Letter to the Editor

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Biological variation of serum iron from the European biological variation study (EuBIVAS)

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To the Editor,

Over the last 20 years, the physiology of iron trafficking and metabolism has been thoroughly studied. Because the free molecule is hazardous, intricate regulatory mechanisms have evolved to ensure adequate intestinal absorption, transportation, utilization, and disposal of iron in mammals [1].

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Estimates of within-subject (CV_I) and between-subject (CV_G) biological variation (BV), have several applications in laboratory and clinical medicine, including the assessment of significance of change in serial measurements in an individual (the reference change value; RCV) and the setting of analytical performance specifications (APS) [2]. The BV model for establishing APS is mainly recommended when model 1, the clinical outcome model, is not applicable or data are lacking, for measurands under strict homeostatic control [3]. There are many proteins involved in iron homeostasis by finely regulating its metabolism, and iron is characterized by large within-day variation [4]. APS based on currently available BV data for iron are far larger than the state of the art delivered by today's methods. In this case, APS may be defined as a compromise between BV and state of the art criteria [5]. To aid in this, reliable BV data are required.

The aim of this letter is to present BV data for serum iron derived from European Biological Variation Study (EuBIVAS) [6], established by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Working Group on BV.

Briefly, EuBIVAS involved six European laboratories, enrolling 91 presumably healthy volunteers (38 males, 43 fertile/pre-menopausal and 10 post-menopausal females) [6]. The serum samples drawn weekly for ten consecutive weeks by each laboratory (April – June 2015) were sent frozen on dry ice to San Raffaele Hospital in Milan, Italy, where they were stored at −80 °C until analysis (December 2017–January 2018). All samples from the same subject were analyzed in duplicate within a single run, using an ADVIA 2400 Clinical Chemistry System (Siemens Healthineers), Siemens reagents (Iron_2, ferrozine principle) and calibrators, and Bio-Rad control materials (Liquid Assayed Multiqual level 1, level 2). The protocol was approved by the Institutional Ethical Board/Regional Ethics Committee. CV_I was estimated using CV-ANOVA for all participants, males, females in fertile and menopausal age (< and

>50 years old). Outlier analysis, homogeneity of analytical CV (CV_A) and CV_I, and trend analysis were performed as previously described [7]. CV_G estimates were calculated on natural log-transformed data for which normality assumption was verified, after assessment for outliers between individuals. Mean values and BV estimates between subgroups were considered significantly different if the associated 95% CI did not overlap. RCVs were calculated using the log normal approach [7]. Data analyses were performed using Microsoft Excel 2010 and IBM SPSS statistics, version 23.

To fulfill criteria for variance homogeneity of the data, 3.8% of results were excluded. This included the exclusion of three subjects; one Norwegian male subject (28 years) identified by the Cochran test as an outlier and two Turkish female subjects due to hemoglobin values <110 g/L. No outliers were identified between individuals in any subgroup. In total, 1,590 analytical results were included in the analysis (Table 1). Iron data for the whole population, males and female subgroups were normally distributed and no trends were identified by regression analysis. The Shapiro–Wilk test results indicated that the data, i.e. subjects' mean values, were normally distributed.

The CV_I estimates derived from males (24.9%, 95% CI; 23.2–27.2) and post-menopausal females (25.6%, 95% CI; 22.4–30.1) were similar. The CV_I estimate (38.3%, 35.6–41.4) for fertile age females, however, was significantly higher than those derived from males and post-menopausal females. This suggests that applying an overall CV_I estimate for both males and females in all ages may not be appropriate. Significant differences in iron concentrations and CV_G between males and females were also observed (Table 1). The APS and RCV were calculated using the mean values and BV estimates obtained from males (Table 1). The RCVs were based on CV_A estimate derived in our study from duplicate analysis of study samples. When in clinical practice, each laboratory must calculate its own RCVs using relevant CV_A estimates from their own routine.

The EFLM BV Database includes 16 BV studies on iron in serum and plasma [9]. Only four of these studies [10–13] (Table 2) fulfil the inclusion criteria for meta-analysis to deliver global BV estimates [8], meaning that they were derived from healthy adult subjects with biweekly to monthly samplings. All four were categorized according to Biological Variation Data Critical Appraisal Checklist (BIVAC) [8] with a grade C, indicating lower compliance with the checklist. The following BIVAC quality items (QIs) were not fulfilled, namely, those for steady state (QI 7), outliers (QI 8), normality assumptions (QI 9), and variance homogeneity (QI10). Only one, a recently published study, had assessed subgroups and reported different CV_I estimates in males and females [12]; however, here the 95% CI slightly overlapped (Table 2). The same study [12] was the only that reported CV_G estimates, which overlapped with the EuBIVAS estimates (Table 2).

When including the EuBIVAS in the meta-analysis, the CV_I estimate increased from 20.7% (95% CI; 19.8–27.3) to 27.7% (95% CI; 19.8–28.4), as consequence of the high number of participants in the EuBIVAS and it being fully BIVAC compliant. Only the Cokluk study had previously

Table 1: Within-subject (CV_I) and between-subject (CV_G) biological variation estimates with 95% CIs, analytical performance specifications (APS) for imprecision (CV_{APS}) and bias (B_{APS}) and reference change values (RCV) for serum iron[a].

Iron, µmol/L	Number of individuals	Total number of results	Mean number of samples/ individual	Mean number of replicates/ sample	Mean value (95% CI)	CV_A, % (95% CI)[b]	CV_I, % (95% CI)	CV_G, % (95% CI)	CV_{APS}, %[c]	B_{APS}, %[d]	RCV, % (decrease/ increase)[e]
All subjects	88	1,590	9.16	1.95	16.3 (16.0–16.7)	1.9 (1.8–2.0)	28.0 (26.7–29.6)				
Males	37	673	9.27	1.93	17.9 (17.5–18.4)		**24.9 (23.2–27.2)**	**19.1 (14.5–26.0)**	12.45	7.85	−43.5/ +76.9
Females <50 years	41	758	9.32	1.97	15.5 (14.9–16.0)		38.3[f] (35.6–41.4)	35.3 (27.5–46.9)			−57.7/ +136.1
Females >50 years	10	193	9.80	1.94	15.2 (14.3–16.0)		25.6 (22.4–30.1)	29.2 (18.7–57.3)			−44.0/ +78.5

[a]Results were reported for males, females <50 years (pre-menopausal), and females >50 (post-menopausal) subgroups. Results in bold were used to estimate APS and RCVs. [b]Analytical variation (CV_A) estimates were based on CV-ANOVA of duplicate analysis of all study samples. [c]CV_{APS}=0.50 CV_I. [d]B_{APS}=0.25 $(CV_I^2 + CV_G^2)^{0.5}$. [e]RCV were calculated delivering asymmetric values for rise and fall at the probability level of 95% for significant unidirectional change, applying CV_A estimates based on duplicate measurement of all study samples. [f]To note that CV_I estimates higher than 33% indicate a skewed distribution of the data, while the normality distribution is necessary for CI and RCV to be calculated directly [8].

Table 2: Overview of studies reporting within-subject biological variation (CV_I) and between-subject biological variation (CV_G) data for serum/plasma iron fulfilling the criteria for meta–analysis in the EFLM biological variation database, with their associated biological variation data critical appraisal checklist (BIVAC) grade[a].

Study reference	Population subgroup	BIVAC grade[b]	CV_I % (95% CI)	CV_G % (95% CI)
Carobene A et al. [10]	7 subjects 25–40 years	$C_{10,12}$	**27.6**	NA
	9 subjects 40–60 years	$C_{10,12}$	**24.1**	NA
	10 subjects 78–96 years	$C_{10,12}$	24.1	NA
Dimitri G et al. [11]	5 subjects 25–31 years	$C_{4,8,10,13}$	**22.8** (18.9–28.7)	NA
Cokluk E et al. [12]	21 subjects 18–50 years	$C_{5,8}$	**27.3** (23.7–32.1)	**32.3** (23.5–47.8)
	10 males 18–50 years	$C_{5,8}$	22.0 (18.1–28.2)	21.2 (12.8–39.9)
	11 females 18–50 years	$C_{5,8}$	33.8 (28.0–42.7)	37.9 (24.4–67.7)
Costongs G et al. [13]	274 subjects	$C_{4,8,10,13}$	**19.8** (19.1–20.6)	NA
EuBIVAS (present study)	88 subjects	A	28.0 (26.7–29.6)	
	37 males 22–59 years	A	24.9 (23.2–27.2)	19.1 (14.5–26.0)
	41 females 21–49 years	A	38.3 (35.6–41.4)	35.3 (27.5–46.9)
	10 females >55–69 years	A	25.6 (22.4–30.1)	29.2 (18.7–57.3)

EuBIVAS, European biological variation study. NA, not available; In bold the estimates included in the meta-analysis, according to BIVAC criteria. [a]The item quality is rated according to the BIVAC, which are scored from A to D depending on the quality of information [8]. [b]An overall grade A indicates full compliance with all the 14 BIVAC quality items. A grade B is awarded if the lowest QI score achieved is B. Similarly, the study is graded C if the lowest quality item score is C. An overall BIVAC grade $C_{8,12}$ (subscript numbers) indicates that QI numbers 8 and 12 were scored as C. [c]CV_I estimate not included because obtained from elderly people.

reported data on CV_G, and the meta-analysis derived CV_G estimate decreased from 32.3% (23.4–47.7) [9] to 26.1% (26.0–26.7) after inclusion of the EuBIVAS data.

Our findings confirm the high within- and between-individual variability of serum iron as also reported in previous publications [10–13] (Table 2), highlighting its limited clinical value [4]. Iron in fact, when suspecting iron deficient anemia, should not be measured alone as a test of first level, but together with other markers of iron deficiency like serum ferritin, the most sensitive and specific test to identify isolated iron deficiency, as it reflects low iron stores [14].

As expected, there was a clear difference between the mean serum iron concentrations in males and females. Less well known is the much higher variability that was observed in fertile age women in our study. We found differences in CV_I and CV_G between males and fertile females, as well as between fertile and post-menopausal women (Table 1), even when the serum iron concentrations in the post-menopausal group were similar to that of fertile women. The difference could be related to the increased and variable iron demand caused by the blood loss during menstruation or indicate a higher random BV variation in fertile age women [4]. Unfortunately, data on ferritin were not available for analysis. However, we could not demonstrate any correlation between the personal intra-individual variation (CVp, calculated as proposed in [15]) of iron with serum iron concentrations or to CVp of transferrin or of soluble transferrin receptor (data not shown).

In summary, our study provides updated BV data for iron derived from the large-scale EuBIVAS and can be used as aid in setting APS and other BV applications for iron.

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