Clinical Chemistry 64:9
1380–1393 (2018)

The EuBIVAS: Within- and Between-Subject Biological Variation Data for Electrolytes, Lipids, Urea, Uric Acid, Total Protein, Total Bilirubin, Direct Bilirubin, and Glucose

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BACKGROUND: The European Federation of Clinical Chemistry and Laboratory Medicine European Biological Variation Study (EuBIVAS) has been established to deliver rigorously determined data describing biological variation (BV) of clinically important measurands. Here, EuBIVAS-based BV estimates of serum electrolytes, lipids, urea, uric acid, total protein, total bilirubin, direct bilirubin, and glucose, as well as their associated analytical performance specifications (APSs), are presented.

METHOD: Samples were drawn from 91 healthy individuals (38 male, 53 female; age range, 21–69 years) for 10 consecutive weeks at 6 European laboratories. Samples were stored at −80 °C before duplicate analysis of all samples on an ADVIA 2400 (Siemens Healthineers). Outlier and homogeneity analyses were performed, followed by CV-ANOVA on trend-corrected data, when relevant, to determine BV estimates with CIs.

RESULTS: The within-subject BV (CV_I) estimates of all measurands, except for urea and LDL cholesterol, were lower than estimates available in an online BV database, with differences being most pronounced for HDL cholesterol, glucose, and direct bilirubin. Significant differences in CV_I for men and women/women <50 years of age were evident for uric acid, triglycerides, and urea. The CV_A obtained for sodium and magnesium exceeded the EuBIVAS-based APS for imprecision.

CONCLUSIONS: The EuBIVAS, which is fully compliant with the recently published Biological Variation Data Critical Appraisal Checklist, has produced well-characterized, high-quality BV estimates utilizing a stringent experimental protocol. These new reference data deliver revised and more exacting APS and reference change values for commonly used clinically important measurands, thus having direct relevance to diagnostics manufacturers, service providers, clinical users, and ultimately patients.

Biological variation (BV)[12] data have many applications in the diagnosis and monitoring of disease and for quality assessment in clinical laboratory medicine. BV components encompass the within-subject BV (CV_I), defined as the fluctuation of a measurand around a homeostatic set point in a steady-state condition, and the between-subject BV (CV_G), defined as the variability of a measurand between the homeostatic set points between different healthy participants *(1)*. Applications of BV data include the index of individuality (II) for the evaluation

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Received February 14, 2018; accepted May 30, 2018.
Previously published online at DOI: 10.1373/clinchem.2018.288415

[12] Nonstandard abbreviations: BV, biological variation; CV_I, within-subject biological variation; CV_G, between-subject biological variation; II, index of individuality; RCV, reference change values; APS, analytical performance specification; EFLM, European Federation of Clinical Chemistry and Laboratory Medicine; EuBIVAS, European Biological Variation Study; IP, inorganic phosphate; CV_A, analytical variation; CV_{APS}, analytical performance specification for imprecision; BIVAC, Biological Variation Data Critical Appraisal Checklist; ALT, alanine aminotransferase.

of the utility of conventional population-based reference intervals in a diagnostic context, assessment of significance of change in serial measurements observed within a participant [reference change values (RCVs)] enabling decision processes in a monitoring context, and the setting of analytical performance specifications (APSs) to ensure that the methods of measurement are fit for purpose *(1, 2)*. The European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) First Strategic Conference in 2014 proposed that laboratory tests can be allocated to different models for the determination of APS *(3)*. The 3 models identified were based on (*a*) the effect on the clinical outcome, (*b*) BV data, or (*c*) the state-of-the-art of measurement. A clinical outcome model is preferred when cutpoints are recommended by guidelines to classify patients into different groups of either therapeutic or interventional strategies, or both *(4)*. However, relevant studies are often not available, or available only from studies applied in specific clinical settings that may preclude their use for APS derivation in the general laboratory. In this situation, the BV data model presents the best available approach to setting APS as an interim measure. When measurands are under strict homeostatic control, a BV model is recommended and considered particularly useful for setting APS *(4)*.

Numerous stakeholders, such as manufacturers of diagnostic tests, laboratory medicine providers, and clinical users, depend on availability of BV data given the range of applications for the data *(2)*. This delivers a requirement for access to robust and well-characterized BV data to enable safe and effective application of the data across time and healthcare systems delivering services to a variety of patient populations. Concerns have been raised around the quality of existing BV studies *(2, 5, 6)* and, consequently, around the robustness of data sets underpinning estimates of BV collated and made available in the online BV database last revised in 2014 *(7)*. This raises a potential safety and effectiveness issue because of application of some existing BV estimates in clinical practice. To address these concerns and the emergent need for new BV data using the latest generation of analytical methods, the EFLM Working Group on Biological Variation *(8)* has designed and implemented the European Biological Variation Study (EuBIVAS) to deliver BV estimates based on best methodological practice and contemporary analytical technologies *(9)*. In this study, a bank of specimens collected from 91 healthy individuals from 5 European countries has been established to provide robust and high-quality BV data for the highest possible number of measurands. This article presents BV data derived from the EuBIVAS for 17 measurands in serum: (*a*) electrolytes: sodium (Na), potassium (K), chloride (Cl), magnesium (Mg), calcium (Ca), and inorganic phosphate (IP); (*b*) lipids: total cholesterol, HDL cholesterol, calculated LDL cholesterol, calculated non-HDL cholesterol, and triglycerides; and (*c*) glucose, urea, uric acid, total protein, total bilirubin, and direct bilirubin.

Materials and Methods

SAMPLE COLLECTION AND HANDLING

The EuBIVAS involved 6 European laboratories from Italy, Norway, Spain, the Netherlands, and Turkey. Ninety-one healthy volunteers were enrolled (38 male and 53 female; age range, 21–69 years) (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue9). Ten women were between 55 and 69 years of age (with none in the age-group 50–54 years). Two men were >50 years of age. Participants completed an enrollment questionnaire to provide information on lifestyle and health status, which was further verified by a set of laboratory tests as previously described in detail *(9, 10)*. All laboratories followed the same protocol for the preanalytical phase *(9)*. Fasting blood samples were drawn for 10 consecutive weeks (April–June 2015). Seventy-seven participants completed all 10 collections, 10 completed 9, 2 completed 8, and 2 completed 7. The serum samples collected by each laboratory were sent frozen in dry ice to San Raffaele Hospital in Milan, Italy and were stored in a freezer at −80 °C until analysis in 2016 *(9)*. The EuBIVAS protocol was approved by the Institutional Ethical Review Board of San Raffaele Hospital in agreement with the World Medical Association Declaration of Helsinki and by the Ethical Board/Regional Ethics Committee for each center collecting samples.

ANALYTICAL METHODS

All analyses were performed using the ADVIA 2400 Clinical Chemistry System (Siemens Healthineers) at San Raffaele Hospital (see Table 2 in the online Data Supplement). LDL cholesterol was calculated by Friedewald formulae *(11)*. Non-HDL cholesterol was calculated as total cholesterol − HDL cholesterol *(12)* using total cholesterol and HDL cholesterol data after adjustment for trends, outliers, and variance homogeneity analysis. Reference materials for glucose (NIST 965b at 4 concentration levels) and fresh-frozen serum pools at 2 different concentrations with target values for Na, K, total cholesterol, and HDL cholesterol were analyzed in triplicate on 3 different days before starting the sample measurements. The reference material target values were assigned by the Standardization Laboratory at San Raffaele Hospital for Na and K by flame atomic emission spectrometry using IL943, concentration calculation through bracketing *(13, 14)*, and for total cholesterol and HDL cholesterol by the Abell–Kendall reference method *(15)*. The Standardization Laboratory participates in the IFCC external quality assessment scheme for

reference laboratories in laboratory medicine *(16)* and is a reference laboratory of the Cholesterol Reference Method Laboratory Network *(17)*.

DATA ANALYSIS

Calculation of CV_I estimates was performed using CV-ANOVA, an ANOVA method when data are first transformed using the CV transformation *(18)*. CV-ANOVA is a nonparametric procedure that has been shown to be a relatively robust, largely distribution-independent procedure for estimating analytical CV (CV_A) and CV_I in 3-level nested random models *(18)*. Before CV-ANOVA, assessment of outliers, variance homogeneity, normality, and steady state were performed as detailed below, with outlier identification and removal performed for replicates and samples on the CV-transformed data. Homogeneity of CV_A (between-replicates) was examined using the Bartlett test *(19)* and of CV_I by the Cochran test *(20)*. The Shapiro–Wilk test was used to verify the normality of the residuals *(21, 22)*. To examine whether participants were in steady state, linear regression on the 10 pooled mean group sample concentrations was performed for each measurand. Participants were considered in steady state if the 95% CI of the slope of the regression line included 0. For measurands where trends were identified, data were adjusted to account for this by applying the inverse of the regression formula before CV-ANOVA. Larger individual systematic changes were identified by the homogeneity test of the CV_I (Cochran test) *(20)*. CV_G was estimated by ANOVA on natural log-transformed data after applying the Dixon *q*-test *(23)* to detect outliers between individuals, and the Shapiro–Wilk test to verify the normality assumption *(22)*. For total and direct bilirubin, results from individuals with total bilirubin >1.0 mg/dL for women and >1.1 mg/dL for men, i.e., a presumable Gilbert disease diagnosis, were excluded before calculation of CV_G.

For all measurands, components of BV were estimated for men and women separately. To evaluate differences in mean concentrations between participants from the different countries or between premenopausal and postmenopausal women, data were visually inspected. If the inspection revealed possible differences, BV components were estimated for the relevant subgroups; in the case of women, the arbitrary cutoff of 50 years was used to discriminate between likely premenopausal and postmenopausal women. The 95% CIs for BV estimates were calculated as described by Sahai *(24)*, and the lack of overlap of the 95% CI of the individual CV_I and CV_G estimates was used to indicate significant differences between subgroups.

To calculate APS and RCV, the overall BV estimate was used unless estimates were significantly different between the subgroups of all men and women <50 years, in which case the lowest BV estimate was applied. Also, if mean concentrations between the subgroups all men and women <50 years were considered significantly different as judged by the lack of overlap of the 95% CIs, the lower of the 2 subgroup CV_G estimates was applied. Estimates achieved in women >50 years were not used for APS calculations owing to the small number of participants in this group. APS for the analytical imprecision (CV_{APS}) and analytical bias (B_{APS}) were calculated according to:

$$CV_{APS} = 0.5CV_I$$

$$B_{APS} = 0.25(CV_I^2 + CV_G^2)^{0.5}$$

RCVs were identified for an increase and a decrease in the measurand using the log normal approach delivering asymmetric values for rise and fall *(25)* as shown below:

$$SD_{A,log}^2 = \log_e(CV_A^2 + 1)$$

$$SD_{I,log}^2 = \log_e(CV_I^2 + 1)$$

$$SD^* = \sqrt{SD_{A,log}^2 + SD_{I,log}^2}$$

$$RCV\% = 100\% \times e^{((\pm Z_\alpha \times \sqrt{2} \times SD^*) - 1)}$$

where $SD_{A,log}$ is the analytical SD based on the back-log transformation of CV_A estimates from duplicate measurement of study samples from all participants; the $SD_{I,log}$, the within-subject SD based on the CV_I estimates (in CV%); and the SD*, the combination of the 2 *(18)*, for a $Z_\alpha = 1.65$ for the probability level of significant unidirectional change set at 95%. To assess the performance of the RCV, changes in measurands in consecutive samples for all the included study participants were determined. This was performed by estimating the percent change from sample 1 to sample 2, sample 2 to sample 3, etc. for each participant, in total 9 week-to-week changes for each of the 91 participants if they had 10 samples included. Thereafter, the percentage of differences exceeding the RCV (increase, decrease) was determined for each measurand. RCVs based on CV_I estimates from the online 2014 BV database *(7)* using the CV_A from duplicate analysis of the EuBIVAS samples were assessed in the same manner. The II was calculated for each measurand using the overall CV_I and CV_G estimates and the formula defined by Harris *(26)*:

$$II = \frac{\sqrt{CV_A^2 + CV_I^2}}{CV_G}$$

Data analyses were performed using Excel 2010, XLSTAT, statistical software for Excel *(27)*, and R 3.4.3, tidyverse version 1.2.1 *(28)*.

Results

The median number of participants per center was 15 (range, 12–19) (see Table 1 in the online Data Supplement). Participants were generally physically active, and

approximately 3% were regular smokers *(9)*. Their median BMI was 22.5 kg/m^2 (range, 17.6–32.5 kg/m^2) (see Table 1 in the online Data Supplement). Assessment of variance homogeneity for CV_I led to exclusion of 3 participants for IP, 2 participants for Na, triglycerides, and glucose, and 1 participant for total cholesterol, HDL cholesterol, total bilirubin, and direct bilirubin, respectively (Table 1; see also Table 3 in the online Data Supplement). With a limited number of exceptions, exclusions represented a single measurand from single individuals (see Table 3 in the online Data Supplement). For the total study population, the median number of results excluded for all measurands owing to outlier and homogeneity variance testing was 1.7% (range, 0%–5.6%) (see Table 3 in the online Data Supplement). The highest number of excluded results for the overall group was delivered for IP at 5.6% and total cholesterol at 3.4% of the total, leaving 1589 (IP) and 1640 results (total cholesterol) to estimate the CV_I. A significant negative trend in the measurand concentration for all participants or subgroups related to sex and/or age was observed for total cholesterol (all study participants and male participants), LDL cholesterol (all study participants, all male participants, and female participants <50 years), and for Cl, a positive trend (all study participants). Differences in mean concentrations between men and women were apparent for all measurands except K, Cl, and Mg (Table 1). Additionally, there were differences in mean measurand concentrations between women <50 and >50 years of age for lipid markers and urea (Table 1 and Figs. 1–3; see also Figs. 1–13 in the online Data Supplement). No differences in mean concentrations between participants from the different countries were evident (data not shown).

CV_I estimates for all measurands except urea and LDL cholesterol were lower than the estimates available in the online 2014 BV database (Table 1) *(7)*, with implications for the corresponding APS and RCV (Table 2). Women had significantly higher CV_I estimates of uric acid than men, and women <50 years had significantly lower and higher CV_I estimates for triglycerides and urea, respectively, than men (Table 1). The CV_I estimates for total cholesterol and LDL cholesterol were significantly higher in women <50 years than in women >50 years of age. The II was ≤0.6 for all measurands except for electrolytes exclusive of Na (Table 3). The EuBIVAS-based RCV identified, on average, 5% of differences as exceeding the RCV, ranging from 4.2% (LDL cholesterol) to 7.5% (triglycerides) (Table 3). For RCV based on the online 2014 BV estimates, on average, 4% of differences were identified, ranging from 0.8% (direct bilirubin) to 8.5% (urea) (Table 3). When applied to the subgroups men/women or men/women <50 years/women >50 years as applicable, RCV based on global EuBIVAS estimates was closer to achieving the 5% goal than RCV based on the online estimates (see Table 4 in the online Data Supplement). Analysis of reference materials was within the expanded uncertainty limits for all relevant measurands (see Table 5 in the online Data Supplement). Analysis of Bio-Rad internal control materials provided CV_A estimates slightly lower for electrolytes and slightly higher for lipids and glucose (see Table 6 in the online Data Supplement) than those delivered by CV-ANOVA based on results of duplicate measurements of study samples (Table 1). Based on assessment of internal quality control materials, there were no systematic trends in the analyses during the study period.

Discussion

ESTIMATES OF BV

The EuBIVAS is highly powered *(29)* and has been rigorously executed in terms of control of the preanalytical and analytical phases, with study design and data handling described in detail *(9)*. Thus, the EuBIVAS estimates are well characterized, providing a level of understanding and confidence around the data to potential users. Comparisons of the CV_I estimates obtained from the EuBIVAS with those in the online 2014 BV database indicate, except for urea and LDL cholesterol, that all are lower *(7)*. The online estimates are not accompanied by measures of uncertainty *(7)*, which is problematic when attempting to deliver a direct objective comparison of these historic data of variable quality with the new EuBIVAS data. However, for all electrolytes and lipid components, except for triglycerides and LDL cholesterol, the online CV_I values were higher than the upper limits of the 95% CIs of the EuBIVAS estimates (Table 1). The studies fulfilling the inclusion criteria for the online 2014 BV database vary in number and quality for the different measurands *(30)*. For total cholesterol, the online estimate represents the median of 46 different studies. It might be expected that so many studies would enable identification of a point estimate of the CV_I that reflects the actual value. However, study designs are heterogeneous with variations in study population, sampling intervals, and applied statistical methods, all of which affect the veracity of the BV estimates. Therefore, confidence in the veracity of the EuBIVAS should follow from the fact that it was designed and executed to fulfill all Biological Variation Data Critical Appraisal Checklist (BIVAC) quality items that are required for production of valid BV data *(31)*. Many of the historical studies fail to meet this new quality standard. For example, a review of the 23 studies included in the online 2014 BV database for serum alanine aminotransferase (ALT) showed that only 2 of these were BIVAC compliant and had been performed in similar populations with comparable sampling intervals *(31)*. Metaanalysis of these 2 studies delivered a significantly lower CV_I estimate for ALT than the estimate in the online 2014 BV database, whereas the

Table 1. Within- and between-subject BV estimates for electrolytes, lipids, proteins, and glucose with 95% CIs, accompanied by the corresponding BV estimates in the online 2014 BV database.[a]

	Number of individuals	Total number of results	Mean number of samples/ individuals	Mean number of replicates/ samples	Mean value (95% CI)[b]	CV_A% (95% CI)[c]	CV_I % (95% CI)	CV_G% (95% CI)	Online 2014 BV database[d] CV_I%	Online 2014 BV database[d] CV_G%
Na, mmol/L										
All	89	1664	9.38	1.99	142.7[b] (142.6–142.8)	0.40 (0.38–0.42)	**0.53** (0.50–0.57)	1.21 (1.06–1.43)	0.6	0.7
M	38	699	9.26	1.97	143.8 (143.6–143.9)		0.50 (0.45–0.55)	**0.95** (0.78–1.25)		
F	53	991	9.38	1.99	141.9[b] (141.8–142.1)		0.54 (0.49–0.59)	1.08 (0.90–1.35)		
K, mmol/L										
All	91	1706	9.40	1.99	4.28 (4.27–4.29)	0.41 (0.40–0.44)	**3.92** (3.73–4.13)	**4.08** (3.61–4.98)	4.6	5.6
M	38	707	9.34	1.98	4.29 (4.27–4.31)		3.93 (3.65–4.27)	4.06 (3.21–5.35)		
F	53	1003	9.47	2.00	4.27 (4.26–4.29)		4.00 (3.76–4.28)	4.12 (3.56–5.44)		
Cl, mmol/L										
All	91	1713	9.45	1.98	105.6 (105.5–105.7)	0.40 (0.38–0.42)	**0.98** (0.93–1.04)	**1.34** (1.17–1.60)	1.2	1.5
M	38	708	9.39	1.97	105.5 (105.3–105.6)		0.96 (0.89–1.06)	1.34 (1.07–1.76)		
F	53	1005	9.49	2.00	105.7 (105.6–105.9)		0.99 (0.92–1.06)	1.35 (1.13–1.71)		
Ca, mg/dL										
All	91	1700	9.38	1.98	8.97[b] (8.96–8.99)	0.85 (0.81–0.89)	**1.81** (1.72–1.92)	2.73 (2.36–3.22)	2.1	2.5
M	38	708	9.39	1.97	9.06 (9.03–9.08)		1.74 (1.61–1.91)	**2.57** (2.06–3.35)		
F	53	1000	9.45	1.99	8.92[b] (8.89–8.94)		1.96 (1.83–2.12)	2.64 (2.20–3.33)		
Mg, mg/dL										
All	91	1713	9.46	1.98	2.01 (2.01–2.04)	2.48 (2.37–2.61)	**2.88** (2.68–3.09)	**5.79** (5.06–6.86)	3.6	6.4
M	38	708	9.39	1.97	2.03 (2.01–2.04)		2.64 (2.35–2.98)	5.28 (4.33–6.99)		
F	53	1005	9.51	1.99	2.01 (2.00–2.02)		3.04 (2.77–3.32)	6.16 (5.14–7.69)		
IP, mg/dL										
All	88	1589	9.26	1.94	3.62 (3.4–3.65)	2.87 (2.74–3.03)	**7.67** (7.24–8.09)	10.5 (9.2–12.6)	8.15	10.8
M	37	645	8.95	1.90	3.49 (3.46–3.56)		8.64 (7.89–9.39)	**10.2** (8.4–14.0)		
F	53	993	9.42	1.98	3.68 (3.65–3.71)		7.66 (7.15–8.21)	10.2 (8.3–12.6)		
Total cholesterol, mg/dL										
All	90	1640	9.22	1.95	191.0 (189.0–192.9)	0.98 (0.93–1.03)	**5.18** (4.92–5.46)	17.4 (15.1–20.4)	5.95	15.3
M	38	686	9.18	1.93	191.8 (188.7–192.6)		5.17 (4.80–5.64)	17.7 (14.6–23.2)		
F<50	43	804	9.42	1.97	176.7 (174.7–179.0)		6.11 (5.70–6.61)	**13.4** (10.9–17.1)		
F>50	9	172	9.56	2.00	232.0 (225.0–235.8)		4.09 (3.52–4.86)	14.6 (9.8–28.5)		

Continued on page 1385

Table 1. Within- and between-subject BV estimates for electrolytes, lipids, proteins, and glucose with 95% CIs, accompanied by the corresponding BV estimates in the online 2014 BV database.[a] *(Continued from page 1384)*

	Number of individuals	Total number of results	Mean number of samples/ individuals	Mean number of replicates/ samples	Mean value (95% CI)[b]	CV_A% (95% CI)[c]	CV_I % (95% CI)	CV_G% (95% CI)	Online 2014 BV database[d] CV_I%	Online 2014 BV database[d] CV_G%
HDL cholesterol, mg/dL										
All	90	1642	9.24	1.95	63.8 (63.0–64.5)	0.56 (0.54–0.59)	**5.67** (5.41–5.99)	25.1 (21.5–29.3)	7.3	21.2
M	38	673	9.03	1.93	52.9[b] (51.8–53.7)		5.66 (5.24–6.15)	**20.4** (16.5–26.5)		
F<50	43	804	9.42	1.97	68.8 (67.6–69.9)		6.24 (5.84–6.76)	21.1 (17.1–26.8)		
F>50	10	193	9.80	1.94	79.6 (78.1–81.2)		5.39 (4.67–6.29)	11.4 (7.7–21.0)		
LDL cholesterol, mg/dL										
All	88	1618	9.31	1.95	106.4 (104.7–108.2)	1.77 (1.68–1.86)	**8.46** (8.05–8.94)	28.2 (24.4–33.0)	7.8	20.4
M	36	654	9.25	1.93	114.8 (112.0–118.6)		8.55 (7.89–9.30)	27.0 (21.9–35.4)		
F<50	42	783	9.38	1.98	92.2 (90.4–94.0)		9.24 (8.59–9.99)	**22.5** (18.2–28.5)		
F>50	10	193	9.80	1.94	133.8 (129.1–138.6)		6.82 (6.05–8.18)	21.6 (14.7–39.5)		
Non-HDL cholesterol, mg/dL										
All	89	1594	9.06	1.96	127.0 (125.1–128.9)	1.47 (1.41–1.55)	**6.88** (6.53–7.27)	26.4 (22.9–30.9)	–	–
M	38	659	8.82	1.94	138.5 (135.3–141.6)		6.41 (5.96–7.02)	25.4 (20.9–33.2)		
F<50	43	800	9.37	1.97	108.0 (105.9–110.1)		8.33 (7.75–9.00)	**23.5** (19.2–29.7)		
F>50	9	172	9.56	2.00	150.2 (144.6–155.7)		5.11 (4.40–6.09)	22.0 (14.8–42.2)		
Triglycerides, mg/dL										
All	89	1667	9.40	1.98	87.6[b] (85.0–89.4)	1.80 (1.72–1.89)	19.8 (18.9–20.9)	40.3 (34.6–48.1)	19.9	32.7
M	37	684	9.32	1.97	104.5 (100.0–108.9)		22.7 (21.0–24.6)	42.9 (34.3–58.3)		
F<50	43	813	9.47	2.00	77.0[b] (74.3–79.7)		**19.0** (17.8–20.6)	**38.5** (31.1–50.1)		
F>50	10	195	9.80	1.98	85.9[b] (81.4–90.3)		19.6 (17.2–23.2)	25.0 (16.3–48.0)		
Glucose, mg/dL										
All	89	1658	9.35	1.99	83.3 (82.8–83.7)	0.58 (0.56–0.61)	**4.7** (4.4–4.9)	8.1 (7.1–9.7)	5.6	7.5
M	38	699	9.26	1.97	85.7 (85.0–86.4)		4.6 (4.3–5.0)	8.6 (7.0–11.3)		
F	51	956	9.39	1.99	81.5 (81.0–81.9)		4.6 (4.4–5.0)	**7.1** (6.0–9.0)		
Urea, mg/dL										
All	91	1660	9.20	1.97	32.3[b] (31.9–33.0)	2.32 (2.21–2.44)	14.1 (13.4–14.8)	22.5 (19.8–27.0)	12.1	18.7
M	38	641	8.55	1.97	36.0 (35.3–36.8)		**12.5** (11.5–13.6)	**19.4** (15.5–25.2)		
F<50	43	789	9.21	1.99	28.0[b] (27.4–28.5)		15.7 (14.6–16.9)	19.7 (15.9–25.4)		
F>50	10	195	9.80	1.98	36.8 (35.7–37.9)		12.9 (11.3–15.3)	15.9 (10.4–29.1)		

Continued on page 1386

Table 1. Within- and between-subject BV estimates for electrolytes, lipids, proteins, and glucose with 95% CIs, accompanied by the corresponding BV estimates in the online 2014 BV database.[a] *(Continued from page 1385)*

	Number of individuals	Total number of results	Mean number of samples/ individuals	Mean number of replicates/ samples	Mean value (95% CI)[b]	CV_A% (95% CI)[c]	CV_I % (95% CI)	CV_G% (95% CI)	Online 2014 BV database[d] CV_I%	Online 2014 BV database[d] CV_G%
Uric acid, mg/dL										
All	91	1687	9.31	1.98	4.81[b] (4.75–4.88)	0.86 (0.82–0.90)	8.32 (7.9–8.8)	23.6 (20.4–27.5)	8.6	17.5
M	38	679	9.00	1.97	5.68 (5.6–5.77)		**7.7** (7.1–8.4)	**14.9** (12.1–19.4)		
F	52	974	9.38	1.99	4.21 (4.14–4.28)		9.2 (8.7–9.9)	19.1 (16.0–24.1)		
Total protein, g/dL										
All	91	1694	9.37	1.97	7.24 (7.22–7.26)	0.97 (0.93–1.02)	**2.6** (2.5–2.7)	4.6 (4.0–5.4)	2.75	4.7
M	38	703	9.37	1.95	7.35 (7.32–7.38)		2.5 (2.3–2.7)	**4.2** (3.4–5.5)		
F	53	1004	9.49	1.99	7.22 (7.20–7.25)		2.9 (2.7–3.1)	4.5 (3.7–5.6)		
Total bilirubin, mg/dL										
All	90	1683	9.47	1.95	0.69 (0.66–0.70)	2.98 (2.85–3.13)	**20.9** (19.9–22.0)	26.6 (22.5–31.6)	21.8	28.4
M	37	682	9.38	1.93	0.81 (0.77–0.84)		21.7 (20.1–23.6)	25.3 (19.6–32.2)		
F	53	1003	9.55	1.97	0.60 (0.58–0.62)		21.6 (20.2–23.1)	**24.4** (20.1–31.3)		
Direct bilirubin, mg/dL										
All	90	1681	9.46	1.95	0.23[b] (0.23–0.24)	6.05 (5.77–6.35)	**20.9** (19.8–22.0)	31.1 (26.4–36.9)	36.8	43.2
M	37	681	9.35	1.94	0.28 (0.27–0.29)		20.9 (19.3–22.8)	30.1 (23.5–40.5)		
F	53	1003	9.53	1.97	0.21[b] (0.20–0.21)		21.0 (19.6–22.6)	**27.8** (23.2–35.9)		

[a] Results are partitioned between men (M) and women (F) or women below (F<50) and above the age of 50 years (F>50). Results in bold indicate that these estimates have been used for calculations of APSs and RCVs (see Table 2).
[b] Normality assumption was not fulfilled based on the Shapiro–Wilk test on normality of the residuals ($P > 0.01$).
[c] CV_A estimates were based on CV–ANOVA of duplicate analysis of all study samples.
[d] The online 2014 biological variation database – http://www.westgard.com/biodatabase1.htm#1.

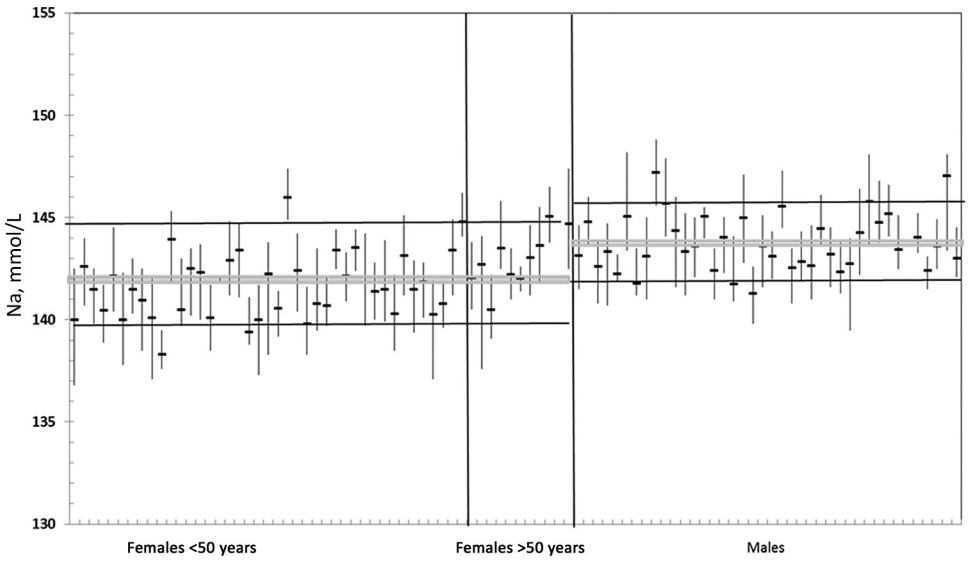

Fig. 1. Median values and range for sodium (Na) for each participant after exclusion of outliers, ordered by sex and age.
Continuous lines indicate the 95% CI of the mean and the 5th and 95th percentiles for women and men.

recently published EuBIVAS ALT estimate was even lower *(10)*. Although differences between the online and EuBIVAS estimates for measurands included in the current publication were smaller than for ALT, this highlights a general need for high-quality BV studies and the application of appropriate methods when pooling estimates from studies of different quality and study design. Metaanalysis of BIVAC-compliant studies offers different approaches to this effect, with a higher weight being given to studies of the highest quality *(31)*. The largest differences between the online and EuBIVAS estimates were observed for HDL cholesterol, glucose, urea, and direct bilirubin. LDL cholesterol and urea were the only measurands for which the overall EuBIVAS es-

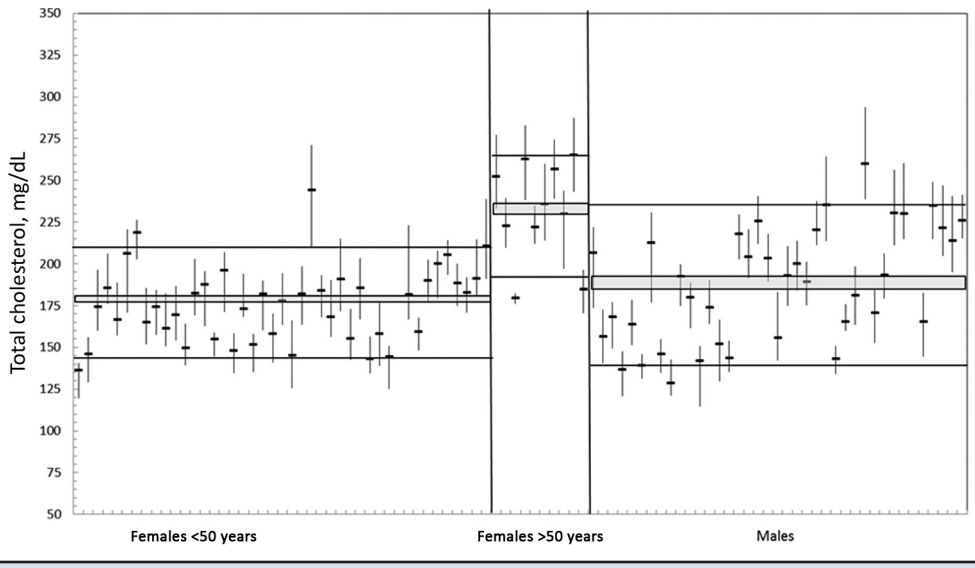

Fig. 2. Median values and range for total cholesterol for each participant after exclusion of outliers, ordered by sex and age.
Continuous lines indicate the 95% CI of the mean and the 5th and 95th percentiles for women <50 years, women >50 years of age, and men.

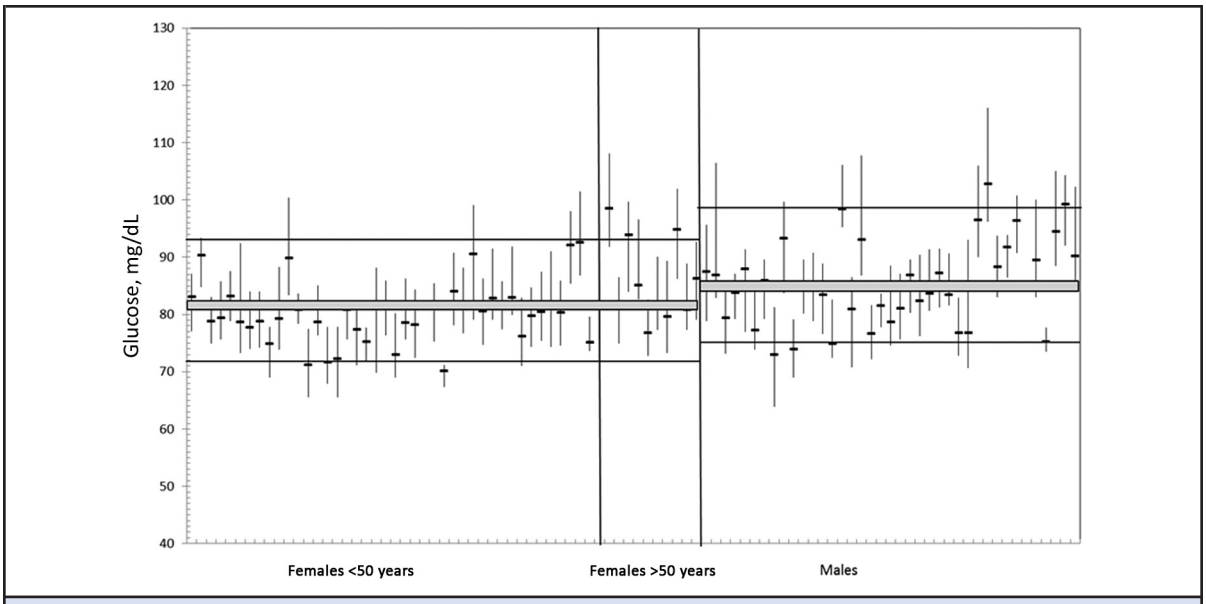

Fig. 3. Median values and range for glucose for each participant after exclusion of outliers, ordered by sex and age.
Continuous lines indicate the 95% CI of the mean and the 5th and 95th percentiles for women and men.

timates were higher than those online *(7)*, with the latter being based on estimates from 6 and 20 different studies, respectively. For urea, the EuBIVAS CV_I estimate was significantly lower in men, with the estimate of this subgroup being similar to that online. Thus, it is possible, depending on the populations in which previous studies have been performed, that differences in study populations may have contributed to this finding. The CV_I estimate for direct bilirubin was about half the online estimate reported for conjugated bilirubin, which was based on 2 older studies *(32, 33)*. In our study, the CV_I estimates achieved for total and direct bilirubin were close to identical. There are differences between bilirubin methods as to the degree of interference from hemolysis and lipemia *(34)* and whether bilirubin covalently bound to albumin is included in the measurement. Generally, it might be expected that generational changes in analytical methods might influence BV estimates because changes in analytical specificity may affect the measurand that is measured and the accuracy of results.

Seasonal variations in cholesterol concentrations are well known *(35, 36)*. In our study, with sampling taking place during the spring, significant negative trends in concentrations were observed for cholesterol (all participants and male participants) and LDL cholesterol (all, male, and female participants <50 years) during the study period. Additionally, although not significant by the set criteria, decreasing trends in concentrations were also apparent for HDL cholesterol and triglycerides, as well as the other subgroups for total and LDL cholesterol. However, the trend correction had little impact on the CV_I estimate, as exemplified for total cholesterol, for which it reduced the CV_I estimate from 5.3% to 5.1%. Sex differences were observed for total cholesterol, triglycerides, and urea between all men and women <50 years, and in the case of uric acid, all women. The estimates in the online 2014 BV are not sex-stratified. A recent study reported sex-related differences in CV_I estimates in an elderly population for several measurands, including triglycerides, uric acid, and urea *(37)*. The study did not assess sex differences in the young adult control group, and because measures of uncertainty are lacking, these comparisons cannot be made. The same study also noted differences between younger and elderly study participants for many measurands, but their patient group was far older (range, 80–92 years) than ours, and a different approach for evaluating differences was applied. In our study, CV_I estimates were found to be significantly lower only for total and LDL cholesterol in women >50 years of age when compared with those <50 years. However, it is worth noting that the subgroup of women >50 years of age consists of only 10 or, in the case of total cholesterol, 9 participants (see Table 3 in the online Data Supplement). Smaller study populations produce more uncertain estimates *(29)*. Additionally, excluding many data points after outlier and variance homogeneity testing, such as for IP, will also make the final estimate less representative of the population from which it is derived. For measurands for which it is necessary to exclude many data points because of outliers and variance

Table 2. II, RCV, EuBIVAS-based APS for imprecision (CV_{APS}) and bias (B_{APS}) and desirable APS for imprecision and bias as reported in the online 2014 BV database.

	EuBIVAS				Online 2014 BV database[b]	
Measurand	II	RCV, %[a] decrease; increase	CV_{APS}, %[a]	B_{APS}, %[a]	CV_{APS}, %	B_{APS}, %
Na	0.5	−1.5; 1.6	0.3	0.3	0.3	0.2
K	1.0	−8.8; 9.6	2.0	1.4	2.3	1.8
Cl	0.8	−2.4; 2.5	0.5	0.4	0.6	0.5
Ca	0.7	−4.5; 4.8	0.9	0.8	1.1	0.8
Mg	0.7	−8.5; 9.2	1.4	1.6	1.8	1.8
IP	0.8	−17.3; 21.0	3.8	3.2	4.1	3.4
Total cholesterol	0.3	−11.5; 13.0	2.6	3.6	3.0	4.1
HDL cholesterol	0.2	−12.4; 14.6	2.8	5.3	3.7	5.6
LDL cholesterol	0.3	−18.2; 22.2	4.2	6.1	3.9	5.5
Non-HDL cholesterol	0.3	−15.1; 17.8	3.4	6.1	–	–
Triglycerides	0.5	−35.6; 55.3	9.5	10.7	10.0	9.6
Glucose	0.6	−10.4; 11.6	2.4	2.1	2.8	2.3
Urea	0.6	−25.5; 34.2	6.4	5.8	6.1	5.6
Uric acid	0.4	−16.5; 19.7	3.9	4.2	4.3	4.9
Total protein	0.6	−6.3; 6.7	1.3	1.2	1.4	1.4
Total bilirubin	0.5	−38.5; 62.6	10.5	11.0	10.9	9.0
Direct bilirubin	0.5	−39.4; 65.1	10.5	11.4	18.4	14.2

[a] Based on bolded EuBIVAS estimates in Table 1.
[b] The online 2014 biological variation database – http://www.westgard.com/biodatabase1.htm#1.

heterogeneity issues, it is important to check the study population for subgroups and/or to consider at what point the data set will no longer be representative, with implications for the relevance of the BV estimates and associated measures. The graphical illustrations of mean measurand concentrations of participants when ordered by age and sex (Figs. 1–3; see also Figs. 1–13 in the online Data Supplement) may indicate additional subgroups for some measurands. However, these would consist of only a small number of individuals, which would affect the reliability and applicability of results. Therefore, additional subgroup analysis was not performed.

Many of the CV_G estimates, especially for lipids, achieved in our study were higher than the estimates available in the online 2014 BV database *(7)*. However, when assessing the sex-stratified CV_G estimates, EuBIVAS estimates for, e.g., total cholesterol, HDL cholesterol, and glucose for subgroups were lower than the online estimates (Table 1). For electrolytes Mg, Cl, Ca, and IP, the overall EuBIVAS CV_G estimates were lower than those online, but the online estimates were within the CIs of the EuBIVAS CV_G estimates. Opposite situations were observed for Na and K, whose CV_G results appear to be significantly higher and lower than the historical data, respectively (Table 1). Differences in the CV_G may be multifactorial in causality. For Na, the estimates in the online 2014 BV database represent the median of 21 studies that may have been performed in smaller or potentially more homogeneous populations than the EuBIVAS, which encompasses 5 different countries. However, this should apply also to K. For K, the lower CV_I and CV_G may depend on the very well-controlled preanalytical phase *(9)*, avoiding hemolysis and the short time interval between blood drawing and centrifugation. For most of the included measurands, differences in the mean concentrations between men and women were observed (Table 1). Most of these were expected, except for the 1.9-mmol/L difference for Na. However, sex differences in median values of around 1 mmol/L have been reported for Na in reference interval studies, especially within populations between the ages of 20 and 50 years *(38–42)*. Additionally, the EuBIVAS was not designed to deliver conventional population-based reference intervals. The II was ≤0.6 for most measurands except electrolytes, indicating that reference intervals have low utility for these measurands (Table 2)

Table 3. Performance of RCVs based on within-subject BV estimates from the EuBIVAS and the online 2014 BV database.[a]

	EuBIVAS RCV		Online 2014 BV database RCV[b]	
Measurand	Decrease, %	Increase, %	Decrease, %	Increase, %
Na	5.2	5.3	4.4	4.0
K	5.3	6.1	3.3	3.8
Ca	6.1	4.3	3.6	3.0
Cl	4.5	4.4	1.9	2.2
Mg	4.9	5.2	2.6	2.9
IP	6.2	6.2	5.2	5.3
Total cholesterol	5.9	5.5	4.4	3.8
HDL cholesterol	5.6	5.1	2.7	1.7
LDL cholesterol	4.5	4.2	5.3	5.0
Non-HDL cholesterol	4.4	6.0	–	–
Triglycerides	6.3	7.5	6.3	7.4
Glucose	6.2	6.6	4.0	4.6
Urea	5.9	5.1	8.5	8.2
Uric acid	5.3	4.8	4.9	4.5
Total protein	5.8	4.8	4.5	4.5
Total bilirubin	6.0	6.0	5.7	5.7
Direct bilirubin	6.4	6.0	0.8	1.2

[a] For best performance, the RCV should identify 5% of differences in measurand concentrations between consecutive week-to-week measurements of EuBIVAS subjects as exceeding the RCV.
[b] The online 2014 biological variation database – http://www.westgard.com/biodatabase1.htm#1.

(26). For example, the CV_G was substantially lower for triglycerides in subgroups such as women >50 years, delivering an II of 0.8, as compared with 0.5 for the overall group. This indicates that for some of these measurands, sex- and age-partitioned reference intervals may be of value *(43)*.

APPLICATION OF BV DATA FOR APS

The BV model for setting APS is 1 of 3 proposed models *(4)*. If APSs based on BV are met, the analytical variability will be small compared with the BV, reducing the analytical "noise." Although differences in the CV_I estimates for electrolytes obtained from this study and those available online are small, the use of EuBIVAS estimates will further increase the gap between the desirable performances and the available analytical quality. For electrolytes, EuBIVAS-derived APS will deliver strict requirements. The CV_A estimates determined from duplicate measurements (Table 1) were higher than the EuBIVAS-based APS for imprecision (Table 2). This indicates that current analytical methods may fail the APS for imprecision, especially when considering that duplicate measurements with all samples for the same participant performed in the same run are likely to deliver a lower CV_A estimate than what can be expected from long-term quality control analysis. Thus, basing the APS on high-quality BV data such as the EuBIVAS presents a challenge to the in vitro diagnostic industry, which would require improving the analytical imprecision for electrolyte measurands, especially for Na and Mg.

For lipids and glucose in a diagnostic setting, clinical outcome models have been suggested as the best approach for setting APS if available *(4)*. Outcome models are typically best used for specific medical situations, detailed in, for example, clinical guidelines. When using APS in a medical laboratory or for external quality assessment/proficiency testing, this is challenging because a variety of different medical scenarios are possible or relevant. The alternatives then remain to choose APS from an outcome model based on the most common clinical situation or the clinical setting that gives rise to the strictest APS. As of today, data for clinical outcome models are scarce; therefore, the BV model may presently be the best approach. Generally, EuBIVAS-based APSs are lower than APS based on historical estimates for lipids and most of the other measurands included in our study. The CV_A estimates for imprecision obtained in our study are clearly lower than the CV_{APS} for most measurands except

electrolytes (Tables 1 and 2), indicating that the analytical system applied in our study may be within these requirements. However, considering our strict study protocol with CV_A estimates being based on duplicate analysis of all samples used in the study to deliver the BV data, a routine setting with long-term analysis may give rise to CV_A estimates exceeding the EuBIVAS APS. The APSs detailed in Table 2 are based on the lowest BV estimate when estimates were significantly different between subgroups and/or the lowest CV_G estimate if subgroup mean concentrations were different. Furthermore, formulae used in our study to calculate APS are termed "desirable," and formulae delivering less strict (minimum) and more strict (optimum) APS are also available. Thus, it is important when setting APS to consider which type of population the laboratory serves and how strict an approach is sought.

APPLICATION OF BV DATA FOR RCV

The lower CV_I estimates identified in our study for most included measurands have implications for interpretation of changes in concentrations over time and the application of RCVs. The RCVs provided in Table 2 are based on the CV_A from duplicate measurements of samples from EuBIVAS participants, as these were the data available in our study. Therefore, our RCVs are likely to be lower than when based on relevant CV_A estimates, i.e., from long-term quality control data. For use in the routine laboratory, local RCVs calculated using the laboratory's own estimates of CV_A must be provided as appropriate. Because we identified sex- and age-related differences in CV_I estimates for, e.g., lipid components, sex- and age-specific RCVs must be considered. This is relevant for monitoring total cholesterol concentrations, for which a significantly lower CV_I estimate was obtained for postmenopausal than premenopausal women. This delivers different RCVs: An RCV for an increase in cholesterol concentration would be 15.5% for a premenopausal woman and 10.3% for a postmenopausal woman. The RCVs in our study are calculated with $Z_\alpha = 1.65$, i.e., 1-sided change in the form of either an increase or a decrease for the probability level of significant change set at 95%. This means that in a situation with 2 consecutive measurements in a healthy individual, these RCVs should, in parallel to a 1-sided 95% reference interval, identify 5% of changes in concentrations greater than the normal expected variation. Other RCVs would be more relevant depending on the clinical question and which probability level is wanted. In our study, RCVs are calculated applying the ln-RCV approach providing asymmetrical limits (Table 2) *(25)*. The ln-RCV is generally the recommended method for calculating RCV because percentage differences between measurements have a skewed distribution like the ln-normal distribution *(25)*. This approach is also superior to the classical RCV calculation, defined for bidirectional changes as $Z_\alpha\sqrt{2}(CV_I^2 + CV_A^2)^{0.5}$, in that it is not possible to derive paradoxical decreases of >100%. For best performance, the ln-RCV approach assumes log-normal distributed data or estimates of $CV_I < 12\%$ *(18, 25)*. For several measurands in our study, the normality assumption was not achieved for the overall and/or subgroup data, as detailed in Table 1. Typically, the normality assumption will be more easily achieved with a small data set, whereas for larger data sets, the normality assumption is less important *(21)*. CV_I estimates for triglycerides, urea, and direct bilirubin were >12%. Thus, to verify the performance of the EuBIVAS-delivered RCVs, the real concentration changes from week to week for all 91 study participants were reviewed. This showed that the EuBIVAS-based RCVs generally identified close to 5% of differences as exceeding the RCV, i.e., in line with what they were designed to deliver (Table 3; see also Table 4 in the online Data Supplement). RCVs based on estimates from the online 2014 BV database typically identified fewer differences as exceeding the RCV (Table 3; see also Table 4 in the online Data Supplement). For Cl, HDL cholesterol, Mg, and direct bilirubin, <3% were identified. Assuming that the EuBIVAS study population is generally representative, this indicates that RCVs based on the online estimates would, for some measurands, also miss similarly sized clinically relevant changes and in reality represent RCVs with a higher probability level. That EuBIVAS RCVs performed better than those based on the online estimates is to be expected because their performance was tested on the EuBIVAS data set from which they were derived; nevertheless, it demonstrates the applicability of this type of RCV calculation. Additionally, reviewing the observed percent of differences falling outside the RCV sheds light on the distribution and homogeneity of the data set and the suitability of these RCVs in clinical practice. Adequate interpretation of changes in concentrations of electrolytes, lipids, and glucose is highly important because these markers play a central role as diagnostic markers, risk markers, and in providing a basis for therapeutic decisions. Thus, the availability of robust CV_I estimates and, when relevant, sex- and age-specific RCVs may have direct impact on patient care and follow-up. Furthermore, if basing APS on EuBIVAS estimates, this may deliver lower specifications for analytical imprecision, which as a next step will lead to lower RCV. The impact of this on patient outcomes remains to be assessed.

STUDY LIMITATIONS

The analyses were performed using only 1 manufacturer's reagents. Reagents from different manufacturers may perform differently, but it is unlikely that this will affect the BV estimates if the measurands are the same. LDL cholesterol was not measured but calculated by Friedewald formulae

(11). Although all the analyses of the same participant were performed in the same run and any contribution from within-run analytical variability on estimates of BV has been eliminated by the study design and statistical approach, the different participants were measured on different days.

Conclusions

In this study, estimates of CV_I and CV_G for electrolytes, lipids, urea, uric acid, total protein, total bilirubin, direct bilirubin, and glucose were obtained by analyzing sera from a multinational cohort consisting of healthy individuals from 5 European countries. The CV_I estimates obtained from the EuBIVAS were homogeneous and, except for urea and LDL cholesterol, lower than the estimates of the online 2014 BV database. Furthermore, sex differences in CV_I estimates were evident for some measurands. These data highlight the applicability of the EuBIVAS BV estimates and that they may be used to determine APS at a global level. This has implications for the delivery of correct diagnosis and monitoring for several clinically important measurands. The more stringent CV_{APS} for several measurands included in our study, which especially for Na and Mg exceeds the current method CV_A performances, entails a challenge for the in vitro diagnostic industry.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: Siemens Healthineers donated all reagents, control materials, and calibrators used for the measurements.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or final approval of manuscript.

Acknowledgments: The authors thank Siemens Healthineers, which allowed us to work on a dedicated Siemens ADVIA 2400 and donated all reagents, control materials, and calibrators used for the measurements. The authors also thank all the participants who donated blood.

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