

## Editorial

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# Folate and vitamin B12 assays after recalibration to the WHO International Standard 03/178: making the interpretation as simple as possible, but not simpler

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The recent alignment of serum folate and vitamin B12 (B12) assays to the WHO International Standard (IS) code 03/178 has produced a significant shift in the average vitamin measured values and triggered studies on the re-evaluation of reference intervals (RI) for both biomarkers [1–4]. This also yielded the opportunity to update recommendations about the cost-effective application of both tests and the interpretation of marker concentrations in serum [5, 6]. Authoritative guidelines, which define the risk for vitamin deficiency according to B12 and folate testing, indeed reported as interpretative criteria, RI estimated by using assays not traceable to the WHO IS 03/178 [7]. For both tests, cost-effectiveness is maximized when the request is applied to subjects at risk for deficiency and when the deficiency itself may represent a life-threatening condition or invalidate the treatment effect (e.g. in pregnant women or hemodialysis patients) [5, 6]. On the contrary, tests should not be ordered to monitor or evaluate the effect of vitamin supplementation. By applying these indications for an appropriate testing strategy, it was demonstrated an ~50% saving in laboratory costs, without compromising the patient safety [5, 6].

In general, for serum B12 the capability of the test to rule out the deficiency at the individual level is low when a single threshold value, such as the lower reference limit (LRL), is adopted. Multiple thresholds have been validated by clinical studies establishing the relationship between marker concentrations, the probability of deficiency and the need for supplementation [5]. Accordingly, the establishment of RI is not helpful for interpreting B12 concentrations in serum. Furthermore, the correct RI derivation by individual laboratories is very challenging because serum B12 levels are strongly conditioned by the selection criteria used to enrol the “apparently healthy” population, by some pre-analytical factors (e.g. time of sample drawing) and can be affected by many variables, such as dietary habit, vitamin supplements, use of the contraceptive pill, drugs, etc., which are sometimes difficult to control [5]. Thus, the experimental establishment of serum B12 RI by Solé-Enrech et al. [3], performed with the

aim of improving the interpretation of vitamin concentrations in individuals suspected for deficiency, is of limited value.

On the contrary, the evaluation of the inter-assay variability of B12 measurement at serum concentrations around the established thresholds for the risk of deficiency may be useful in indicating if these cut-offs may be generalized or require an adjustment according to the employed method. The more recently published data about the comparability among current B12 assays agree in showing a good concordance among the most widely used measuring systems, except for results by Beckman Coulter DxI Unicel that were negatively biased [8, 9]. Results from external quality assessment schemes (EQAS) can also aid to assess the current status of harmonization of B12 measurements. Data from the national Qualimedlab EQAS ([www.qualimedlab.it](http://www.qualimedlab.it)) (2016–2018 exercises) for the four most popular assays (Abbott Architect, Beckman Coulter Access/Dx, Roche Modular/Elecsys/cobas and Siemens Advia Centaur) confirm the marked negative bias for Beckman Coulter systems (–22% in average), with other methods fulfilling the quality goal for desirable inter-assay bias ( $\pm 17.7\%$ ) based on biological variation of B12 [9]. According to all these data, we can conclude that the harmonization of B12 results obtained by the commercially available measuring systems is currently acceptable enough, with few exceptions.

For serum folate testing, a single threshold, set at LRL, is usually employed to screen for vitamin deficiency in non-fortified countries. Consequently, the prevalence of folate deficiency is strictly dependent from the correctness of cut-off establishment and from the accuracy of folate assays [6]. Data for the US National Health and Nutrition Examination Survey (NHANES) first showed that the actual prevalence of folate deficiency in the population may significantly shift with the reformulation of assays [10]. If tracing back calibration of commercial assays to a new reference material changes results, this may alter their relation to existing RI, with a consequent misleading test interpretation. This is exactly what happened with the introduction of WHO IS 03/178 as a calibrator of commercial systems, which caused a significant

negative shift in the folate measured values [6]. Using the reformulated Roche assay, at serum folate concentrations around the LRL, we experienced a difference of ~50% vs. the old Roche assay [6]. Therefore, we observed marked changes in the distribution of folate results of the population tested in our laboratory after the adoption of the recalibrated assay, with a wide increase of test results suggesting a vitamin deficiency. Similar results were obtained by other authors [2]. New experimental data from reference individuals should, therefore, be urgently obtained with the recalibrated assays in order to accurately redefine the reference distribution and estimate proper RI.

Table 1 lists the design and results of available studies dealing with the re-evaluation of folate RI [1–4]. All studies employed the Elecsys Folate III (Roche Diagnostics) reagents traceable to WHO IS 03/178 applied on cobas e601/e801 platforms. Comparing the RI provided by these studies, there are relevant differences that may be ascribed to the study design, sample size, definition of the reference population, type of enrolment and criteria for subject recruitment, pre-analytical factors (fasting,

time of blood drawing, detection of sample hemolysis), and measurement protocols. As previously mentioned, the cut-off reported by the running guidelines to define the state of folate deficiency, i.e.  $<3 \mu\text{g/L}$  (or  $<4 \mu\text{g/L}$  when using homocysteine concentrations as the metabolic indicator) [7], dated back to the use of assays not yet standardized to WHO IS 03/178 and, in contrast to what was claimed by Cluitmans and van den Ouweland [4], cannot be used as comparators to judge the reliability of RI obtained in the studies listed in Table 1.

In 2016, Kristensen et al. [9] showed that differences among folate results obtained from different manufacturers were within the limit for desirable bias ( $\pm 19.2\%$ ), which is, however, a relatively large acceptance criterion, as a result of the wide biological variation of the analyte. At that time, only Abbott reported traceability to WHO IS 03/178, while the other assays claimed traceability to different US pharmacopeia (USP) convention or in-house manufacturer calibrators. Since then, to the best of our knowledge no updated comparative data have been published. EQAS could help us in understanding the current status of folate measurements, but non-commutability of

**Table 1:** Characteristics of published studies estimating the reference interval for serum folate measured with Roche Folate III assay, calibrated against WHO International Standard 03/178.

Authors [ref.]	Sample size (n)	Studied population	Exclusion criteria	Measurement protocol	Estimated RI (2.5th–97.5th percentile limits), $\mu\text{g/L}$
Ferraro et al. [1]	322	Blood donors, median age 45.4 years	Hb and MCV $\neq$ RI, supplementation with folic acid, HI $\geq 25$ (Abbott Architect)	Serum stored at $-80^\circ\text{C}$ , measurements performed in four runs using a single reagent lot	1.3–9.8
Hepburn et al. [2]	9797	Outpatients, median age 54 years	$<18$ or $>90$ years of age, folate concentrations $<2$ or $>20 \mu\text{g/L}$ , Hb, MCV and B12 $\neq$ RI <sup>a</sup>	Samples analysed on the day of collection	2.4–17.5
Solé-Enrech et al. [3]	120 126	Mainly healthy hospital workers and residents	Low dietary consumption, malabsorption, increased requirements or losses of vitamins <sup>a</sup>	Samples analysed on the day of collection	2.0–13.9, $<45$ years 2.9–16.6, $\geq 45$ years
Cluitmans and van den Ouweland [4]	130	Outpatients, median age 55 years	Pregnant or lactating women, supplementation with folic acid, homocysteine $>15 \mu\text{mol/L}$ , HI $\geq 50$ (Roche cobas)	Serum stored at $-80^\circ\text{C}$ until measured	3.0–19.0

RI, reference interval; Hb, haemoglobin; MCV, erythrocyte mean corpuscular volume; HI, hemolytic index; B12, vitamin B12. <sup>a</sup>HI not evaluated.

control materials may be a very limiting issue, at least for some commercial assays [9].

A final important aspect relates to the general potential inconsistency of the use of LRL (or another single cut-off rule) for detecting folate deficiency. According to the estimation of biological variation of folate in serum, the lower within-person variation with respect to the inter-individual variation implies a low index of individuality (i.e. ratio of within- to between-subject variances) [6]. Under this condition, an isolated dichotomized interpretation of folate results, resorting to a decision limit for deficiency, can be misleading and the longitudinal monitoring of serial folate changes be more effective in classifying individuals with relation to their vitamin status [6].

In conclusion, the meritorious work for reducing the inter-assay disagreement between folate and B12 results, started with the availability of the WHO IS 03/178 and the implementation of traceability to it, must be continued. The release and adoption of other suitable reference materials, like the Joint Committee on Traceability in Laboratory Medicine (JCTLM)-listed SRM 1955 from the National Institute of Standards and Technology (NIST), should be encouraged, together with the regular monitoring of the performance of commercial measuring systems through the participation to EQAS using proper commutable materials, aiming to improve the accuracy of results. This will make the interpretation of folate and B12 values more consistent, but not simpler, giving the complexity of issues around diagnosis and treatment of their deficiency.

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