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REVIEW ARTICLE

## Generation of data on within-subject biological variation in laboratory medicine: An update

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### Abstract

In recent decades, the study of biological variation of laboratory analytes has received increased attention. The reasons for this interest are related to the potential practical applications of such knowledge. Biological variation data allow the derivation of important parameters for the interpretation and use of laboratory tests, such as the index of individuality for the evaluation of the utility of population reference intervals for the test interpretation, the estimate of significant change in a timed series of results of an individual, the number of specimens required to obtain an accurate estimate of the homeostatic set point of the analyte and analytical performance specifications that assays should fulfill for their application in the clinical setting. It is, therefore, essential to experimentally derive biological variation information in an accurate and reliable way. Currently, a dated guideline for the biological variation data production and a more recent checklist to assist in the correct preparation of publications related to biological variation studies are available. Here, we update and integrate, with examples, the available guideline for biological variation data production to help researchers to comply with the recommendations of the checklist for drafting manuscripts on biological variation. Particularly, we focus on the distribution of the data, an essential aspect to be considered for the derivation of biological variation data. Indeed, the difficulty in deriving reliable estimates of biological variation for those analytes, the measured concentrations of which are not normally distributed, is more and more evident.

**Abbreviations:** ANOVA: analysis of variance; CA-125: carbohydrate antigen 125; CgA: chromogranin A; CI95%: confidence intervals at 95%; CRP: C-reactive protein; cTnT: cardiac troponin T; CV<sub>A</sub>: analytical coefficient of variation; CV<sub>G</sub>: between-subject biological coefficient variation; CV<sub>I</sub>: within-subject biological coefficient of variation; CV<sub>T</sub>: within-subject total coefficient of variation; DCCT: Diabetes Control and Complications Trial; EFLM: European Federation of Clinical Chemistry and Laboratory Medicine; FLC: immunoglobulin-free κ and λ light chains; HbA<sub>1c</sub>: glycated hemoglobin; HE4: human epididymis protein 4; ICC: intraclass correlation coefficient; IFCC: International Federation of Clinical Chemistry and Laboratory Medicine; IH: index of heterogeneity; II: index of individuality; IQC: internal quality control program; IUPAC: International Union of Pure and Applied Chemistry; LoD: limit of detection; ME: IFCC-NGSP master equation; n: number of specimens required for an analyte to ensure that the mean result be within desired percentage closeness to the individual's homeostatic set point; NGSP: National Glycohemoglobin Standardization Program; NSE: neuron-specific enolase; P1: baseline result; P2: the following value that was significantly lower or higher than P1; RCV: reference change value; RI: reference interval; S<sup>2</sup><sub>A</sub>: average within-run analytical variance; S<sup>2</sup><sub>G</sub>: between-subject biological variance; S<sup>2</sup><sub>I</sub>: average within-subject biological variance; S<sup>2</sup><sub>I+A</sub>: average within-subject total variance; S<sup>2</sup><sub>T</sub>: total variance of all measurements; SEQC: Spanish Society of Clinical Chemistry and Molecular Pathology; STARD: Standards for Reporting of Diagnostic Accuracy; TE: total error

### Keywords

Intra-individual biological variation, inter-individual biological variation, within-subject biological variation, between-subject biological variation, normality test, analytical goals, index of individuality, reference change value

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## Biological variation and derived indices

### Introduction to biological variation

The total variation ( $CV_T$ ) of a laboratory result is made up of three components, namely, preanalytical variation, analytical variation ( $CV_A$ ) and within-subject biological variation ( $CV_I$ )<sup>1</sup>. The preanalytical variation concerns all phases covering the preparation of the subject for sampling, and the collection, transport, storage and handling of biological samples. It can be assumed that, once all potential preanalytical factors have been identified, they can be minimized and the contribution of the preanalytical variation component to  $CV_T$  can be considered negligible.  $CV_A$  is associated with the analytical performance in terms of measurement errors. This variability should be strictly controlled, but, unlike the preanalytical variation, it cannot be completely removed. Finally,  $CV_I$  represents the physiological changes of analyte concentration that occurs in a specified biological fluid in each individual due to biological factors<sup>1</sup>. At each moment, the concentration of a constituent in a biological fluid, e.g. blood or urine, in a single individual is the result of dynamic control. This control is due to mechanisms that tend to maintain the analyte concentration around an average value that is optimal for the organism, namely the “homeostatic set point.”  $CV_I$  reflects this random fluctuation of the analyte concentration around the homeostatic set point. In contrast, the between-subject biological variation ( $CV_G$ ) expresses the difference among homeostatic set points for the same analyte in different individuals under the same physiological conditions<sup>1</sup>. By definition, the biological variation components are not reducible and are typically associated with a given analyte. As  $CV_I$  tends to vary from individual to individual, in general, the average of  $CV_I$  values derived in a group of individuals is considered. This should be obtained experimentally through a rigorous experimental protocol that includes the elimination of preanalytical variation, the careful estimate of  $CV_A$ , and the robust statistical derivation of  $CV_I$  and  $CV_G$ , expressed as coefficient of variation. Biological variation studies of different body constituents have received increasing attention in recent decades because of the potential practical application of such knowledge. Biological variation data are now considered a required element to judge the potential clinical significance of a biomarker.

### Applications of data on biological variation

Biological variation data for an analyte permits the derivation of important information for the correct application and clinical interpretation of its measurement<sup>2</sup>. In particular, the evaluation of utility of population reference intervals (RIs) for the interpretation of test results, the estimate of the significance of changes of results in an individual and the calculation of the number of specimens required for an analyte to ensure that the mean result is within the desired closeness to the individual’s homeostatic set point ( $n$ ) can be derived. Finally, it is possible to derive analytical performance specifications for a given measurement to assess its suitability for clinical use.

### Analytical performance specifications

Defining analytical performance specifications for each analyte measurement is essential to make its determination clinically usable and to ensure that the measurement error does not have an impact on the result<sup>3</sup>. In a conference held in 1999 in Stockholm under the auspices of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and International Union of Pure and Applied Chemistry (IUPAC), a hierarchy of sources for deriving the analytical goals of a laboratory measurement and defining quality specifications in laboratory medicine was defined<sup>4</sup>. In 2014, a follow-up conference held in Milan revisited the Stockholm consensus and investigated the extent to which the originally advocated hierarchy was still valid or the need to change or expand it<sup>5</sup>. Although the essence of the hierarchy established in Stockholm was still supported, new perspectives prompted its simplification and explanatory additions. The recommended approaches for defining analytical performance specifications should primarily rely on the effect of analytical performance on clinical outcomes or on the biological variation of the measurand<sup>6</sup>. Since analytical specifications derived from experimental studies assessing the clinical impact of methodological performances are difficult to obtain in practice, biological variation constitutes the most reliable source of useful information in the definition of analytical targets for the majority of the analytes<sup>7</sup>. The landmark paper by Fraser et al.<sup>8</sup> proposed specific formulae to calculate analytical performance specifications at three quality levels, i.e. minimum, desirable and optimal, for imprecision, bias and total error (TE), from biological variation data. Some authors have questioned this approach for deriving allowable TE and have proposed an alternative model in which the maximum allowable bias and imprecision are interrelated<sup>9</sup>. Even with some limitations, the classical Fraser model remains the most widely used approach for the definition of analytical goals in laboratory medicine<sup>10</sup>. It should also be mentioned that, in specific clinical monitoring situations, alternative approaches to deriving specifications could be adopted<sup>11</sup>.

### Index of individuality (II)

From the biological variation components, it is possible to derive the II, a parameter that allows the assessment of the utility of conventional population-based RIs derived from apparently healthy individuals for the interpretation of laboratory tests<sup>12</sup>. The formula to derive II uses the three following variance components: average within-run analytical variance ( $S^2_A$ ), average within-subject biological variance ( $S^2_I$ ) and between-subject biological variance ( $S^2_G$ ) (Table 1). During his pioneering studies on biological individuality, Eugene Harris proved that, if II is  $<0.6$ , the analyte shows high individuality and the variation of values for the individual occupies only a small part of the RI, making the latter barely useful for result interpretation<sup>13</sup>. In these cases, there is the risk of improperly considering physiological the values of an analyte in an individual that are significantly far from its homeostatic set point but still included within RI<sup>14,15</sup>. On the contrary, if II is  $>1.4$ , the analyte shows low individuality and the value dispersion in each individual

Table 1. Symbols and formulae related to biological variation data.

Abbreviations	Definitions	Formulas
$S^2_T$	Total variance of all measurements	$(S^2_A + S^2_I + S^2_G)$
$S^2_{I+A}$	Average within-subject total variance	
$S^2_A$	Average within-run analytical variance	
$S^2_I$	Average within-subject biological variance	$(S^2_{I+A} - 1/2S^2_A)$
$S^2_G$	Between-subject biological variance	$([(2kr-1)/2k(r-1)] \{S^2_T - S^2_A - [(2kr-2)/(2kr-1)] S^2_I\},$ where $k$ is the number of specimens per subject and $r$ is the number of subjects)
$CV_T$	Within-subject total coefficient of variation	
$CV_A$	Analytical coefficient of variation	
$CV_I$	Within-subject biological coefficient of variation	
$CV_G$	Between-subject biological coefficient of variation	
II	Index of individuality	$(S^2_{I+A}/S^2_G)$
RCV	Reference change value	$(2.77 (CV_A^2 + CV_I^2)^{0.5})$
$n$	Number of specimens required to ensure that the homeostatic set point estimate is within a desired percentage of closeness (D)	$[1.96^2 (CV_A^2 + CV_I^2)/D^2]$
IH	Index of heterogeneity (ratio of the observed $CV_T$ to the theoretical CV)	$(CV_T/[(2/k - 1)^{1/2} \times 100])$ , where $k$ is the number of specimens per subject

covers most of the dispersion among individuals that is represented by the RI, making it a useful tool for the test interpretation.

#### Reference change value (RCV)

For analytes showing high individuality (i.e.  $II < 0.6$ ), for which comparison of results with the RI is not recommended, a better interpretation strategy is the monitoring of longitudinal time changes of test results by the RCV application. The RCV, which was introduced by Harris and Yasaka in 1983<sup>16</sup>, is defined as the change needed between two serial results from the same individual to be statistically significantly different, taking into account the measurement error. The RCV is a fundamental parameter for the monitoring of longitudinal changes of analyte concentrations in an individual and it is especially useful for those analytes that show a high individuality and for which the RI has limited use in the interpretation of results. This situation occurs for most analytes assayed in laboratory medicine since  $CV_I$  is frequently much smaller than the respective  $CV_G$ <sup>17</sup>.

The estimation of RCV has sparked wide debate and discussion regarding the statistical approach that should be applied to derive it. In Fraser's traditional approach, RCV is derived from the  $CV_I$  estimation and, consequently, requires a normal distribution of biological data (Table 1)<sup>15</sup>. However, not all biological constituents show a symmetric variation of their concentrations in individuals, even after their logarithmic transformation. Therefore, when the biological behavior of an analyte does not meet assumptions needed to apply statistical parametric models, the derivation of RCV becomes complex<sup>18</sup>. To overcome the issue of data distribution, we recently proposed and preliminarily validated an alternative statistical model to the parametric one to assess the significance of the difference between serial measurements in an individual<sup>19</sup>. In particular, we compared the traditional parametric and the newly proposed non-parametric statistical models by selecting three analytes, i.e. glycated hemoglobin (HbA<sub>1c</sub>), chromogranin A (CgA) and C-reactive protein (CRP), that show a normal, a bimodal and a skewed

distribution of their individual concentrations, respectively. For each analyte, we used both models to derive the following value (P2) that was significantly lower or higher than the baseline value (P1). For HbA<sub>1c</sub>, P2 results obtained by the two methods overlapped. For CgA, when P1 concentrations were in the physiological range, P2 values obtained by the parametric method and the new one were similar, while, when P1 concentrations were equal to or higher than the upper reference limit, the P2 estimate differed significantly. Finally, for CRP at every tested concentration, P2 values derived from the two statistical approaches differed markedly, and those obtained by the traditional parametric method were unreliable and clinically impractical<sup>19</sup>. These results showed that when biological analyte concentrations present a bimodal or skewed distribution, the new statistical approach appears to be more reliable in assessing differences between serial measurements. In addition, for some analytes such as cardiac troponins, the application of RCV as an absolute (instead of relative) difference between serial samples has been advocated, and this approach could be suitable in these cases.

#### Number of specimens required to estimate the individual's homeostatic set point (n)

In principle, the greater the number of samples measured for each individual in biologically stable conditions, the more accurate the estimate of the homeostatic set point will be, especially if the analytical method is imprecise and/or the analyte shows a high  $CV_I$ . To calculate  $n$ , it is possible to use the rearrangement of the usual standard error of the mean formula given in Table 1<sup>2</sup>.

### Experimental protocol for deriving biological variation data

#### Premise

In the previous section, we briefly summarized the importance of the knowledge of biological variation data in laboratory medicine. It is, therefore, essential that these data are accurately derived through a standardized, well-defined

and robust experimental protocol. Currently, an international guideline describing the way for deriving biological variation data is lacking, and the only standard about this topic is the landmark publication by Fraser and Harris in 1989<sup>2</sup>. However, this paper is more than 25 years old and, in some aspects, its content needs to be updated according to recent developments in the field.

As clearly shown in the Ricos database<sup>11</sup>, there are numerous experimental studies in the literature that derive biological variation data for many analytes (approximately 240 articles dealing with more than 350 analytes). The main problem is high heterogeneity among experimental protocols used by different authors that necessarily affects the biological variation estimates obtained<sup>20–23</sup>. The availability of an updated standard would, therefore, allow investigators not only to carry out new experimental studies for deriving biological variation data in a correct and standardized way but also to objectively review the published literature and identify the high-quality studies. The Biological Variation Working Group (WG-BV) established by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) has recently published a checklist<sup>24</sup>, built in accordance with the Standards for Reporting of Diagnostic Accuracy (STARD) guideline<sup>25</sup>, that contains the minimum information that should be obtained from a biological variation study in order to be able to judge its quality. As the scope of this checklist is for consultation in the preparation of a publication related to a biological variation study, it considers very briefly the practical aspects that should characterize a biological variation experimental protocol. In this section, we provide a practical guideline for producing correct biological variation data, by updating the Fraser and Harris original recommendations<sup>2</sup> and expanding all aspects included in the checklist published by the EFLM WG-BV.

### Preanalytical phase

As mentioned above, the ideal experimental protocol for deriving biological variation data should minimize as much as possible the preanalytical factors to make the preanalytical variation component negligible. In this regard, it is necessary to strictly control each step from the preparation of the subject for sample collection to the time of analytical measurements.

#### Selection of subjects

There has been an extensive debate on which subjects should be enrolled to correctly derive biological variation data of an analyte<sup>26,27</sup>. In principle, we need to agree (or not) on the biological variation theory that defines the *physiological* fluctuation of an analyte around its homeostatic set point and, consequently, recommends the enrollment of ostensibly healthy subjects. The presence of disease, even if stable and well controlled, may modify the experimentally obtained information by amplifying such fluctuation<sup>21</sup>. Indeed, Fraser and Harris stated that “the [enrolled] subjects should be apparently healthy and undertaking and maintaining their usual lifestyles”<sup>2</sup>. More recently, Fraser has supported the possibility of obtaining acceptable biological variation estimates without undertaking his classic experimental protocol, by also enrolling subjects affected by the disease, provided

that this is stable<sup>15,28</sup>. However, the difficulty of defining disease stability *a priori* makes this approach less than ideal and poorly recommendable in practice.

Age is another critical aspect to be considered in the enrollment of subjects. Although earlier studies<sup>29</sup> showed no differences in CV<sub>I</sub> of common biochemistry analytes among different age groups, more recent publications have shown that a close correlation between CV<sub>I</sub> and age can be present, at least for some quantities<sup>30,31</sup>. In general, it is recommended to enroll adult subjects aged between 20 and 50 years, unless the clinical use of the analyte being considered is intended for specific age intervals (e.g. children). It is also important to report the ethnicity of the enrolled population in a biological variation study.

The enrolled subjects should be selected so as to minimize pre-analytical variables. In this regard, it is necessary to consider as exclusion criteria: (1) unusual habits and lifestyles, (2) taking medications (including contraceptives and over-the-counter drugs), (3) alcohol intake (>10g of ethanol/day) and (4) smoking. In addition to these common preanalytical variables, it is always necessary to identify more specific variables related to the evaluated analyte (e.g. the menstrual cycle regularity for deriving biological variation data of a gynecological marker).

The total number of subjects to be enrolled is not defined, but it is intuitive that the higher the number, the better the biological variation average estimate will be. It is clear, however, that it is more difficult to complete the experimental protocol and perform analyses under the recommended conditions if the number of subjects (and consequently of samples) is high. In summary, the number of enrolled subjects should be a compromise between an ideal high number and a smaller number that is more easily manageable within the recommended experimental design. A minimum of 10 subjects for each identified subgroup is considered sufficient to obtain a good biological variation estimate for as-yet-unstudied analytes<sup>32</sup>. The term “subgroup” here identifies a specific sample of the general population for which it is important to separately derive biological variation data. Usually, the subgroups considered in a biological variation study are those of the two genders (males and females), but for specific analytes (e.g. ovarian cancer markers), additional subgroups (e.g. pre- and post-menopause women) could be considered.

#### Sample collection and storage

It is strongly recommended to standardize and define, in advance and in writing, the criteria for sample collection, transport, aliquoting and storage. Sample collection should be performed at fixed time intervals and, in the case of blood, draws should be performed by the same phlebotomist at the same hour of the day (i.e. in the morning). At the time of collection, the subjects should be fasting for at least 12 h without having exercised in at least the preceding 24 h. As an analyte might be affected by posture, it is appropriate that subjects remain at rest before blood drawing for a time period of between 5 and 10 min, preferably in a sitting or supine position<sup>33</sup>. To reduce the likelihood of sample hemolysis, the use of 20- or 21-gauge needles is recommended<sup>34</sup>. The tube

type should be chosen on the basis of the analyte to be evaluated. The use of tubes with no anticoagulants and without gel separator is recommended, providing that the measurand can be determined in serum. The use of tubes containing anticoagulants or gel separator may introduce a source of preanalytical variation<sup>22</sup>. In any case, anticoagulants for sample collection should be thoroughly validated before they are used in a biological variation protocol, by using appropriate experimental and statistical approaches<sup>35,36</sup>.

Once collected, all samples should be processed in the same manner. Blood samples should be centrifuged within 1 h of collection, but not before 30 min from drawing. Sample integrity is important as inadequate preparation of the specimen blood tube or insufficient specimen centrifugation time may result in false analyte values due to the non-analyte reaction from the presence of red blood cells, fibrin clots or other floating debris. Subsequently, it is important to immediately aliquot the samples into specified tubes for freezing (secure closure cap). It is also recommended to determine the interference indices (hemolysis, lipemia and icterus) on an aliquot of each mother tube in an automatic system to be able to exclude samples showing altered values of these parameters<sup>37</sup>.

Finally, aliquoted specimens must be immediately stored at  $-80^{\circ}\text{C}$  until analyzed. Specific sample processing before freezing may be necessary. For example, in a study for the biological variation derivation of tartrate-resistant acid phosphatase, a marker of osteoclast activity, serum aliquots were acidified with 20  $\mu\text{l}$  of 5 mol/l acetate buffer, pH 5.0, per mL of serum before freezing to preserve the enzyme activity<sup>38</sup>. Only when all specimens of all enrolled subjects are available is it possible to proceed with the analytical phase. A different experimental design may be necessary when the analyte under study is unstable. In this case, samples must be assayed on the day of collection, and the between-run  $\text{CV}_A$  must be estimated from assays of a quality control material having a concentration near the mean of the subjects studied; then the between-run  $\text{CV}_A$  must be subtracted from the  $\text{CV}_T$  to obtain the biological variation<sup>39</sup>.

#### *Study duration*

The biological variation estimate may depend on the selected time interval between samples collected from the individual and on the study duration. To obtain a reliable  $\text{CV}_I$  estimate, it is important to collect samples at regular time intervals and to determine the study duration, which must be neither too short (a few days) nor so long (years) as to be influenced by additional causes (e.g. seasonal variation)<sup>40</sup>. In principle, the sample time interval and the study duration should be related to retesting times used for the measurements of the specific analyte in clinical practice.

#### **Analytical phase**

##### *Definition of the measurand and selectivity of the analytical method*

We have previously defined biological variation as an intrinsic characteristic of the analyte. When we derive the biological variation of an analyte, it is important to make sure

that the analyte in question coincides with the analytically determined measurand. For this reason, it is essential, before starting an experimental biological variation study, to check if: a) the measurand has been uniquely defined by professional organizations and b) the analytical method used in the experimental study is selective (i.e. analytically specific) enough for the measurand as it has been defined.

In general, it is possible to distinguish two types of analytes measured in the laboratory, the first that is represented by well-defined chemical entities (e.g. electrolytes and metabolic products such as cholesterol, creatinine) and the second that is composed of heterogeneous molecules in human body fluids<sup>41</sup>. When a measurand definition for a given analyte is officially recognized at the international level, this should be considered in a biological variation study<sup>42</sup>. Accordingly, it is essential to focus attention on the selectivity of the analytical method used for the study, as this characteristic is an important qualifier of biological variation. In principle, if the methodology used has different selectivity for the measured analyte, one could expect that the biological variation, a property closely associated with the characteristics of the analyte itself, could change significantly<sup>43</sup>. Establishing the metrological traceability of the measurement results undoubtedly helps to understand the measurements, and for those analytes for which a reference measurement system is available, the use of a traceable analytical procedure for the derivation of their biological variation data is mandatory. However, this is necessary but may not be sufficient. A classic example is that of creatinine, the determination of which can be performed by either alkaline picrate-based or enzymatic methods. Both groups of methods are currently made traceable to the reference system<sup>44</sup>. However, it is well known that alkaline picrate-based assays are non-specific for creatinine and that some endogenous and exogenous substances in serum, particularly proteins, can also be measured. Traceability implementation *per se* is unable to correct for those analytical non-specificity issues, so the two assay principles measure two different things<sup>45</sup>. On the other hand, it is possible to derive biological variation data for analytes for which measurement standardization is not available, provided that the measurand determined by the analytical method used in the study is clearly defined. The information regarding metrological traceability of the analytical method used should always be reported in a biological variation study. If, at the time a new test is placed into service, the measurand is not yet known or well-defined, published biological variation studies should indicate that results are applicable to the specific assay used and not necessarily to the biomarker itself.

##### *Sensitivity of the analytical method*

In addition to being selective, the analytical method used in a biological variation experimental study should also be sufficiently sensitive to the analyte to allow its reliable determination in enrolled apparently healthy subjects. For many analytes, this requirement is easily achieved; however, for some, the use of assays providing high analytical sensitivity is required<sup>22,46</sup>. For instance, studies that tried to assess the biological variation of cardiac troponin T (cTnT) were implausible as the majority<sup>47</sup> or a significant number<sup>48</sup>

of cTnT results in selected individuals were lower than the assay limit of detection (LoD). Before beginning a biological variation study, it is therefore appropriate to evaluate whether the LoD of the analytical method is suitable for the concentrations to be measured during the experimental study<sup>22,49</sup>.

### Sample analysis

Only when all samples of all enrolled subjects are available is it possible to proceed with their analysis, which should be performed in the same analytical run, in duplicate and in random order. This protocol is the ideal experimental model<sup>50</sup>. Performing all measurements in the same analytical run allows the elimination of the between-run  $CV_A$  component, which is difficult to assess correctly; this approach limits the influence on the obtained  $CV_T$  of results to only the within-run  $CV_A$ , and reduces the chance of error in the biological variation calculation obtained by subtraction of the  $CV_A$ . Duplicate measurements of any sample aliquot permit a direct estimate of the within-run  $CV_A$ . This is much better than deriving it from the laboratory internal quality control program (IQC), often obtained on non-commutable materials potentially displaying a different behavior from biological samples<sup>51</sup>. Finally, this approach allows the use of a single lot of reagents and the involvement of a single analyst, which is beneficial in reducing the imprecision of the analytical method. As mentioned above, the only disadvantage of this experimental model is the limited number of samples that can be analyzed and, consequently, of subjects that can be studied.

Before and after the analytical run, it is essential to check the alignment of the analytical system used with quality control materials applying the validation range stated by the manufacturer<sup>3</sup>.

### Statistical analysis

After assaying all samples, it is recommended that the results be tabulated in a clear and ordered way. Before starting the statistical analysis, it is advisable to carefully checking the rough results. Particularly, it is important to make sure that all concentrations are above the LoD of the analytical method. Then, it is possible to proceed with the evaluation of outliers and the distribution of the data. Figure 1 displays the recommended flow chart.

### Tests for outliers

The first statistical evaluation that must be performed on the results is outlier analysis. The importance of this phase is related to the fact that even a single abnormal observation, which could be caused by an analytical error or a sample exchange, may markedly influence the estimation of variability components. For outlier identification, it has been recommended to use Cochran's test among observations (derived from duplicate measurements) and  $S^2_{I+A}$  (average within-subject total variance) values, and Reed's criterion among mean concentration values of subjects<sup>2</sup>.

Cochran's test examines the ratio of the maximum variance to the sum of variances. This test presumes that each variance

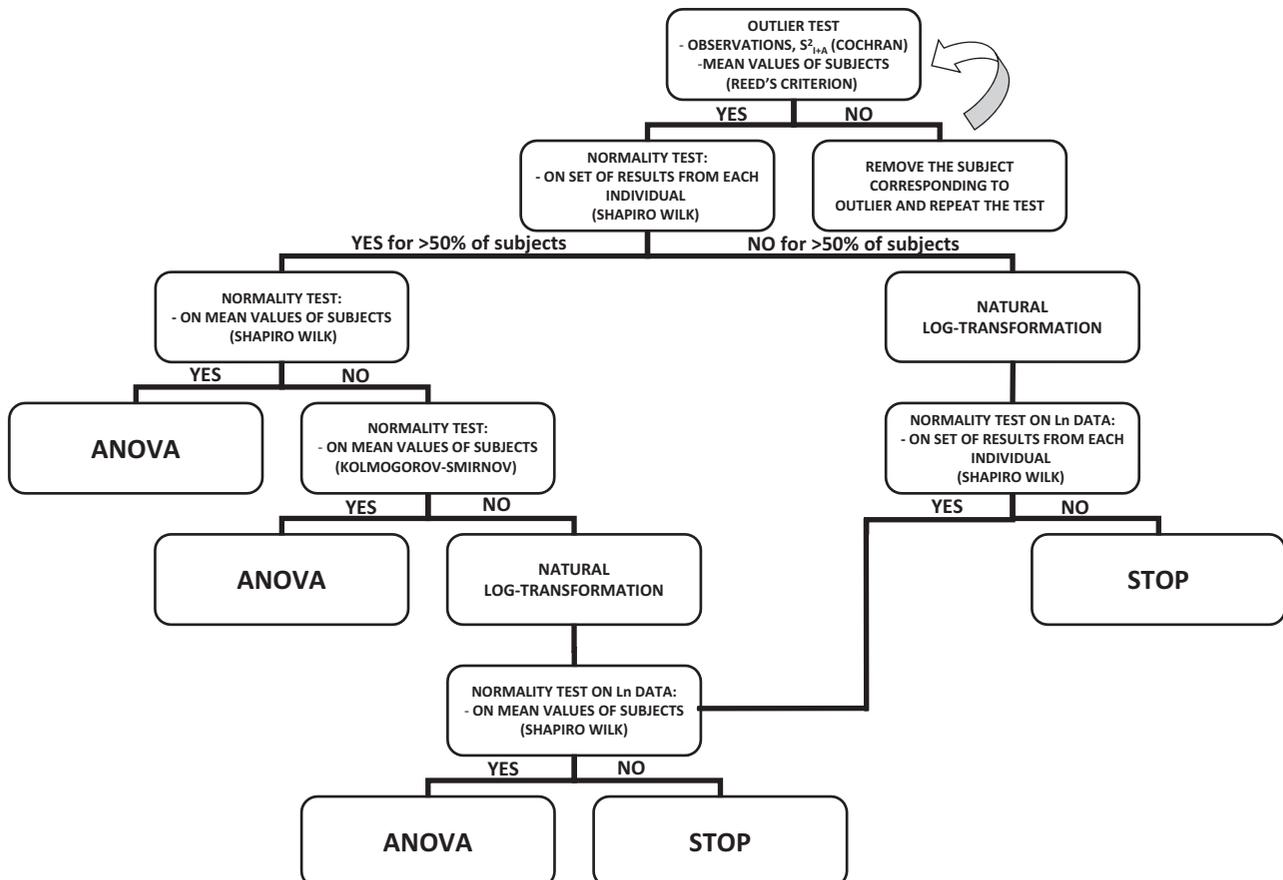


Figure 1. Recommended flow chart for the evaluation of outliers and data distribution in a biological variability study.

is based on an equal number of observations and that only one of the variances being tested appears unusually large. In terms of the current application, the test also assumes that the errors of duplicate determinations and/or of  $S^2_{I+A}$  values are normally distributed about the true value for that specimen and/or for those subjects<sup>2</sup>. Reed's criterion considers the difference between the extreme value and the next highest (or lowest) value and rejects the extreme if this difference exceeds one-third of the range of all values. This test, a rule-of-thumb simplification of a family of tests proposed by Dixon, also assumes that the true distribution (in this case, the distribution of the population of the mean concentration values of the subjects) is normal<sup>2</sup>. To simplify the statistical calculations, if an outlier is identified, regardless of the level at which it belongs (observations,  $S^2_{I+A}$  values or average values of the subjects), it is advisable to exclude all data of the corresponding subject.

### Normality tests

The biological variation derivation as CV requires that data are normally distributed. Some formulas used for the biological variation data applications, including that for the RCV calculation, also require the normality condition. It is, therefore, essential, after outlier exclusion, to proceed with the evaluation of data distribution. To determine if a dataset is modeled by a normal distribution and to compute how likely it is for a random variable underlying the data set to be normally distributed, it is necessary to apply some normality tests.

At first, a statistical normality test (Shapiro–Wilk test)<sup>52</sup> should be applied separately to the set of results from each individual to check data distribution and to validate the normality hypothesis. If the normal distribution assumption is rejected for most of the analyzed subjects (i.e. >50%), it is recommended that the natural logarithmic scale transformation of all data be used.

The same test must be used to evaluate the distribution of mean concentration values of all subjects and, if necessary, separately for each subgroup. If the test rejects the hypothesis of normality, it is recommended that the normality evaluation be repeated by a different statistical test (e.g. Kolmogorov–Smirnov test)<sup>53</sup>. If even this test confirms a skewed distribution, a natural logarithmic scale transformation should be applied. It is also essential to repeat the Shapiro–Wilk test on the log-transformed values to experimentally confirm the normality of the transformed data distribution. If the data transformation does not resolve the skewed distribution issue, the authors are forced to stop the calculations. On the other hand, if the normal distribution is in fact confirmed, it is possible to derive the variance components from the transformed data. These data must, however, be converted back before calculating the CVs to make these latter applicable to laboratory practice.

### Tests for comparing populations

To compare the mean and  $S^2_{I+A}$  values of two subgroups (e.g. males versus females), an unpaired Student's *t*-test and an *F*-test, respectively, should be performed. As the Student's *t*-test is a parametric statistic, if the normality assumption for the distribution of mean concentration values of the subjects is

not accepted, the non-parametric Wilcoxon–Mann–Whitney test<sup>54</sup> on untransformed median values should be performed. If by applying the proper statistical test, the mean values of the two subgroups are significantly different and the RI, on the basis of II, is useful, the interpretation of test results should be based on RI differentiated by subgroup. If the *F*-test shows that  $S^2_{I+A}$  values related to two subgroups are statistically different, it is important that all the parameters derived from  $CV_I$  (II, RCV, *n*, analytical performance specifications, etc.) are calculated separately for each subgroup.

### Index of heterogeneity

To assess the heterogeneity of within-subject variances, it is recommended to estimate the index of heterogeneity (IH) that is the ratio of the observed  $CV_T$  to the theoretical CV, which is  $[2/(k-1)]^{1/2}$ , where *k* is the number of specimens collected per subject. The SD of the difference between this ratio and its expected value of unity (under the hypothesis of no heterogeneity of true within-subject variances) is  $1/(2k)^{1/2}$ . A significant heterogeneity is present if the ratio differs from unity by at least twice this SD<sup>2</sup>. In this case, it is necessary to consider that the estimated RCV using the experimentally obtained  $CV_I$  is not ubiquitously valid, but it may be used as a simple figure to guide clinical decision-making. On the contrary, if IH is not significant, the average of the observed within-subject variances can be used for calculating a reference difference between two successive measurements, which is valid in different individuals<sup>2</sup>. Other acceptable tests for assessing variance homogeneity are Bartlett's Chi-square test<sup>55</sup>, Cochran's test<sup>56</sup>, Levene's test<sup>57</sup>, Brown–Forsythe's test<sup>58</sup> and Fligner–Killeen's test<sup>59</sup>.

### Analysis of variance (ANOVA)

The ANOVA of results obtained from replicates analysis is a reliable non-parametric procedure for estimating the variance components of interest. The preliminary phase of this analysis consists in the derivation of  $S^2_A$ , estimated as the average variance between duplicate measurements of the analyte, and of  $S^2_{I+A}$ , estimated as the weighted average of the variance of means of duplicate measurements for each subject. The  $S^2_I$  is obtained from  $S^2_A$  and  $S^2_{I+A}$  by the formula shown in Table 1. The final component of variance for which an estimate is desired is  $S^2_G$ . To estimate this quantity, when the number of specimens varies from one subject to another, a nested component of variance analysis should be carried out. When the number of specimens collected from each enrolled subject is the same, this estimate may be calculated as shown in Table 1 without going through a formal ANOVA. All the above-mentioned variance components are then transformed into CVs using the corresponding mean value.

### Biological variation result reporting

The mean concentration values, the estimates of  $CV_A$  and biological variation components (as CV) for all subjects, and separately for the subgroups, and the derived indices (II, RCV, *n*, etc.) should be tabulated in a clear format to allow their easy identification. We suggest also tabulating the derived

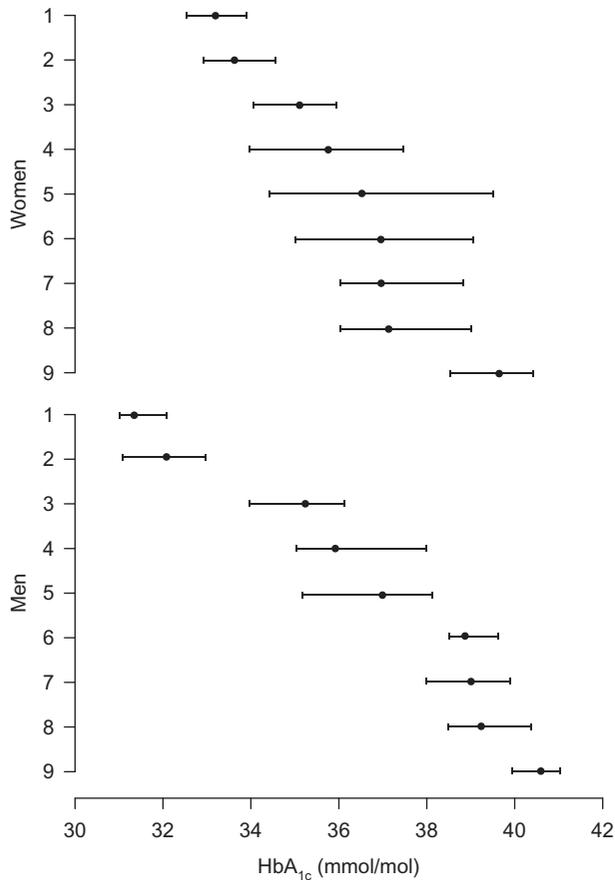


Figure 2. Representation of individual parametric mean and absolute range of values in a group of subjects evaluated for HbA<sub>1c</sub> biological variation. From<sup>43</sup>.

analytical performance specifications at the three quality levels (minimum, desirable and optimal) in a separate table. The CV<sub>I</sub> data should be reported with corresponding confidence intervals at 95% (CI95%). In this regard, if the determinations are performed in duplicate, the calculation of the CI95% can easily be derived from the average number of samples and subjects<sup>60</sup>. The terminology, symbols and units of measurement should conform to recommended standards<sup>61</sup>.

In the results section, the number of subjects (and of samples) included in statistical calculations after the identification and exclusion of outliers should be clearly reported. A biological variation study should also graphically report the individual parametric mean and absolute range of values in the individuals that were studied. Figure 2 shows an example of this graph.

## Discussion and conclusion

In recent decades, biological variation studies of different analytes have received increasing attention due to the practical application of such knowledge in defining important parameters for the interpretation and use of laboratory tests. Considering the importance of biological variation data in laboratory medicine, it is essential to experimentally derive them in an accurate and reliable way. Currently, a dated guideline for the biological variation data production and a more recent checklist that assists researchers in producing

high-quality publications are available<sup>2,24</sup>. Here, we have updated the approach to derive biological variation data in order to facilitate compliance with the checklist recommendations. In this regard, we have analyzed in detail all aspects that should be considered in an experimental protocol for the biological variation data production of an analyte.

Currently, the most commonly used information on the biological variation of biochemical and hematological analytes is that compiled by the Spanish Society of Clinical Chemistry and Molecular Pathology (SEQC), which is freely available<sup>10</sup>. This database lists the average CV<sub>I</sub> and CV<sub>G</sub> components derived from data available in the literature as well as the desirable targets for analytical imprecision, bias and TE for each analyte. The criteria used for the production of this database have recently been published<sup>62</sup>. In spite of being highly consulted, the content of this database has been criticized and the need to improve the information by applying more stringent criteria in the selection and review of available biological variation studies has been recognized<sup>21,22</sup>. To this end, a new study group has recently been created by EFLM under the auspices of the Task Force on Performance Specifications in Laboratory Medicine<sup>5</sup>. Another EFLM group is dealing with the most appropriate model for deriving analytical performance specifications for laboratory measurements<sup>63</sup>. In the preliminary discussion, the biological variation model is considered very useful if the measurand has strict homeostatic control, whereas it is probably not appropriate for analytes that show no homeostatic control or that are present in blood but reflect no physiologic role.

The statistical management of data derived in biological variation studies is an often-neglected problem. The difficulty in deriving reliable estimates of biological variation for those analytes, the measured individual concentrations of which are not normally distributed, is more and more evident. When an analyte shows a skewed biological variation data distribution, the log-transformation may be appropriate to solve problems related to a non-Gaussian distribution of values. However, this may create confusion in interpreting the RCV obtained as the use of log-transformed laboratory test results is impractical for individual patient care. Furthermore, this approach does not always solve the distribution problems or work. For instance, for CRP, the symmetric distribution of individual data is seldom achieved even by logarithmic transformation. deGoma et al. employed an alternative model for calculating RCV in the case of CRP<sup>64</sup>. Although their methodological approach tried to overcome the limitation of parametric protocols, some important pitfalls were highlighted<sup>18</sup>. In particular, with regard to the statistical analysis of data, the authors applied a linear mixed effects model for longitudinal data that had previously been adopted by Glynn et al. in the study of intra-class correlation coefficient (ICC) of CRP<sup>65</sup>. However, as highlighted above, in the case of CRP, the log-transformation of results, which is instrumental in using the ICC estimate, is unable to assure the normalization of data distribution, making the proposed approach weak. To succeed in solving these problems, we have recently proposed a new non-parametric statistical model for the interpretation of differences in serial test results from an individual<sup>19</sup>. In addition to the distribution problem, the proposed approach

also overcomes the problem of correlation between within-subject serial measurements, which may cause an overestimation of CV<sub>I</sub>, allowing for the first time the establishment of reliable interpretative criteria for assessment of results of biologically complex analytes such as CRP, and potentially contributing to set aside previously raised perplexities about their clinical utility<sup>66</sup>. A validation of the new proposed model in specific clinical contexts is definitely required before its final application.

### Declaration of interest

The authors report no declarations of interest.

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## Appendix

### Practical Examples

#### Pre-analytical phase

The following examples relate to some important preanalytical aspects that should be carefully considered in the biological variation experimental protocol: subject selection<sup>31</sup> and study duration<sup>19</sup>.

#### Selection of subjects

Serum human epididymis protein 4 (HE4) is a biomarker recently proposed for diagnosis and monitoring of ovarian cancer<sup>67</sup>. In planning a study for estimating the biological variation of this tumor marker, all potential sources of preanalytical variability previously identified for HE4 measurements were taken into account and excluded in enrolled patients<sup>68</sup>. As it was unclear if menopausal status *per se* might influence serum HE4 concentrations, in recruiting individuals to derive HE4 biological variation data, we selected ostensibly healthy women in premenopause ( $n = 14$ ; age interval, 25–53 years) and in post-menopausal ( $n = 14$ ; age interval, 50–68 years) and analyzed them as separated subgroups<sup>31</sup>. Furthermore, to avoid potential HE4 changes in response to different phases of the menstrual cycle and the effect of oral

contraceptives<sup>69,70</sup>, the selected pre-menopausal women had regular menstrual cycles and were not using hormonal contraceptives. Blood samples were obtained monthly for four consecutive months. In women in pre-menopause, the blood was collected between the 12th and 14th d of the menstrual cycle; this corresponded to their ovulation period and avoided the potential influence of different phases of the menstrual cycle on the biological fluctuation of HE4 concentrations.

### Study duration

CRP is an acute-phase protein produced by the liver in response to inflammation and infection<sup>71</sup>. Seasonal variation has been demonstrated for the protein concentrations in blood<sup>72</sup>. It, therefore, seems reasonable to adjust the duration of a study evaluating the biological variability of CRP to between approximately 1 and 3 months, with a frequency of sample collections of 1–2 weeks<sup>19</sup>.

## Analytical phase

### Measurand definition and method selectivity

HbA<sub>1c</sub> is the “gold standard” for the monitoring of diabetes mellitus and the knowledge of its blood concentrations is essential in the assessment of the degree of glycometabolic control in diabetic patients and in the prediction of risk for vascular complications in these subjects<sup>73</sup>. In the process of HbA<sub>1c</sub> standardization that IFCC started 20 years ago, the measurand has been defined as hemoglobin molecules having a specific hexapeptide in common, the stable adduct of glucose to the N-terminal valine of the hemoglobin  $\beta$ -chain ( $\beta$ N-1-deoxyfructosyl-hemoglobin)<sup>74</sup>. Starting from this measurand definition, IFCC has developed a complete reference measurement system for HbA<sub>1c</sub> through 1) the implementation of two equivalent reference methods (HPLC/mass spectrometry- and HPLC/capillary electrophoresis-based) that are highly specific for the measurand as defined; 2) the characterization of primary and secondary calibrators and 3) the organization of an international network of laboratories performing one or both reference procedures<sup>75</sup>. Before the establishment of the IFCC reference measurement system for HbA<sub>1c</sub>, the National Glycohemoglobin Standardization Program (NGSP) was created in the United States to harmonize HbA<sub>1c</sub> results through the implementation of assay traceability to the ion-exchange HPLC method, originally employed in the Diabetes Control and Complications Trial (DCCT)<sup>76</sup>. In the NGSP system, HbA<sub>1c</sub> was, therefore, roughly defined as the area under the curve of the corresponding chromatographic peak obtained with the method mentioned above<sup>42</sup>. To transfer the clinical experience gained with the NGSP-aligned assays to the new IFCC system, a relationship between IFCC and NGSP systems was established by defining the so-called IFCC-NGSP “master equation” (ME) [NGSP (%) = 0.09148 × IFCC (mmol/mol) + 2.152], which expresses the correlation between the two systems<sup>77,78</sup>. The presence of a significant intercept (2.152) in the ME equation denoted, however, the different analytical selectivity of the two measuring systems (NGSP and IFCC), as expected from the difference in the measurands as defined above.

Weykamp et al.<sup>79</sup> first demonstrated with the simple mathematical reasoning that the biological variation estimates related to the NGSP and IFCC definitions of HbA<sub>1c</sub> may differ. To verify this hypothesis experimentally, we carried out a study to derive biological variation parameters for HbA<sub>1c</sub> by performing measurements using an assay for which we had previously ascertained its perfect alignment to the IFCC reference system<sup>43</sup>. The results obtained when compared with those of previously available studies, which assayed a measurand different from that defined by IFCC<sup>21</sup>, showed that the biological variation components were higher using the IFCC system than the NGSP system, giving rise, consequently, to different analytical specifications.

### Sensitivity of assays used

The use of assays capable of accurately measuring very low analyte concentrations sometimes present in healthy subjects is fundamental for generating reliable biological variation data. In evaluating the reliability of the available information on the biological variation of CRP, we

turned our attention to the LoD reported in publications evaluating the analytical sensitivities of assays used in different studies to measure the very low CRP concentrations detected in healthy individuals<sup>22</sup>. Only half of the studies directly or indirectly described the LoD of the analytical method used. Furthermore, as only standard CRP assays, which were able to consistently measure CRP elevation in inflammatory conditions but were unreliable for detecting low protein concentrations in a healthy cohort, were available until the second part of the 1990s; all older studies suffered from insufficient analytical sensitivity. In a recent study evaluating the biological variation of CRP, we measured this marker using a highly sensitive latex immunoturbidimetric assay (Roche Diagnostics, Mannheim, Germany), with a LoD of 0.15 mg/L<sup>19</sup>. The study involved the collection of 110 serum specimens (five from each of 22 apparently healthy volunteers), each assayed in duplicate. One case was eliminated because three CRP values out of five were <LoD.

## Statistical analysis

### Identifying outliers

For the biological variation estimate of the serum immunoglobulin free  $\kappa$  and  $\lambda$  light chains (FLC) and  $\kappa/\lambda$  FLC ratio calculation, we collected five blood specimens from each of 21 enrolled volunteers and each sample was measured in duplicate<sup>80</sup>. In Tables A1–A3, we report, as an example, the calculations for outlier identification performed for  $\kappa$  FLC with Cochran’s statistical test applied to duplicate observations and  $S^2_{I+A}$  values and for Reed’s criterion applied to the average  $\kappa$  FLC values of subjects, respectively. By consulting the table for Cochran’s test optimized (for  $p=0.01$ ), one sees that, with a total number of  $\sim 100$  values and 2 degrees of freedom, the maximum/sum ratio should be  $<0.1424$ <sup>81</sup>. Having experimentally obtained a value of 0.154 for this ratio on all observations (Table A1), the highest detected variance (0.21125 mg/L) has to be considered as an outlier and the subject displaying this variance (number 2) eliminated from analyses. After removing the outlier, Cochran’s test was performed again; the maximum/sum ratio was 0.079, which, on the basis of the table for Cochran’s test optimized, allowed the data to be accepted. Cochran’s test was also applied to  $S^2_{I+A}$  values (Table A2). By consulting the table for the Cochran’s test optimized (for  $p=0.01$ ), one notes that, with 20 values and 4 degrees of freedom, the maximum/sum ratio should be  $<0.2654$ <sup>81</sup>. Having experimentally obtained a value of 0.28819 for this ratio for  $S^2_{I+A}$  values, the maximum variance (3.88997) should be considered as an outlier and the corresponding subject (number 16) should be eliminated from analyses. Cochran’s test was then performed again; the maximum/sum ratio was 0.2611, which allowed the data to be accepted. Finally, for the application of Reed’s criterion for identification of outliers among mean values of subjects, the average  $\kappa$  FLC values (the mean of the five duplicate means) of each individual were considered in ascending order (Table A3). To exclude the presence of outliers, the differences between the second lowest value (6.20 mg/L) and the lowest (5.88 mg/L) and between the highest value (13.41 mg/L) and the second highest (9.85 mg/L) should not be  $>2.51$  mg/L, which is the difference between the maximum (13.41 mg/L) and the minimum value (5.88 mg/L), divided by 3. The first difference (0.32 mg/L) was indeed well below the limit, but the second one (3.56 mg/L) was much higher. The subject with the highest  $\kappa$  FLC mean value was then eliminated and Reed’s criterion repeated; this time a difference between the highest value (9.85 mg/L) and the next highest one (9.66 mg/L) of 0.19 mg/L was obtained, which is lower than one-third of the difference between the two extreme values (1.32 mg/L). At the end of all steps, after removing all the outliers, the number of subjects with results usable for the estimate of biological variation components for serum  $\kappa$  FLC was 18<sup>80</sup>.

### Testing normality of distributions

Reported examples relate to the biological variation studies of three analytes with different data distributions.

In studying HbA<sub>1c</sub> biological variation in a group of 18 subjects<sup>19,43</sup>, we evaluated the frequency distribution of HbA<sub>1c</sub> values characterizing the subject population. The Shapiro–Wilk test accepted the hypothesis of normality of the data distribution in the great majority of subjects. The

Table A1. Calculations for Cochran's statistical test for outlier identification applied to the duplicate observations for serum  $\kappa$ -free light chains obtained in a biological variation study (80).

Subject no.	Sample	$(r_2 - r_1)^2/2$ (mg/L)
1	A	0.00500
	B	0.00320
	C	0.00005
	D	0.02420
	E	0.00125
2	A	0.21125
	B	0.00180
	C	0.08405
	D	0.00080
	E	0.00720
3	A	0.00080
	B	0.00320
	C	0.01445
	D	0.00125
	E	0.03125
4	A	0.00245
	B	0.00125
	C	0.06480
	D	0.00125
	E	0.03380
5	A	0.04805
	B	0.00500
	C	0.08405
	D	0.04205
	E	0.00605
6	A	0.00320
	B	0.00320
	C	0.04805
	D	0.01125
	E	0.01805
7	A	0.01805
	B	0.00045
	C	0.00005
	D	0.00405
	E	0.02000
8	A	0.00080
	B	0.00500
	C	0.00045
	D	0.00020
	E	0.00320
9	A	0.00005
	B	0.00500
	C	0.00125
	D	0.01125
	E	0.00125
10	A	0.00045
	B	0.02000
	C	0.00045
	D	0.00045
	E	0.03125
11	A	0.00500
	B	0.00020
	C	0.04500
	D	0.01620
	E	0.00845
12	A	0.02000
	B	0.00045
	C	0.00180
	D	0.00500
	E	0.02420
13	A	0.02000
	B	0.00080
	C	0.00605
	D	0.00405
	E	0.00500
14	A	0.00125
	B	0.00605
	C	0.05120

(continued)

Table A1. Continued

Subject no.	Sample	$(r_2 - r_1)^2/2$ (mg/L)
15	D	0.00020
	E	0.00405
	A	0.00720
	B	0.00020
	C	0.00045
16	D	0.00405
	E	0.00000
	A	0.00080
	B	0.00000
	C	0.00980
17	D	0.00020
	E	0.00500
	A	0.00980
	B	0.01445
	C	0.01280
18	D	0.01620
	E	0.00245
	A	0.01620
	B	0.01620
	C	0.00125
19	D	0.03125
	E	0.00005
	A	0.00245
	B	0.00005
	C	0.01280
20	D	0.02000
	E	0.01125
	A	0.00045
	B	0.01125
	C	0.00605
21	D	0.02205
	E	0.00845
	A	0.00605
	B	0.00045
	C	0.00005
	D	0.00845
	E	0.00005
	Total number of variances	105
	Sum of variances	1.36905 mg/L
	Maximum variance	0.21125 mg/L
Maximum/sum ratio	0.154	

r1, replicate 1; r2, replicate 2.

condition of normality was also accepted for the distribution of mean HbA<sub>1c</sub> values of all subjects and of male and female subgroups.

In studying the biological variation of carbohydrate antigen 125 (CA-125), a biomarker for detecting ovarian cancer recurrence and monitoring the therapeutic response, the Shapiro–Wilk test accepted the hypothesis of normality for the distribution of within-subject marker values for all subjects, while for the distribution of individual mean CA-125 values of enrolled subjects, the normality test failed<sup>31</sup>. The Kolmogorov–Smirnov test confirmed the skewed distribution. Consequently, a natural log scale transformation was applied before calculation. The Shapiro–Wilk test was repeated for the CA-125 individual mean values obtained from log-transformed data and the hypothesis of normality was finally accepted. The obtained  $S^2_I$ ,  $S^2_G$  and overall mean were converted back via inverse natural logarithmic function before deriving biological CVs<sup>31</sup>.

In the study evaluating the biological variation of CRP quoted above<sup>19</sup>, the Shapiro–Wilk test failed to accept the hypothesis of normality for the distribution of 70% of within-subject values. Therefore, we transformed all data by natural log-scale and repeated the normality test. Even after logarithmic transformation, the Shapiro–Wilk test failed to accept the hypothesis of normality for the distribution of 25% of within-subject CRP values. The inability to normalize the distribution of CRP values prevented the biological variation data derivation for this analyte by the traditional protocol based on a parametric calculation of CVs.

Table A2. Calculations for the Cochran's statistical test for outlier identification applied to the within-subject variances obtained in a biological variation study of serum  $\kappa$ -free light chains (80).

Subject no.	$S^2_{I+A}$ (mg/L)
1	0.54219
3	2.50866
4	0.31316
5	2.09345
6	0.17169
7	0.46693
8	0.15303
9	0.13124
10	0.13100
11	0.28817
12	0.22058
13	0.82346
14	0.22063
15	0.09914
16	3.88997
17	0.46514
18	0.15857
19	0.39579
20	0.2546
21	0.17045
Total number of variances	20
Sum of variances	13.4978 mg/L
Maximum variance	3.88997 mg/L
Maximum/sum ratio	0.28819

### Comparing populations

During an experimental study of biological variation of total copper, ceruloplasmin and copper/ceruloplasmin ratio (Cu:Cp) in serum, mean analyte values and  $S^2_{I+A}$  values of male and female subjects were compared by the unpaired Student's *t*-test and the *F*-test, respectively, using the MedCalc statistical program (MedCalc Software bvba, Ostend, Belgium)<sup>82</sup>. Mean Cu:Cp values was slightly higher ( $p=0.02$ ) in women than in men, whereas no sex-dependent difference was found for copper and ceruloplasmin concentrations. However,  $S^2_{I+A}$  values of the studied parameters were not different between the genders; this allowed the derivation of only one  $CV_I$  value for the whole population.

Table A3. Average values for each of the enrolled subjects, displayed in ascending order, in a biological variation study of serum  $\kappa$ -free light chains to be used for the application of Reed's criterion (80).

Mean (mg/L)
5.88
6.20
6.28
6.68
7.20
7.61
7.75
7.94
8.00
8.05
8.44
8.50
8.56
8.66
9.01
9.08
9.66
9.85
13.41

### Assessing the index of heterogeneity

CgA and neuron-specific enolase (NSE) are biomarkers for neuroendocrine tumors. For the derivation of their biological variation, we collected five blood specimens from each of 22 apparently healthy volunteers on the same day of the week, every 2 weeks for 2 months<sup>37</sup>. With five data points for each subject, the within-subject data are homogeneous if IH is  $<0.632$ . However, the calculated IH (0.768 for CgA and 0.805 for NSE) did not fulfill the homogeneity condition. Consequently, the RCV obtained for CgA and NSE were not directly transferable to the entire population for the clinical interpretation of marker results, but were just suggestive of the extent that changes in results should reach to indicate the presence of some external sources of variation (e.g. disease progression or therapy effectiveness).