40]	
Immunoluminometric	In-house	Wood		9.4-9.8[41]	

CV = coefficient of variation. Superscript numbers in the table refer to references from which the data are derived and/or where information about the method can be found.

Table II. Data on biological variation in low levels of C-reactive protein.

Period	Individuals	Age (years, average or range)	No.	Geometric mean (mg/l)	CVc (%)	IQR (%)	CVi (%)	Correlation (Spearman)	Interclass correlation	% in upper class	Reference
1 × 5 days	Healthy volunteers	22-55	143	0.87	112	167	18	0.904	0.502	11	Riese[49]
10 × 2 weeks	Healthy volunteers	20-46	19	1.4*	99*§§	–	63*§§	–	–	–	Clark and Fraser[48]
8 × 3 weeks	Healthy volunteers	–	26	1.7*	102*§§	–	42.2*§§	0.46-0.86§	–	–	Macy et al.[18]
9 × 3 weeks	Healthy volunteers	31	20	0.75	108§§	68§§	74§§	0.30-0.61§	0.08-0.92§	10	de Maat et al.[32]
5 × 4 weeks	Healthy volunteers	42	26	0.69	96§§	104§§	82§§	0.45-0.83§	0.18-0.35§	11	de Maat et al.[50]
5 × 4 weeks	Angina pectoris patients	66	26	1.57	85§§	99§§	67§§	0.66-0.90§	0.34-0.61§	23	de Maat et al.[32]
1 × 4 weeks	Smoking volunteers	35	15	1.51	131	359	34	0.781	< 0.3	40	de Maat et al.[45]
1 × 6 weeks	Postmenopausal type II diabetics	61	19	2.11	137	234	30	0.844	0.791	42	Brussaard et al.[51]
1 × 3 months	First and second trimester of pregnancy	20-40	36	2.44	85	143	24	0.658	0.491	39	In-house§§§
1 × 3 months	Users of oral contraceptives	20-40	94	2.12	122	147	41	0.535	0.418	38	Kluft et al.[52]
1 × 3 months	Users of oral contraceptives	20-40	36	1.00	120	139	35	0.694	0.625	10	Gevers Leuven et al.[53]
1 × 3 months	Type II diabetics	45-75	61	3.14	106	190	21	0.765	0.643	44	Van de Ree§§§
1 × 6 months	Menopausal	48-53	26	0.80	131	255	45	0.574	0.333	15	Jespersen§§§
1 × 6 months	Random selected volunteers	18-75	88	1.04	120	163	37	0.716	0.540	18	Broekmans§§§
1 × 12 months	Healthy elderly	60-80	64	1.37	99	130	32	0.724	0.363	20	Schuit et al.[47]
1 × 3 years	Healthy volunteers	45-64	704	~1.6	–	–	–	–	0.53	–	Koenig et al.[54,55]
1 × 5 years	Healthy Japanese	30-69	368	0.10	–	–	–	0.43	–	–	Kayaba et al.[14]
1 × 5 years	MI patients	59.3	214	2.4**	–	–	–	–	0.60	–	Ridker et al.[46]

A period refers to the time between two consecutive blood samples; a number to the number of individuals; the geometric mean is obtained after lg10 transformation unless otherwise indicated; the correlation between two consecutive samples is calculated using Spearman; the interclass correlation is calculated for the example of two classes around a decision limit of 3 mg/l; the percentage of values above the limit is given. The CVc (composite of CVi and interindividual coeffcient of variation) is determined by multiplying the SD of the distribution after natural log transformation by 100, or calculated from reported data. The mean value of the CVi is obtained from the SD of two consecutive samples (a-b)/$\sqrt{2}$ and the calculation of the mean of the variances (SD^2) divided by the geometric mean of the group[56]. CVc = composite coefficient of variation; CVi = intraindividual coefficient of variation; IQR = interquartile range expressed as percentage of the median; MI = myocardial infarction. * non-log value; **median; § range for multiple correlations; §§ multiple samples per individual analyzed[32]; §§§ databases kindly obtained from the author indicated (for details see acknowledgments).

Intraindividual variation. *Acute increases.* Following trauma, an acute increase of a magnitude > 1000-fold in the blood level of CRP is possible[1]. Both the increase and the clearance of CRP are rapid; the biological half-life is less than 1 day. After cessation of the trauma the acute phase increase in CRP levels can resolve quickly. No systematic investigations are known, but a period of 2-3 weeks is sufficient for a return of CRP concentrations to the habitual levels[1]. For such a period there is experimental support from the observations of Macy et al.[18], Wilkins et al.[27] and de Maat et al.[32] in longitudinal studies with 3-week intervals. Moderate increases in CRP levels were present only on isolated occasions.

Figure 1 shows the biological variation in healthy persons and it can be seen that most individuals show only a single outlier in the 6-month study period[32]. The results of Macy et al.[18] and Wilkins et al.[27] are comparable, indicating that in stable individuals there is a constantly low level of CRP with occasional outliers.

Determinants of chronic habitual levels of individuals. The low habitual level of CRP is altered by all the mechanisms that cause an acute phase stimulation and by changes in its major determinants.

Major determinants are age/menopause, sex/phase in menstrual cycle, chronic inflammatory diseases, coronary artery disease, type II diabetes, body mass index (in women), smoking, alcohol consumption, strenuous exercise and the use of drugs such as statins, aspirin, vitamin E, antibiotics, and sex steroids for contraception or hormonal replacement. Details are reported in a separate manuscript[57].

The determinants can be divided according to whether they can be modified (Table III).

In determining the habitual level of an individual, specific attention should be paid to the modifiable determinants. Known major determinants of habitual CRP levels that may vary over 1 month are body weight/diet, oral contraceptive or hormone replacement therapy, strenuous exercise, smoking habits, statin treatment, alcohol consumption, anti-infection treatment, and major dental revision[57].

For statins[46,58], sex steroid treatment[59], aspirin[60], vitamin E[61,62], strenuous exercise[42,47] and alcohol consumption (Sierksma et al., unpublished data) the changes due to intervention have actually been demonstrated; the other effects are inferred from the known associations and require experimental data.

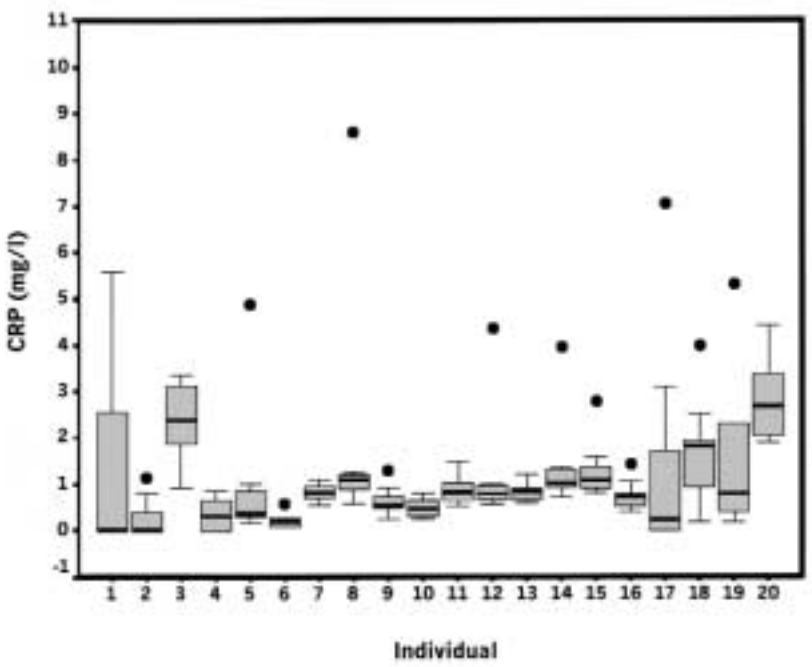

Figure 1. *Box plot of individual C-reactive protein (CRP) levels. Box plot with median, interquartile range and 5-95% confidence interval bars from individuals numbered 1-20. Samples were drawn at 3-week intervals in a 6-month period. Outliers are indicated by the* filled circles*. From de Maat et al.*[32]*, modified.*

Table III. Modifiable and non-modifiable determinants of low, chronic levels of C-reactive protein.

Modifiable	Non-modifiable
Body mass index/diet	Age/menopause
Smoking	Sex
Hormone use	Coronary artery disease
Alcohol	Type II diabetes
Strenuous exercise	
Chronic periodental disease	
Chronic bronchial inflammation	
Medication	
Statins	
Aspirin	
Antibiotics	
Vitamin E	

Longitudinal studies on CRP levels are not often reported and to supplement the literature we analyzed the intraindividual variations in a number of control and placebo studies. In table II, the data on longitudinal variation are summarized.

From table II it can be observed that the longitudinal variation (CVi) is comparable for very short periods lasting a few days versus long periods lasting a number of years. The variation is also very similar for different populations including healthy males or females, users of oral contraceptives, pregnant women, or patients with type II diabetes mellitus.

For clinical routine it can been suggested to work using a threshold (3 mg/l). CRP levels may be classified as low or high according to whether they are inferior to or exceed this threshold. As an example, in the sets of data we evaluated the interclass correlation for two classes of values below and above 3 mg/l. It can be observed that the agreement between longitudinal data, expressed as interclass correlation, is lower than when the data are evaluated as a continuous variable. The interclass correlation has on average a correlation of r = ~0.5, which implies that 20-25% of individuals changed class. Multiple blood samples will improve the allocation.

Consequences

Number of blood samples according to biological, statistical criteria. Most of the data in table II suggest that in order to precisely determine the habitual level of CRP in healthy volunteers according to criteria based on biological variability, one blood sample is sufficient. The criterion of Cotlove et al.[8] (CVi < 0.5*CVc) is met by most larger studies.

Although the longitudinal or intraindividual variability is quite significant (~30%), with a CVc ~120%, it seems sufficient to analyze just one blood sample to position the value adequately in the biologically wide distribution.

It should be noted that for patients this may not always be sufficient. Besides, the conclusion that only one blood sample is required is based solely on biological statistical criteria and does not take into account the occurrence of outliers, which is a particular feature of CRP.

Positioning the habitual value of an individual within smaller categories such as tertiles, quartiles or quintiles is more challenging since the CVi remains large while the distribution of the group is made smaller. It should be noted that for two categories with equal distribution and a decision limit at the middle, the criterion of Cotlove et al.[8] will be met with borderline reliability.

The suggested working scheme for two categories below and above 3 mg/l involves a larger category below 3 mg/l for which most probably one blood sample is sufficient to meet the criterion in most populations. For the smaller category above 3 mg/l the fact that the CRP values are skewed to higher values might result in a sufficiently large distribution in some populations. Alternatively, for this category a second blood sample is obtained and the CVi reduced to $CVi/\sqrt{2}$[56].

These general considerations can be applied to specific populations to decide about the number of samples and the threshold levels that are acceptable.

Outliers and replicate samples. In epidemiological studies on apparently healthy individuals, only a limited number of subjects have a CRP value > 10 mg/l. In some studies these subjects have been excluded from the analysis because of the suspicion of exacerbated or temporarily increased values (e.g. clinically significant infection). However, such increased levels may be chronically present in both apparently healthy individuals and patients. The policy of excluding all subjects with CRP levels > 10 mg/l is not valid for diagnostic approaches for the determination of the habitual level of CRP in individuals. In older subjects, values ranging between 10-20 mg/l are quite often seen (even 50% of subjects over 85 years of age[16]).

When a second blood sample is collected, for example when patients are classified and to exclude outliers, the criterion that the two samples reflect the same habitual level is determined by the CVa and the CVi. A maximum acceptable percentage difference can be calculated using the formula for rise or fall or single-sided deviations: $\sqrt{2}*1.65*\sqrt{(CVa^2 + CVi^2)}$[48]. With a CVa of 6% and an average CVi of 30%, this translates into 71% for non-transformed data.

Decision limits. We concluded above that, on the basis of a single blood sample, it is difficult to categorize individuals reliably in tertiles, quartiles or quintiles. Reliable categorization into two unequal categories still produces problems for the smaller group. The choice of a decision limit should not therefore be too eccentric or high, so as not to reduce the smaller category too much.

The limitations of the use of a single decision limit and one blood sample only are further illustrated in table II by the calculation of the interclass correlation for the two classes of the example with values below and above 3 mg/l. This is particularly clear for the 5-day repeat sample which shows a limited interclass stability (r = 0.502) while correlation between the continuous values is strong (r = 0.904) (Table II).

Practical analytical strategy for individual C-reactive protein levels

Outliers. The experience of de Maat et al.[32] with moderate CRP exacerbation is that on most occasions it was not recognized despite a structured questionnaire specifically asking for changes in the modifiers of CRP and in any recent disease or medical intervention.

On the other hand acute changes may be induced by the following situations which can be recorded by a small questionnaire.

• Question 1: Did you have in the last 2 weeks/or do you now have
- an infection
- common flu
- fever
- a vaccination or immunization
- piercing, tattoo, acupuncture
- backache, headache
- medical or dental treatment.

Modifiable determinants. It is of relevance to know whether in the last 2-3 weeks significant changes occurred in the modifiable determinants of chronic CRP levels.

• Question 2: Did you in the last 2 weeks start or stop or significantly change
- dietary habits
- smoking (only start)
- use of oral contraceptives or hormone replacement therapy
- alcohol consumption
- strenuous exercise
- use of multivitamins
- medication, e.g. statins, aspirin, antibiotics, treatment for bronchial complaints.

Blood sampling approach and decisions after analysis (assumes that a single decision limit is to be used).

• First/single blood sample:

1. take first blood sample and determine the CRP level;
2. below the decision limit: accept this value without repeat sampling;
3. above the decision limit: repeat blood sampling after ~2 weeks or later;
4. > 10 mg/l if age < 70 years and all other cases when > 20 mg/l: discard and start all over again after ~2 weeks or later.

• Second blood sample:

1. take second blood sample after ~2 weeks or later after the first and determine the CRP level;
2. treat as the first sample if repeated because of a CRP level > 20 mg/l, or > 10 mg/l at age < 70 years;

3. treat as the replicate sample in all other cases and if coefficient of variation CV < 71% accept level of second sample as confirmation and use the mean; otherwise perform third sampling after ~2 weeks or later after the second. Inspect questionnaire for possible causes of large fluctuations and changes;
4. is the CRP level again > 20 mg/l or > 10 mg/l at age < 70 years? If affirmative, discard and start all over again after ~2 weeks or later.
• Third blood sample:
- CRP remains > 10 mg/l and questions 1 and 2 provide no information: accept.

Discussion

The CVa of methods for low levels of CRP is low (< 6%) for the majority of the commercially available and published in-house methods. It is generally accepted that the CVa should be less than half the biological CVi. Since the biological CVi is ~30% for CRP, the precision of the assays is adequate[44].

All commercially available and most in-house assays for CRP use the WHO standard or its derivatives for standardization. This provides a good basis for comparing data from different assays and studies. However, recent evaluation of methods has shown some specific differences[12,18,63] which call for the definition of specificity criteria and/or a reference method[64]. Also, there is need for installment of external quality assurance systems to secure continuous and future comparability of data and to allow generalized applicability of decision limits.

The CVi of CRP is rather large averaging ~30%. This longitudinal variability of individuals is however small when considering it in relation to the composite variability (inter- and intraindividual variations together) in most populations of table II. It is within the criterion of Cotlove et al.[8] (the CVi < 0.5*CVc), which implies that the variation in the determination of an individual value is < 12% of the total variability and therefore constitutes a good approach in the determination of the habitual value of an individual.

Thus, although the individual, non-transformed values have an uncommon absolute variability they are sufficiently accurate for positioning within the biological spectrum of CRP levels, comparable to many other biological variables that are used for cardiovascular risk prediction[18]. The criterion is wide also for acceptance of a CRP value in a subsequent blood sample, rendering all non-transformed values within 71% of the level observed in the first blood sample acceptable.

We have used the biological data and the average values of the CVc (~120%) and of the CVi (~30%) to discuss the strategy for analyzing CRP as a risk marker for cardiovascular disease. What is required for the further use of CRP as a biomarker of risk is a decision on whether normal values will be specific for age, gender, etc., or corrected for other determinants or not. The present opinion tends to favor non-corrected values.

An important aspect is the number of blood samples that is taken or required.

To fulfil the criteria set by Cotlove et al.[8], for CRP usually one blood sample is sufficient to determine its habitual level in an individual. Because for CRP the risk of outliers is present, a second sample is indicated if an acute phase reaction in the subject is suspected on the basis of the data from the questionnaire or of a recorded increased level. This may result, for some individuals, in the policy of taking two blood samples, which is also required for individual levels of cholesterol. Hence, cholesterol and CRP levels may be determined on the same occasion. Such a strategy may be adopted in practice and two blood samples always taken for both markers. Another reason to combine these two variables is that no generalized screening policy is available and the strategy for evaluating CRP in all cases where cholesterol is being evaluated may further add to simplification of procedures.

Apart from practical reasons, cholesterol and CRP assessment should be combined even because our focus is on CRP as it is related to cardiovascular disease. It further increases the specificity of CRP assessment for cardiovascular disease and largely circumvents the fact that CRP is a non-specific inflammation marker as well.

The observation that, in risk assessment, cholesterol/HDL cholesterol and CRP are additive further supports the use of combined assessment[65]. It is expected that the treatment consensus presently based on assessment without CRP will soon include CRP for further specificity in case of high cholesterol/HDL cholesterol ratios[2] and the recognition of a high risk group of patients among those with moderately elevated cholesterol/HDL cholesterol ratios[5]. The specific efficacy of treatment in CRP subgroups in the CARE study indicates a similar policy[66].

The discussion regarding the number of blood samples is even more pertinent when a value must be reliably classified within categories. Here we arrived at the conclusion that one blood sample is insufficient to position a CRP value reliably in more narrow categories such as tertiles, quartiles and quintiles. It is therefore proposed to aim at only one decision limit and that reliable classification of values within a smaller, high risk upper class might require at least two blood samples.

To evaluate the use of a decision limit we calculated the interclass stability for the example of the arbitrary decision limit of 3 mg/l (Table II), showing limited class adherence for various levels with r = ~0.5 or with 75-80% class stability. For our proposed strategy of relying on one blood sample below the decision limit, this implies that 20-25% of individuals with a habitual level actually above the limit will be missed (false-negatives).

An approach that more completely includes individuals at risk might be desirable. The situation may be improved by either increasing the reliability (second blood sample) or by selecting a lower practical decision limit [for instance limit minus (1 × SDi) = (3 - 0.9) = 2.1 mg/l]. In this respect it should be noted that when the decision limit is derived from epidemiological studies, the risk is underestimated by the regression dilution bias[67], which for r = ~0.5 is a factor of two[68]. Alternatively, these non-corrected evaluations may apply to a lower practical limit.

The proposal to evaluate a second blood sample above the decision limit decreases the number of false-positives to below 25%.

The above considerations about the biological variability of CRP, the consequences of reliable classification and the degree of misclassifications should be the basis for a final policy or policies, evaluated from medical and economical perspectives and tested in practice.

The availability of therapeutic options such as low dose aspirin[5,60], vitamin E[61,62] and statins[46,58] is important. An improved risk assessment by the inclusion of CRP may further motivate practitioners to utilize the existing repertoire more aggressively and, in particular, may motivate lifestyle interventions more strongly.

Finally, we defined a first practical strategy for assessment of continuously low individual CRP levels taking all available biological data into account. This may serve as a template for further refinement and adjustments.

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