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UNIVERSITÀ DEGLI STUDI DI MILANO

Centre for Metrological Traceability in Laboratory Medicine (CIRME)

site: http://users.unimi.it/cirme

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BIOLOGICAL VARIABILITY: THE CIRME CONTRIBUTION

F. Braga

Centre for Metrological Traceability in Laboratory Medicine (CIRME)

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10th International Scientific Meeting. November 17-18, 2016

TOTAL VARIATION (CV_T)

~~**PREANALYTICAL VARIATION**~~

ANALYTICAL VARIATION (CV_A)

WITHIN-SUBJECT BIOLOGICAL VARIATION (CV_I)

BETWEEN-SUBJECT BIOLOGICAL VARIATION (CV_G)

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Useful applications of Biological Variation data

→ *Derived indices*

- **Index of individuality** = $\sigma^2_{A+I}/\sigma^2_G$
  - If II >1.4: RI is useful for result interpretation
  - If II <0.6: RI is barely useful for result interpretation (→RCV)
- **RCV** = $2.77\ (CV_A^2 + CV_I^2)^{1/2}$
  - If the analyte does not show a normal distribution of data → alternative statistical model *[Clin Chem Lab Med 2015;53:815]*
- **n** = $1.962\ (CV_A^2 + CV_I^2)/D$
  - n° of samples required to ensure that the homeostatic set point estimate is within a desired percentage of closeness (D)

→ *Analytical performance specifications*

IMPRECISION: $\leq 0.25\ CV_I$ (O); $\leq 0.5\ CV_I$ (D); $\leq 0.75\ CV_I$ (M)

BIAS: $< 0.125\ (CV_I^2 + CV_G^2)^{1/2}$ (O); $< 0.25\ (CV_I^2 + CV_G^2)^{1/2}$ (D); $< 0.375\ (CV_I^2 + CV_G^2)^{1/2}$ (M)

TOTAL ALLOWABLE ERROR:

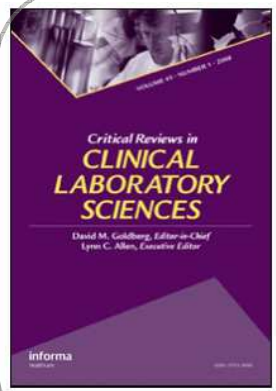
$= 1.65\ (0.25\ CV_I) + 0.125\ (CV_I^2 + CV_G^2)^{1/2}$ (O)

$= 1.65\ (0.5\ CV_I) + 0.25\ (CV_I^2 + CV_G^2)^{1/2}$ (D)

$= 1.65\ (0.75\ CV_I) + 0.375\ (CV_I^2 + CV_G^2)^{1/2}$ (M)

[Fraser, et al. Ann Clin Biochem 1997;34:8-12]

International Guideline describing the experimental protocol for BV estimate



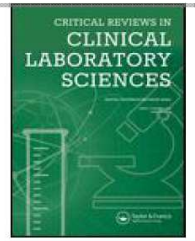
Critical Reviews in Clinical Laboratory Sciences
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400870

Generation and Application of Data on Biological Variation in Clinical Chemistry

Gallum G. Fraser [a]; Eugene K. Harris [b]
[a] Department of Biochemical Medicine, Ninewells Hospital and Medical School, Dundee, Scotland [b] Department of Pathology, University of Virginia Health Sciences Center, Charlottesville, Virginia

Online Publication Date: 01 January 1989

1989



Critical Reviews in Clinical Laboratory Sciences

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

Federica Braga & Mauro Panteghini

2016

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International Checklist for the preparation of a publication related to a BV study

DE GRUYTER

Clin Chem Lab Med 2015; 53(6): 879–885

Opinion Paper

2015

William A. Bartlett*, Federica Braga, Anna Carobene, Abdurrahman Coşkun, Richard Prusa, Pilar Fernandez-Calle, Thomas Røraas, Neils Jonker and Sverre Sandberg, on behalf of the Biological Variation Working Group, European Federation of Clinical Chemistry and Laboratory Medicine (EFLM)

A checklist for critical appraisal of studies of biological variation

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Experimental Protocol for BV Estimate

Critical Reviews in Clinical Laboratory Sciences

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1) PREANALITICAL PHASE

2) ANALITICAL PHASE

3) STATISTICAL ANALYSIS

4) BV RESULT REPORTING

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1) PREANALYTICAL PHASE

➢ Selection of subjects

- **Ostensibly healthy subjects**

  [or subjects affected by the disease, provided that this is stable (?)]

- **Age between 20 and 50 years**

  [unless the clinical use of the analyte is intended for specific age intervals]

- **Exclusion criteria:**

  - unusual habits and lifestyles

  - taking medications (including contraceptives and over-the-counter drugs)

  - alcohol intake (>10 g of ethanol/day)

  - smoking

  [identify more specific variables related to the evaluated analyte!]

- **~ 10 subjects for each identified subgroup**

A specific sample of the general population for which it is important to separately derive biological variation data.

1) PREANALYTICAL PHASE

➢ Sample collection

[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]

- Fixed time intervals, same hour of the day
- Blood draws:
  - Same phlebotomist
  - 20- or 21-gauge needles
  - Tube type chosen on the basis of the analyte

    [if it is possible no anticoagulants and without gel separator]
- Fasted subject for at least 12 h without having exercised in at least the preceding 24 h.

➢ Sample storage

- Centrifuge the blood samples within 1 h of collection, but not before 30 min from drawing
- Aliquot them into specified tubes for freezing (secure closure cap)
- Determine the interference indices
- Store the aliquoted specimens at -80°C until analyzed

Practical example…

HE4

DE GRUYTER Clin Chem Lab Med 2014; 52(11): 1625–1631

Federica Braga*, Simona Ferraro, Roberta Mozzi and Mauro Panteghini

The importance of individual biology in the clinical use of serum biomarkers for ovarian cancer

- Ostensibly healthy women:
  - in pre-menopausal (n=14; age interval, 25–53 years) and
  - in post-menopausal (n=14; age interval, 50–68 years)
- 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

  [to avoid the potential influence of different phases of the menstrual cycle on the biological fluctuation of HE4 concentrations]

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1) PREANALITICAL PHASE

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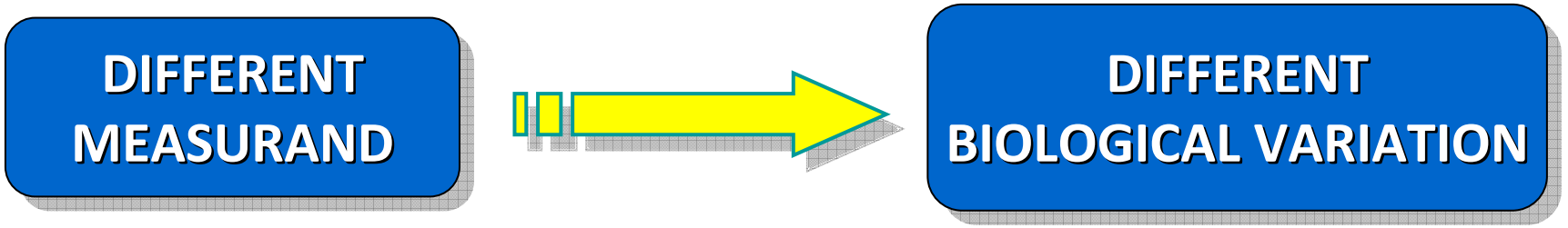
2) ANALYTICAL PHASE

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



It is essential, before starting an experimental BV study, to check if:

a) the *measurand has been uniquely defined* by professional organizations

b) the *analytical method* used in the experimental study *is selective* (i.e. analytically specific) enough for the measurand as it has been defined.

Practical example…

HbA_{1c}

"Hemoglobin molecules having a specific hexapeptide in common, the stable adduct of glucose to the N-terminal valine of the hemoglobin b-chain (bN-1-deoxyfructosyl-hemoglobin)"

[IFCC, 1995]

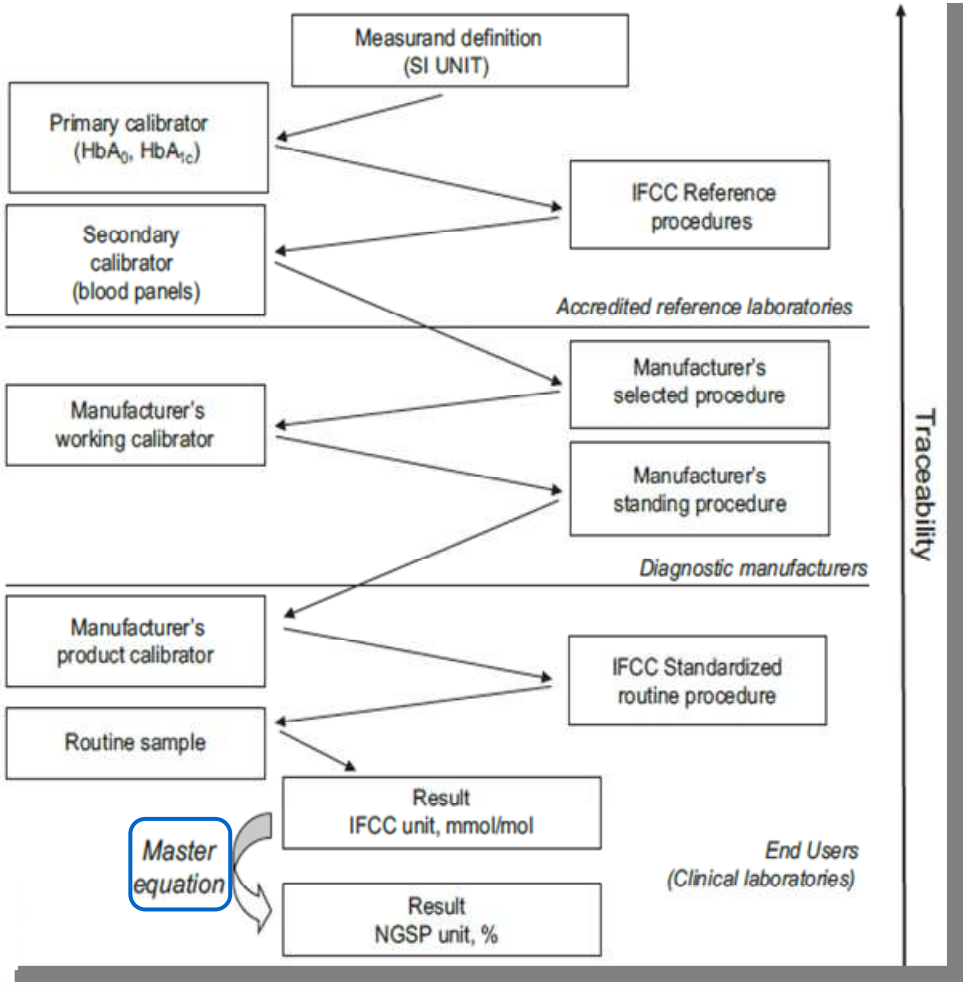
IFCC-NGSP
"Master equation"

NGSP (%) =
0.09148 x IFCC (mmol/mol) + 2.152

[Different analytical selectivity of the two systems!]

[BV estimates related to the NGSP and IFCC definitions of HbA_{1c} may differ!]

Weykamp CW et al.
Clin Chem 2011;57:1204-6



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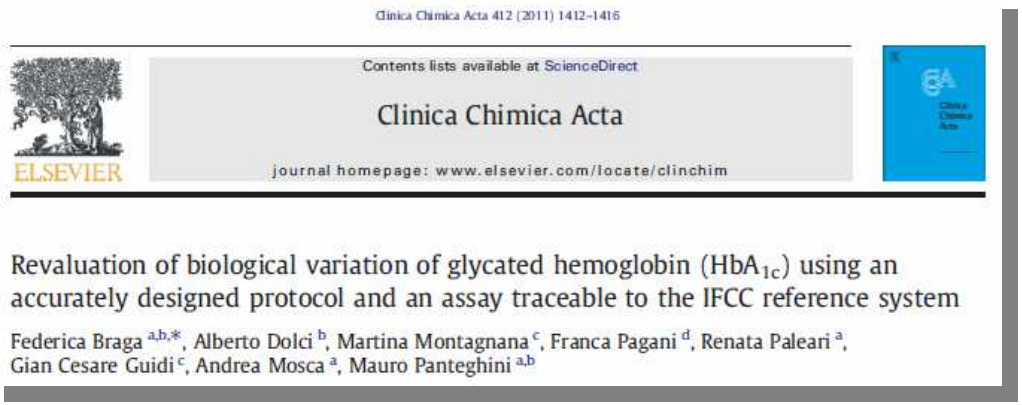
The analytical selectivity is an important quality to derive BV of an analyte!



Clinica Chimica Acta 412 (2011) 1412–1416

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Revaluation of biological variation of glycated hemoglobin (HbA_{1c}) using an accurately designed protocol and an assay traceable to the IFCC reference system

Federica Braga [a,b,*], Alberto Dolci [b], Martina Montagnana [c], Franca Pagani [d], Renata Paleari [a], Gian Cesare Guidi [c], Andrea Mosca [a], Mauro Panteghini [a,b]

We performed measurements using an assay for which we had previously ascertained its perfect alignment to the IFCC reference system

	CV_I%	CV_G%
Our study	2.5	7.1
Ricos database	1.9	5.7

≠ Analytical selectivity

≠ Biological Variation

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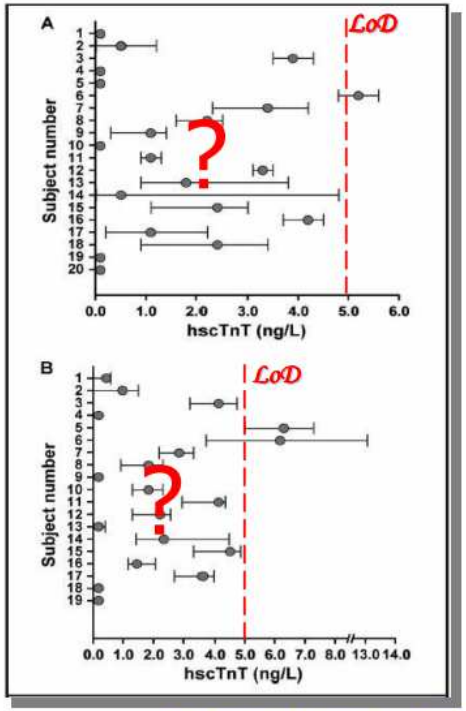
2) ANALYTICAL PHASE

➢ Sensitivity of the analytical method

- The analytical method used in a BV experimental study should be *sufficiently sensitive* to the analyte to allow its reliable determination in enrolled apparently healthy subjects

- Before beginning a BV 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



[Vasile VC et al., Clin Chem 2010;56:1086]

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Practical example…

CRP


Clinica Chimica Acta 413 (2012) 1179–1183

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Invited critical review

Biologic variability of C-reactive protein: Is the available information reliable?

Federica Braga *, Mauro Panteghini

Centro Interdipartimentale per la Riferibilità Metrologica in Medicina di Laboratorio (CIRME), Università degli Studi, Milano, Italy

In evaluating the reliability of the available information on the BV of CRP, *we turned our attention to the LoD* reported in studies measuring the very low CRP concentrations detected in healthy individuals:

- **Only half of the studies directly or indirectly described the LoD of the method**
- **All studies older than 1990 suffered from insufficient analytical sensitivity (only standard CRP assays were available!)**

DE GRUYTER

Clin Chem Lab Med 2015; 53(5): 815–822

Federica Braga*, Simona Ferraro, Francesca Ieva, Anna Paganoni and Mauro Panteghini

A new robust statistical model for interpretation of differences in serial test results from an individual

To derive BV of CRP we used a *highly sensitive latex immunoturbidimetric assay* (Roche Diagnostics, Mannheim, Germany), with a LoD of 0.15 mg/L.

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.

2) ANALYTICAL PHASE

➢ 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

[allows the elimination of the between-run CV_A component, difficult to correctly assess]

✓ duplicate

[permit a direct estimate of the within-run CV_A. This is much better than deriving it from the IQC, often obtained on non-commutable materials]

✓ random order

✓ single lot of reagents and single analyst

✓ check the alignment of the analytical system with QC materials applying the validation range stated by the manufacturer

Experimental Protocol for BV Estimate

Critical Reviews in Clinical Laboratory Sciences

ISSN: 1040-8363 (Print) 1549-781X (Online) Journal homepage: http://www.tandfonline.com/loi/ilab20

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1) PREANALITICAL PHASE

2) ANALITICAL PHASE

3) STATISTICAL ANALYSIS

4) BV RESULT REPORTING

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3) STATISTICAL ANALYSIS

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.*

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3) STATISTICAL ANALYSIS

➢ Tests for outliers

- **Cochran's test**
  - observations (derived from duplicate measurements)
  - S^2_{I+A} (average within-subject total variance)

[examines the ratio of the maximum variance to the sum of variances]

- **Reed's criterion**
  - mean concentration values of subjects

[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]

If an outlier is identified, regardless of the level at which it belongs, it is advisable to exclude all data of the corresponding subject.

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Practical example…

k-FLC

Braga F, et al. "Biological variation of free light chains in serum" Clin Chim Acta 2012;415:10-11

Cochran's test [observations]

[5 blood specimens from each of 21 subjects; each sample measured in duplicate]

Subject no.	Sample	$(r2-r1)^2/2$ (mg/L)
	D	0.00020
	E	0.00405
15	A	0.00720
	B	0.00020
	C	0.00045
	D	0.00405
	E	0.00000
16	A	0.00080
	B	0.00000
	C	0.00980
	D	0.00020
	E	0.00500
17	A	0.00980
	B	0.01445
	C	0.01280
	D	0.01620
	E	0.00245
18	A	0.01620
	B	0.01620
	C	0.00125
	D	0.03125
	E	0.00005
19	A	0.00245
	B	0.00005
	C	0.01280
	D	0.02000
	E	0.01125
20	A	0.00045
	B	0.01125
	C	0.00605
	D	0.02205
	E	0.00845
21	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.

- **Table for Cochran's test optimized (for p =0.01):** with a total number of ~100 values and 2 degrees of freedom, *the maximum/sum ratio should be <0.1425*

- *Experimentally obtained value = 0.154* [the highest detected variance (0.21125 mg/L) considered as an *outlier* and the subject displaying this variance eliminated from analyses]

- After removing the outlier, *Cochran's test was performed again; the maximum/sum ratio = 0.079*, which, on the basis of the table for Cochran's test optimized, allowed the data to be accepted.

Practical example…

k-FLC

Braga F, et al. "Biological variation of free light chains in serum" Clin Chim Acta 2012;415:10-11

[5 blood specimens from each of 21 subjects; each sample measured in duplicate]

Cochran's test [S^2_{I+A}]

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

- Table for Cochran's test optimized (for p =0.01): with a total number of ~20 values and 4 degrees of freedom, *the maximum/sum ratio should be <0.2654*

- *Experimentally obtained value = 0.288* [the highest detected variance (3.88997 mg/L) considered as an *outlier* and the corresponding subject eliminated from analyses]

- After removing the outlier, *Cochran's test was performed again; the maximum/sum ratio = 0.261*, which, on the basis of the table for Cochran's test optimized, allowed the data to be accepted.

Practical example…

k-FLC

Braga F, et al. "Biological variation of free light chains in serum" Clin Chim Acta 2012;415:10-11

Reed's criterion
[mean values of subjects]

[5 blood specimens from each of 21 subjects; each sample measured in duplicate]

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

- The mean values were considered *in ascending order*
- To exclude the presence of outliers, the differences between the second lowest value and the lowest and between the highest value and the second highest should not be >2.51 mg/L [the difference between the maximum and the minimum value divided by 3]
- 6.20-5.88 = 0.32 mg/L; *13.41-9.85 = 3.56 mg/L*
- The subject with the highest k FLC mean value was eliminated as *outlier and the test repeated*
- 9.85-9.66 = 0.19 mg/L [< 1/3 of the difference between the two extreme values (1.32 mg/L)]

After removing all the outliers, the number of subjects with results usable for the estimate of BV for serum k FLC was 18

3) STATISTICAL ANALYSIS

The BV derivation as CV requires that data are normally distributed!

➢ Normality tests

- **Shapiro-Wilk test**
  - separately to the set of results from each individual
    [rejected assumption → natural log scale transformation]
  - mean concentration values of all subjects
    [rejected assumption → different statistical test (Kolmogorov-Smirnov test)
    [rejected assumption → natural log scale transformation]

Repeat the Shapiro-Wilk test on the log-transformed values to experimentally confirm the normality of the data distribution!

[rejected assumption → stop the calculations]
[accepted assumption → to derive the variance components from the transformed data. These data must be converted back before calculating the CVs to make these latter applicable to laboratory practice]

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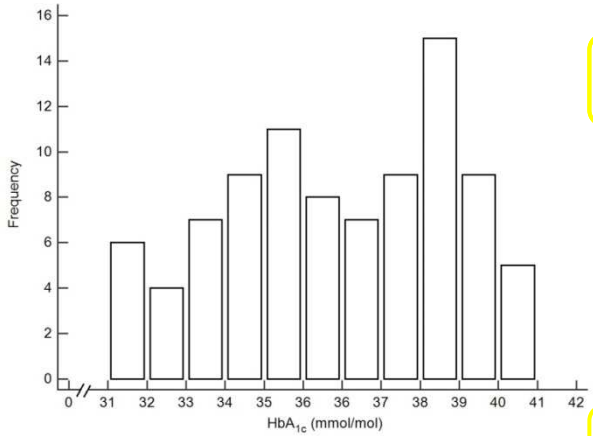
Practical example…

DE GRUYTER Clin Chem Lab Med 2015; 53(5): 815–822

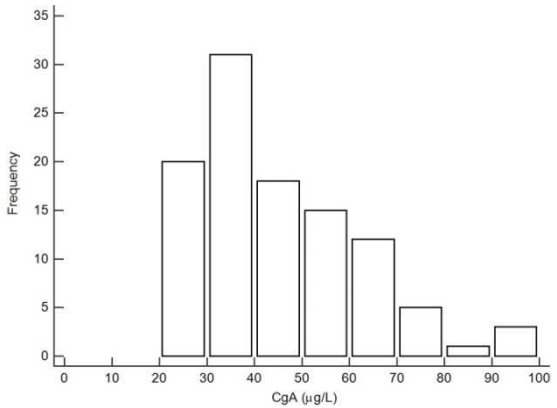
Federica Braga*, Simona Ferraro, Francesca Ieva, Anna Paganoni and Mauro Panteghini

A new robust statistical model for interpretation of differences in serial test results from an individual



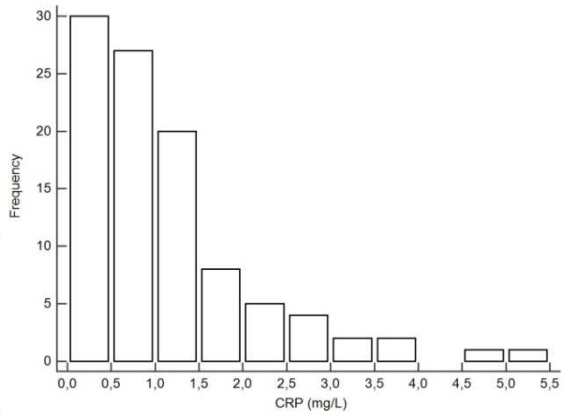
HbA_{1c}

- ***Accepted*** **normality hypothesis in the great majority of subjects.**
- ***Accepted*** **normality hypothesis also for the mean values of all subjects and of male and female subgroups.**



CgA

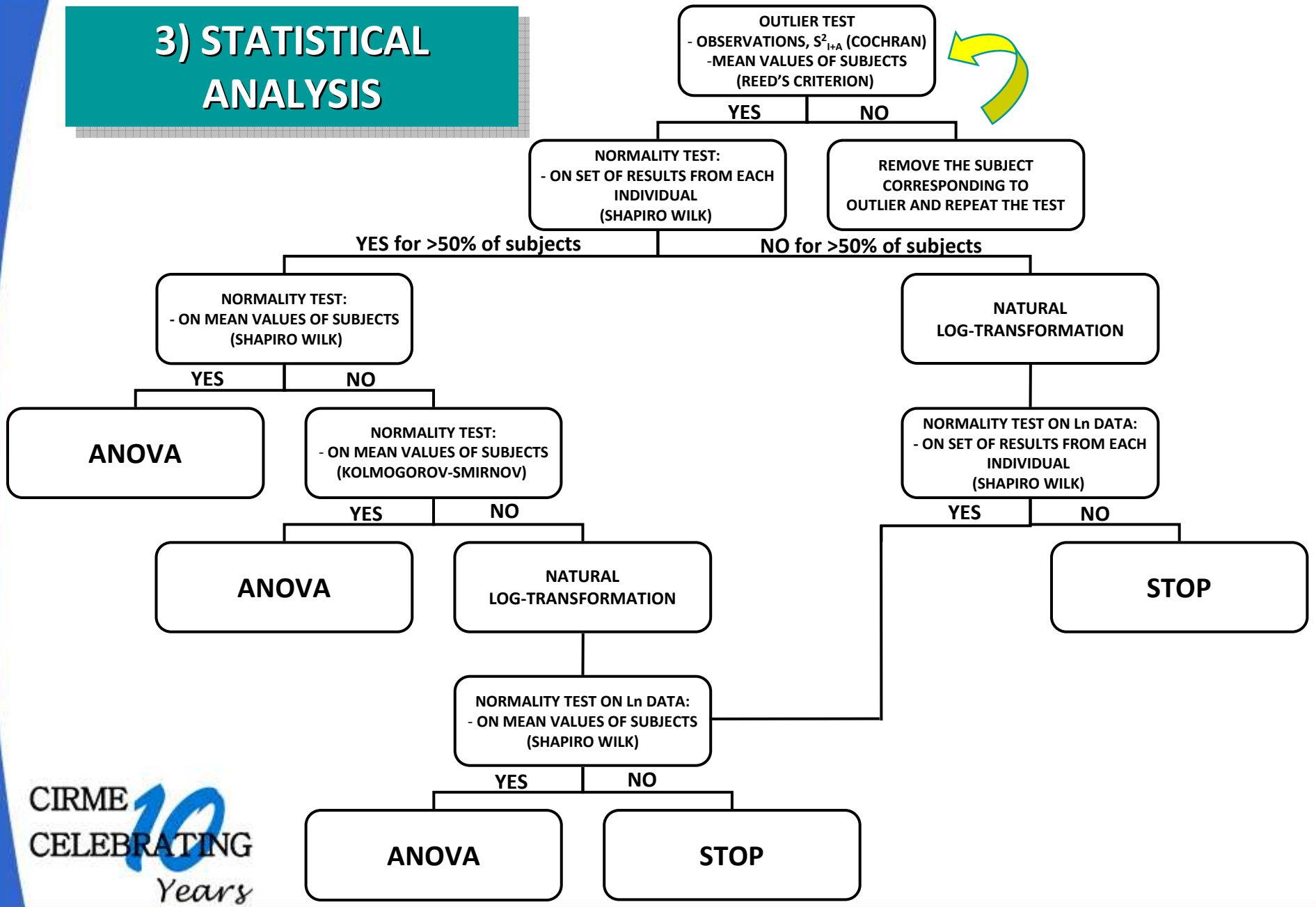
- ***Accepted*** **normality hypothesis in the great majority of subjects.**
- ***Rejected*** **normality hypothesis for the mean values of all subjects (confirmed by Kolmogorov–Smirnov test) → *natural log scale.***
- ***Accepted*** **normality hypothesis for transformed data.**
- **The S^2_I, S^2_G and overall mean converted back via inverse natural log function before deriving biological CVs.**



CRP

- ***Rejected*** **normality hypothesis in the 70% of within-subject values → natural log scale.**
- ***Rejected*** **normality hypothesis for transformed data → stop the calculations**

3) STATISTICAL ANALYSIS



3) STATISTICAL ANALYSIS

➢ Tests for comparing populations

- **Parametric Student's t-test (or non-parametric Wilcoxon-Mann-Whitney)**

  - To compare the *mean values* of two subgroups

  [If they 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]

- **F-test**

  - To compare S^2_{I+A} *values* of two subgroups

  [If they 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]

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Practical example…

Cu, Cp and Cu/Cp

Clinica Chimica Acta 415 (2013) 295–296

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Braga et al. "Biologic variation of copper, ceruloplasmin and copper/ceruloplasmin ratio (Cu:Cp) in serum"

	Cu	Cp	Cu/Cp
Student's t-test	F=M	F=M	F>M *P*=0.02
F-test	F=M	F=M	F=M

3) STATISTICAL ANALYSIS

➢ Index of heterogeneity

= $CV_T/[(2/k - 1)^{1/2}$ x100], where k is the number of specimens per subject.

A significant heterogeneity is present if the ratio differs from unity by at least 2SD, where SD is $1/(2k)^{1/2}$

[If HI is significant: the estimated RCV using the experimentally obtained CVI is not ubiquitously valid, but it may be used as a simple figure to guide clinical decision-making]

[If HI 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]

Other acceptable tests: Bartlett's Chi-square test
Cochran's test
Levene's test
Brown–Forsythe's test
Fligner–Killeen's test

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Practical example…

CgA and NSE

$SD = 1/(2 \times 5)^{1/2} = 0.316$

$2SD = 0.632$

CgA IH = 0.768 (> 0.632)

NSE IH = 0.805 (> 0.632)


Clinical Biochemistry 46 (2013) 148–151

Contents lists available at SciVerse ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Biological variation of neuroendocrine tumor markers chromogranin A and neuron-specific enolase

Federica Braga [a,b,*], Simona Ferraro [a], Roberta Mozzi [a], Alberto Dolci [a], Mauro Panteghini [a,b]

[5 blood specimens from each of 22 subjects]

"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)"

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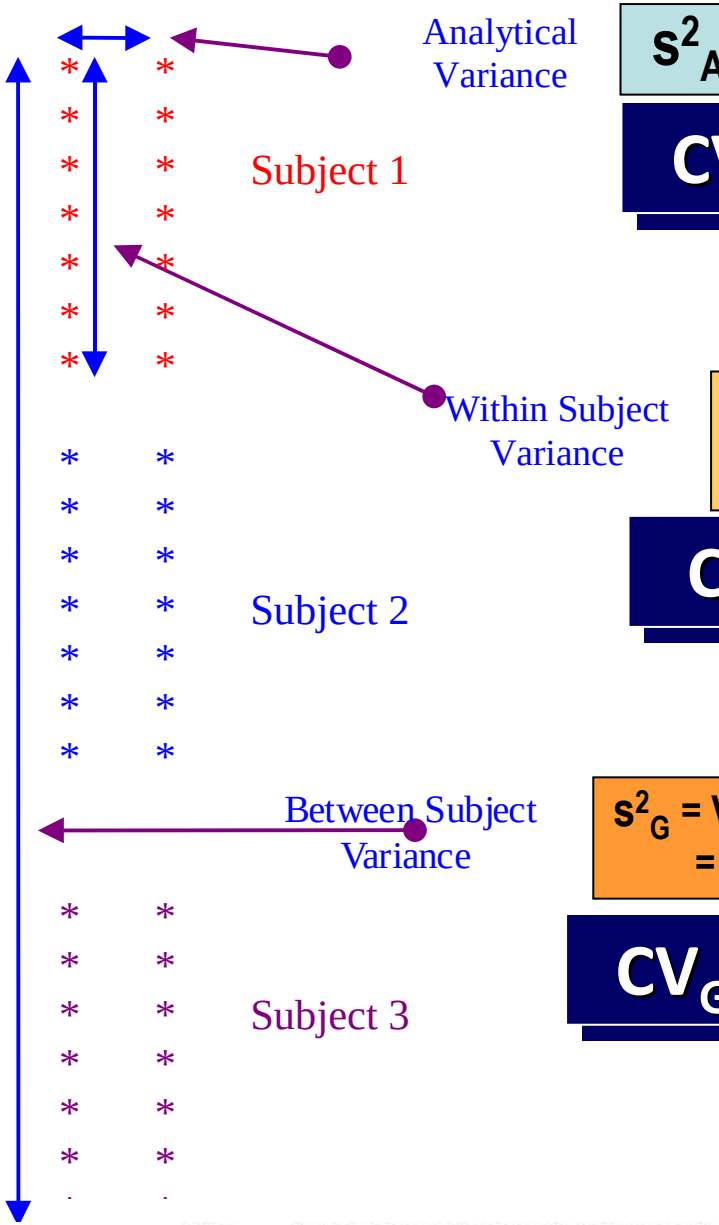
➢ Analysis of variance (ANOVA)

3) STATISTICAL ANALYSIS



s^2_A = Average variance of replicate assays

CV_A

s^2_I = Average biologic within subject variance
= (s^2_{I+A}) - ($s^2_A/2$)

CV_I

s^2_G = Variance of true means among subjects (between subjects)
= [(2kr - 1) / 2k (r - 1)] { ST2 - s^2_A - [(N – 2) / (N – 1)] s^2_I }

CV_G

Where:

r – 1 = Degrees of freedom (number of subjects – 1)

k = Specimens for subject

N = Total specimens = 2kr

ST2 = Total variance of N

Experimental Protocol for BV Estimate

Critical Reviews in Clinical Laboratory Sciences

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

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1) PREANALITICAL PHASE

2) ANALITICAL PHASE

3) STATISTICAL ANALYSIS

4) BV RESULT REPORTING

4) BV RESULT REPORTING

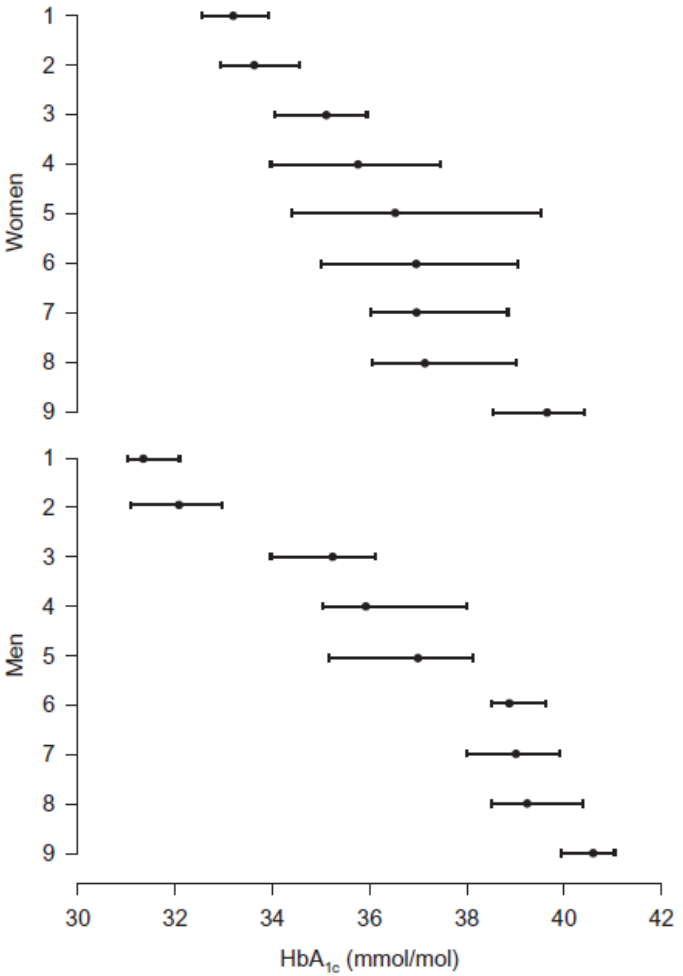
Table 1

Parameter	Group (n)	Mean	CV_A%	CV_I% [CI95%]	CV_G%	II	RCV%	n
	All							
	(Men)							
	(Women)							

Table 2

Analytical goals								
Imprecision as CV%			Bias%			Total error%		
Min	Des	Opt	Min	Des	Opt	Min	Des	Opt

Figure



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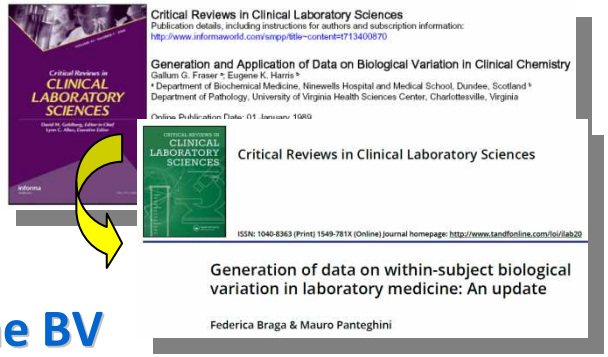
CONCLUSIONS

- Considering the *importance of BV data* in laboratory medicine, it is essential to experimentally *derive them in an accurate and reliable way*
(Particular attention to statistical management!)



- Currently, the most commonly used information on the BV of biochemical and hematological analytes is that compiled by the SEQC (*www.westgard.com/biodatabase1.htm*)

but

The need to improve it by applying *more stringent criteria* in the selection and review of available BV studies has been recognized…

This is the aim of *New Study Group* created by EFLM under the auspices of the Task Force on Performance Specifications in Laboratory Medicine

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EFLM EUROPEAN FEDERATION OF CLINICAL CHEMISTRY AND LABORATORY MEDICINE

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