Review

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Tackling serum folate test in European countries within the health technology assessment paradigm: request appropriateness, assays and health outcomes

Abstract: Several authors have recently claimed an excess in serum folate test ordering, suggesting phasing out it from clinical use. According to studies performed in countries undergoing folic acid fortification policies, it is indeed no more cost-effective to test folate in the face of deficiency prevalence <1%. In this paper, we sought to evaluate request appropriateness, analytical issues, and cost-effectiveness of serum folate determination for clinical purposes in the European context, considering if evidence retrieved in fortified countries may be generalized. Studies performed in non-fortified countries have generally reported a suboptimal folate intake and suggest a remarkable prevalence of folate deficiency. Our internal data suggest that ~20%–25% of the subjects undergoing serum folate test are at risk for deficiency. However, a reliable evaluation of the risk for deficiency implies the knowledge of all issues related to the total testing process of folate measurement as well as the identification of the appropriate population in which to perform the test. The cost-effectiveness of the test is maximized when the request is oriented to subjects suggestive/at risk for deficiency, becoming low if the test is used as a screening tool or for monitoring of vitamin intake/supplementation. Because the individual folate status has a key role in ensuring normal development, physiologic growth, and maintenance of optimal health, the evaluation of its serum levels has to be retained in the clinical use in non-fortified countries, boosting for more appropriate request, and evidence from countries following fortification policies should be cautionary interpreted.

Keywords: assay; biological variability; folate; request; traceability.

Introduction

Recently, several authors have debated the utility of serum folate testing, also in face of the high rate of misuse and of the associated costs [1–5]. From the retrospective analysis of data, a shared position on the utility and cost-effectiveness of folate testing has emerged. The main evidence is well summarized in the systematic review by the Canadian Agency for Drugs and Technologies in Health (CADTH) [6], showing that the folate test is highly requested, but its results do not affect the clinical management of patients, thus resulting in a poor cost-effectiveness [7–9]. The CADTH report is well rooted on the evidence mainly built on American/Australian populations, characterized by a far low prevalence of folate deficiency (ranging from 0.1 to 1%) due to wheat flour folate fortification [6, 10, 11]. Nevertheless, appraisals claim that the rate of folate testing has not significantly changed after fortification to yield a significant shrinkage of costs, although indications for the appropriateness of the request have been clearly reported [6, 12].

Although folate testing in subjects without known risk factors has little clinical significance [13], a wide range of conditions have been identified all potentially impairing folate intake/absorption or increasing requirement (Table 1) [5, 6, 14–17]. For many of these risk conditions, a poor clinical utility of folate testing has, however, been reported, since patients usually exhibit serum

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Table 1: Conditions potentially impairing folate intake/absorption or increasing requirement.

– Inadequate intake (e.g. malnutrition, low intake of folate-rich or folic acid-fortified foods)
– Impaired absorption (e.g. gastrointestinal disease, bariatric surgery patients, laparoscopic biliopancreatic diversion with or without duodenal switch, gastric by-pass, sleeve gastrectomy procedures, inflammatory bowel and Crohn's disease, inflammation of small intestine due to coeliac disease, tropical sprue, use of drugs such as metformin, proton pump inhibitors, oral contraceptives, anticonvulsants, sulfasalazine, triamterene and pyrimethmine)
– Increased requirements (e.g. pregnancy and lactation)
– Lifestyle (e.g. alcohol abuse)
– Genetic factors (mutations in the *SLC46A1* gene, proton-coupled folate transporter deficiency)
– Other clinical conditions associated to: evidence of anemia and/or macrocytosis, neuropsychiatric symptoms (depression, psychosis) or Alzheimer's and non-Alzheimer's dementia, poor proprioception, peripheral neuropathy, history of glossitis and mouth ulceration, autoimmune disease, chronic hemolytic states, chronic kidney disease and renal dialysis, malignancy (e.g. leukemia, non-myeloid malignancies at risk for anemia), situations similar to vitamin B12 deficiency

concentrations well within the reference interval, and this is true in non-fortified countries too [5, 18, 19].

Several US authors are now seeking for phase out folate testing according to the mentioned evidence of low utility and poor cost-effectiveness for both inpatients and outpatients [1, 4, 20]. It is also noteworthy that for several populations of individuals at risk for deficiency, folic acid supplementation is highly recommended or suggested, but folate testing is not required or is not accounted for prior initiating supplementation [8, 9]. Furthermore, it has been reported that the knowledge of low folate results often does not change the clinical management of patients [6]. This is fostered by the lack of evidence on the benefit associated to or consequent to folate testing [1, 6, 20]. On the other hand, it has been shown that the direct costs of folate testing overcome those of supplementation, which is not associated to side effects apart from masking hematological symptoms of vitamin B_{12} deficiency with exacerbation of neurological symptoms [9].

In 2000, a European document focused on the tolerable upper intake level of folate, alerting that the excess of synthetic folic acid supplementation may increase the concentration of unmetabolized folic acid (UMFA). The accumulation of both vitamers may result in various adverse outcomes, such as epileptogenic and neurotoxic effects, decreased efficacy of some chemotherapeutics, decreased zinc absorption and deficiency, and carcinogenicity [21–23]. More recently, other authors wholly disagreed with

these warnings, observing that the relationship of folic acid with adverse effects can be affected by several confounding factors [24]. For instance, the hypothesis of an association of folic acid supplementation with cancer development is rapidly declining, owing to its possible chemopreventive role and no evidence of a relationship with cancer incidence [24, 25]. Currently, the main indication on a possible adverse effect of folic acid upper intake level addresses the surveillance of the elderly for what concerns the possible masking of a vitamin B_{12} deficit [21]. Any possible effect of blood UMFA on health remains to be elucidated, and this is not an easy task, considering that folate assays available for clinical purposes are not able to detect it [26].

Considering the above-described premise, it seems appropriate to review here the cost-effectiveness of serum folate determination for clinical purposes in the European context, considering if the evidence retrieved in countries undergoing mandatory folic acid fortification policy may be generalized to other countries and the related pipeline shared.

Folate testing to quote risk for deficiency in Europe: a public health problem

Evidence from European countries

Although the CADTH report represents the most recent and exhaustive review on optimal methods for folate testing, its clinical utility, cost-effectiveness, and appropriateness of the request, in Europe, we cannot, in principle, rely on recommendations built in folate-fortified countries. Here, the prevalence of folate deficiency is now too low to provide a reliable evidence on the clinical utility of this test. As for all tests used in laboratory medicine, the capability of folate results to correctly identify or exclude a deficiency strongly depends on the prevalence (pretest probability) of the condition in the clinical setting in which the test is applied [27].

A robust and updated estimate of folate deficiency in the European countries is lacking, although several documents have reported on the occurrence and incidence of diseases related to folate deficiency, with strong recommendations on supplementation [28–31]. This concept has recently been reinforced by Mills and Dimopoulos, discussing on the possible introduction of folic acid fortification in Europe and observing that the cost-benefit of this

policy in US has been shown by the consequent dropping of folate deficiency (from 24% to <1%, according to the measurement of serum folate) [32, 33]. The perception that folate deficiency is quite disregarded in Europe emerges from the guidelines retrieved by the CADTH review: out of nine, only two have been developed in Europe, both by the European Federation of Neurological Societies [34, 35]. Some speculations on a remarkable prevalence of folate deficiency may be gathered from national surveys reporting on the nutritional behaviour of the populations in European countries, although the direct measurement of serum folate has been performed only in ~40% of these studies [26, 28, 30]. Accordingly, it has been shown that in the Netherlands, Norway, Sweden, and Greece, the mean (±SD) serum folate concentrations [3.4 (±0.3) µg/L] reflects a poor food-based folate intake, which could also be dependent on the way food manufacturers supplement their grain products [26]. A moderate intake [estimated by a mean (±SD) serum folate of 5.6 (±0.8) µg/L] has been reported for Finland, Ireland, Czech, Italy, and Portugal, whereas favorable folate concentrations in serum (7.3±0.6 µg/L) have been detected in UK, Germany, and Spain [28].

In Italy, there are few updated evaluations on serum folate concentrations in the general population under healthy conditions. The first study, performed on blood donors, dates back to 2000 [36]. The mean folate concentration for all case series (n=201) was ~5.0 µg/L, with no sex-dependent differences. The rate of subjects below the adopted lower reference limit [i.e. 3.0 µg/L by Abbott Diagnostics microparticle enzyme immunoassay (MEIA)] was 9.9%, with no gender dependence [36]. More recently, Zappacosta et al. [37] evaluated serum folate concentrations in blood donors accrued in the middle-southern Italy. On a case series of 240 subjects, the overall mean folate concentration (Abbott MEIA) was 4.8 µg/L, with concentrations being slightly higher in women. From this study, we cannot estimate the percentage of subjects with folate deficiency, but, by defining adequate serum folate concentrations as >6.6 µg/L, the authors observed that only 22.5% of the study participants showed an adequate folate intake. Results from multiple logistic regression showed that female gender, older age, no smoking habit, regular physical activity, and consumption of at least three portions of vegetable/fruits per day promoted higher folate concentrations [37]. Two further studies specifically focusing on the age dependence of folate concentrations in serum have reported that folate deficiency increases with age, showing in the elderly a prevalence of ~5% in the 65–74-year range and of ~10% in individuals >75 years old, respectively [31, 38]. Sofi et al. [39] in evaluating dietary habits and lifestyle of Italian subjects, measured

serum folate in 520 ostensibly healthy subjects, showing mean (±SD) values of 5.4 (±2.6) µg/L. Girelli et al. [29], investigating on the relationship between folate status and activity of 5,10-methylenetetrahydrofolate reductase (MHTFR) as risk factor for cardiovascular disease (CVD), reported similar concentrations in 222 CVD-free controls and found ~24% of apparently healthy subjects with an inadequate folate status on the basis of their MHTFR genotype. This study addressed an additional public health problem related to folate status, not considered by CADTH but relevant for Caucasian populations: the considerable prevalence of *MHTFR* homozygous mutation (10%–18%) and the inverse association between folate concentrations in serum and both homocysteine status and CVD risk. Although the evidence on the relationship between folate status and CVD risk is mild, the correction of hyperhomocysteinemia with folic acid supplementation is widely used in the general population as well as the combined (re)testing of serum folate and plasma homocysteine for therapeutic monitoring [40].

In conclusion, in the European region, the adequacy of folate status appears to be widely variable across countries and some populations appear to be characterized by a suboptimal folate intake. Lacking robust data, we can doubt that the problem of a folate deficiency in the general population may be underestimated and neglected.

Tackling the problem by a health technology assessment (HTA) approach

As discussed above, for several European countries data on folate status in the general population are scarce and dated although public health authorities are engaging in efforts to increase overall folate intake according to the US model, in particular in women of childbearing age for the prevention of neural tube defects (NTD) [41, 42]. In a recent report, Mastroiacovo and Corchia have observed that serum folate concentrations in this women subset are comparable to those found in the US female population of the same age in the 1988–1994 period before the fortification [42]. Notably, these levels are lower than recommended by the World Health Organization (WHO) and far lower than those concentrations minimizing the risk for NTD [43]. In the USA, the folate status and changes in prevalence of folate deficiency occurring after fortification have been strictly monitored: contemporary to a 54.5% increase in folate concentrations, a decrease of NTD cases from 10.7 to 7.0/10,000 cases/year after fortification has been recorded [43]. A cost-benefit analysis has clearly shown that health and economic gains associated

to serum folate increase after folic acid fortification far outweighed the losses [44]. Indeed, the consequent enhanced prevention of myocardial infarction, colorectal cancer, NTD, and vitamin B_{12} deficiency masking resulted in a direct positive impact on health outcomes, such as annual burden of disease, quality-adjusted life years, and costs [44]. Tackling the folate issue by involving key stakeholders in healthcare policy and decision-making such as health management and clinical professions, industry, patient organizations, third-party NTD, and government has been therefore successful [45].

Firmly rooted in research and in the scientific method, HTA aims to support policy makers in making evidence-informed decisions on the application of scientific knowledge in healthcare and prevention [46]. HTA covers a broad range of relevant expertise comprising diagnostic and treatment methods, medical equipment, pharmaceuticals, rehabilitation and prevention methods, and the organizational and supportive systems within which healthcare is provided [47]. In agreement with the HTA approach, the US National Health and Nutrition Examination Survey (NHANES) program has involved a multi-disciplinary panel of 23 experts in folate and vitamin assessment, epidemiology, clinical laboratory science, and biostatistics. The roundtable also had 10 scientists from governmental agencies that conducted and funded folate biomarker measurements in NHANES surveys and developed relevant reference methods and materials [45]. A part from documenting a sharp increase then followed by a slight decrease during the 12-year post-fortification period, the roundtable reported on the quality of folate-related measurement procedures, on the quality of reference procedures and reference materials available or under development, as well as provided public health considerations for the choice of which folate-related biomarkers to measure in the population [45]. Accordingly, US Health Advisory Committees with respect to European counterparts have paid greater attention to the measurement of the actual folate status more than to folate intake in the general population, monitoring folate concentrations in the US population from pre- (1988–1994) to post-fortification (1999–2010) period and estimating or predicting the consequent improvement of health outcomes. Notably, they also focused on the assays available for folate testing and on the analytical issues.

Choice of the test for assessing folate status

The available literature is confusing in defining which test is more appropriate to assess the adequacy of folate intake and status. Even if the measurement of serum folate is currently accepted as the test of choice for the clinical assessment of folate status/deficiency, some authors have proposed to replace this determination with plasma homocysteine or judged more reliable the measurement of folate in red blood cells (RBC) [28]. Although its request has steeply risen because of the relationship with CVD and of a possible correction of its values with synthetic folic acid, plasma homocysteine should be considered as a non-specific functional indicator of folate status [26]. On the other hand, the selection of the sample (serum or RBC) where measuring folate may depend on the features of the population in relation to the exposure to folic acid fortification or supplementation (see below) [48].

Before clarifying the analytical issues related to folate determination in serum and RBC, we should retrieve information on the various vitamers constituting the whole of total folate and their detectability by the available assays [45]. The predominant form of the vitamin naturally found in foods is the tetrahydrofolic (THF) polyglutamate, whereas folic acid is the form used in food fortification/enrichment and dietary supplement. The biologically active THF acid is involved in the synthesis of purines, deoxythymidine, and some amino acids. The main circulating and stable folate form is the 5-methyltetrahydrofolate (5MeTHF), whereas RBCs predominately contain long-chain polyglutamates of 5MeTHF. According to isotope dilution–liquid chromatography–tandem mass spectrometry (ID-LC-MS/MS) studies, 5MeTHF constitutes from 82% to 93% of the total folate found in native serum from healthy individuals [49–51]. Additional forms may be pteroylglutamic acid (PGA), 5-formyltetrahydrofolic acid (5FoTHF), THF, various reduced forms, UMFA, and, possibly, an intermediate product, the 4-α-hydroxy-5MeTHF (hmTHF) [49–51]. The concentration of each form depends on factors such as dietary supplementation and food fortification [52]. Populations exposed to folic acid fortification and known to have a historical prevalence of folic acid supplementation are characterized by far higher median concentrations of 5MeTHF ($\sim$ 17.0 µg/L) and UMFA ($\sim$ 0.4 µg/L) when compared to subjects from countries with no fortification (7.0 and 0.10 µg/L, respectively) [50, 53]. In addition, for RBC folate, the *MTHFR* C677T polymorphism affects folate vitamers distribution and thus the results strictly depend on the genotype [54, 55].

Although there is an increasing interest in detecting and quantifying serum folate forms by ID-LC-MS/MS, in particular to evaluate the effects of fortification, currently only information derived from the measurement of total folate can be used for the clinical assessment of folate status. Thus, considering the wide heterogeneity of

forms present in individual biological samples, it is relevant to know what vitamers may be recognized by the marketed assays and enter in the estimate of "total folate". The laboratory measurement of folate is complicated because not all forms are characterized by biological activity and various inactive degradation products may interfere.

The microbiological assays exclusively enabled the evaluation of biologically active folate species, but, given the poor performance, they were replaced first in NHANES surveys and later in clinical laboratories by competitive protein-binding (CPB) measurement procedures, assuring higher repeatability and reproducibility, but with some problems of bias [45]. Using the National Institute of Standards and Technology (NIST) standard reference material (SRM) 1955, the CPB assay by Bio-Rad resulted to underestimate total folate concentrations of 2.6 and 5.8 μg/L by 25% and 40%, respectively, when compared to ID-LC-MS/MS assigned values [56]. The use of National Institute for Biological Standards and Control (NIBSC) 03/178 International Standard revealed an underestimation of ∼10% for CPB Bio-Rad serum folate results at an assigned total folate concentration of 5.3 μg/L [57]. In general, for the Bio-Rad assay it have been reported an under recovery of 5MeTHF and a proper folic acid recovery, whereas the estimated recovery of other minor folate species was significantly lower or higher than expected [45].

With the further development of CPB assays on different automated platforms and the availability of External Quality Assessment (EQA) data, it became definitively evident that various assays bind different folate species in the sample with different affinities. The currently available CPB assays are built on the use of highly specific folate-binding proteins (FBP) extracting from the sample preferentially 5MeTHF and other vitamers such as folic acid and 5FoTHF [58]. The recovery may, however, vary according to total folate concentrations present in the sample: for instance, the Roche Diagnostics Folate III assay, developed according to what ID-LC-MS/MS detects [59], measures an average composition of 93.3% 5MeTHF, 2.3% folic acid, and 4.4% 5FoTHF in serum samples with total folate <22 μg/L, and an average composition of 81.7% 5MeTHF, 15.7% folic acid and 2.5% 5FoTH for total folate concentrations >22 μg/L (A. Ruschel-Mitscherlich, personal communication). CPB assays have also a tendency to over recover PGA and this suggests an increased bias in total folate measurements for samples containing increased concentrations of PGA. Therefore, CPB assays would be more appropriate for the measurement of sera with total folate <22 μg/L, that is to say when used to test populations not receiving staple foods fortified with PGA

[60]. Finally, the affinity of various CPB assays may be further affected by slight deviations from the optimal pH of 9.3, particularly at the binding phase [61, 62]. Considering all the described issues, it should not be surprising that EQA results for serum folate exhibit a poor inter-method agreement, particularly at clinically significant low concentrations of the analyte [63–65].

The selected calibration regimens may undoubtedly contribute to the lack of method comparability. As anticipated above, two reference materials for folate became available in the last years for implementing traceability of the commercial assays. The NIST SRM 1955 has certified values for 5MeTHF, reference values for folic acid and information values for total folate and 5FoTHF. Out of three level materials, only the level 2 material (with total folate concentration of 5.8 μg/L) provides, however, a reliable quality to be used as calibration material being an unaltered frozen human serum, whereas commutability issues may arise with the use of levels 1 (diluted material) and 3 (fortified material) [56]. Importantly, this material is currently out of stock and information on a new lot release is not available. Being lyophilized, commutability could also be an issue for the other reference material, the NIBSC 03/178 International Standard (available since 2007) [57]. Its total folate concentration, determined using ID-LC-MS/MS, is 5.33 μg/L, made up of 4.3 μg/L 5MeTHF, 0.7 μg/L 5FoTHF, and 0.33 μg/L folic acid [57].

The NHANES experts agree on the alternative use of serum and RBC folate measures to monitor folate concentrations in the US population, warning that the first one reflects recent intakes and may be more appropriate for the clinical detection of deficiency [45]. On the other hand, they suggest that in fortified countries the determination of RBC folate is preferable, being a sensitive and long-term (4 months on average) indicator of folate status and stores [45]. Both serum and RBC assays are more responsive to interventions with folic acid in supplements and fortified foods than to natural food folates (THF polyglutamate), but the increase of serum vs. RBC folate concentrations consequent to folic acid fortification in the US was far higher (+119%–161% vs. +44%–64%, respectively) [66, 67]. In addition, the RBC folate concentrations did not show the expected plateau effect if tissue saturation occurs at higher intakes [68]. On the other hand, RBC folate concentrations fall with vitamin B_{12} deficiency, which makes them useful in the evaluation of folate effects on vitamin B_{12} deficient subjects.

A mandatory prerequisite to obtain reliable folate results is to prevent the loss of 5MeTHF by promptly processing samples according to their stability (2 h at room temperature, 48 h at 2–8 °C or freezing at −70 °C for longer

period) [69]. For RBC folate, the in vitro conversion of 5MeTHF polyglutamates to monoglutamates may further complicate the pre-analytical phase [26]. Furthermore, for serum folate determination fasting is required as well as the rejection of hemolyzed samples [free hemoglobin (Hb) ≥ 0.25 g/L].

A final important aspect is the definition of decision level for serum folate indicating negative folate balance/folate deficiency in the evaluated population. Over the past three decades, the threshold has been widely reviewed, passing from 3.0 to 4.4 µg/L, when the definition accounted for the relationship with a metabolic indicator (i.e. increase of plasma homocysteine). Although the implementation of folate cut-offs had a pivotal role in promoting public health interventions gaged on NHANES surveys [70–72], the use of decision values could be inconsistent and the individual chronic folate deficiency may be stochastically found by multiple serial testing. According to the estimation of biological variation of folate in serum, the far 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), i.e. 0.44 [73]. 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. This approach is also supported by the number of samples needed to theoretically establish an individual's homeostatic set point. According to the current statistical approach [74], the number of specimens that should be collected to ensure that the mean marker result is within ±10% of the individual's homeostatic set point can be obtained according to the following statistics: 1.96^2 (analytical CV^2 + within-subject biological CV^2)/100. Using an average analytical CV of 4.0% and an average intra-individual CV of 21.5% [73], for folate, it can be estimated that each subject should theoretically undergo up to 18 marker measurements to achieve a sufficiently accurate estimate of the folate individual's homeostatic set point, which is clearly unfeasible.

Status of folate testing in Italy

Inpatients and outpatients

Aiming to characterize the prevalence of folate deficiency in the served patient population of our academic metropolitan hospital, we retrieved a 1-year (April 2015–April 2016) case series of consecutive serum folate results from our laboratory information system. Out of 4225 folate values obtained by using Roche Diagnostics Folate III assay (code 04476433190) performed on the Modular EVO platform, ~11.0% were >20 µg/L, the remaining cases exhibiting a median (25th–75th percentile) concentration of 6.4 (4.8–9.0) µg/L. By selecting results below the lower reference limit recommended by the manufacturer of our folate assay (4.6 µg/L), 861 (20.4%) results characterized subjects as at risk for folate deficiency. In this subset of individuals, the median (25th–75th percentile) folate concentration was 3.8 (3.2–4.2) µg/L. The frequency distributions of serum folate concentrations in outpatients [n = 2318; 16.8% < 4.6 µg/L; median (25th–75th percentile) 6.8 (5.1–9.4) µg/L, excluding 285 (12.3%) subjects > 20 µg/L] and inpatients [n = 1907; 24.8% < 4.6 µg/L; median (25th–75th percentile) 6.0 (4.4–8.4) µg/L, excluding 181 (9.4%) subjects > 20 µg/L] are reported in Figure 1.

By analyzing folate ordering, we observed that, with the exception of patients carrying myelogenous leukemia (adding up 9.8% of orders), requests were not preferentially attributable to some specific wards or to specific sets of outpatients. In particular, 8.9% and 10.2% of requests concerned patients admitted to Departments of Internal Medicine and Infectious Disease, while the number of requests coming from other divisions was lower [Neurology and Stroke Unit (7.6%), Nephrology and Dialysis (4.6%), and Gastroenterology (3.5%)]. More than half of the total results below the lower reference limit were partitioned among the Department of Internal Medicine (25.3%), the Department of Infectious Disease (15.4%), and general outpatients (18.1%). Neurology and Stroke Unit (8.7%), Nursing Home Care (7.1%), Outpatients carrying Myelogenous Leukemia (5.7%), Nephrology and Dialysis (4.1%), Gastroenterology (3.7%), Reumatology (2.1%), and Oncology (1.6%) showed a lower prevalence of serum folate reduction.

Considering folate retesting, we found 732 results from 337 retested patients (62.4% outpatients). Each patient had a mean (SD) of 2.2 (0.4) requests/year, in 14.2% of patients, the folate tests being ≥ 3. The average (SD) time interval of retesting was 3.4 (2.5) months between the first two requests and 3.1 (2.0) months between the second and the third one. Figure 2 displays the frequency distribution of absolute and percentage changes between the first two serial measurements of serum folate in retested subjects. Accounting for an average intra-individual CV of 21.5% [73] and the analytical CV of the assay, one can derive the reference change value (RCV) for serum folate. The RCV is defined as the change needed between two serial results from the same individual to be significantly
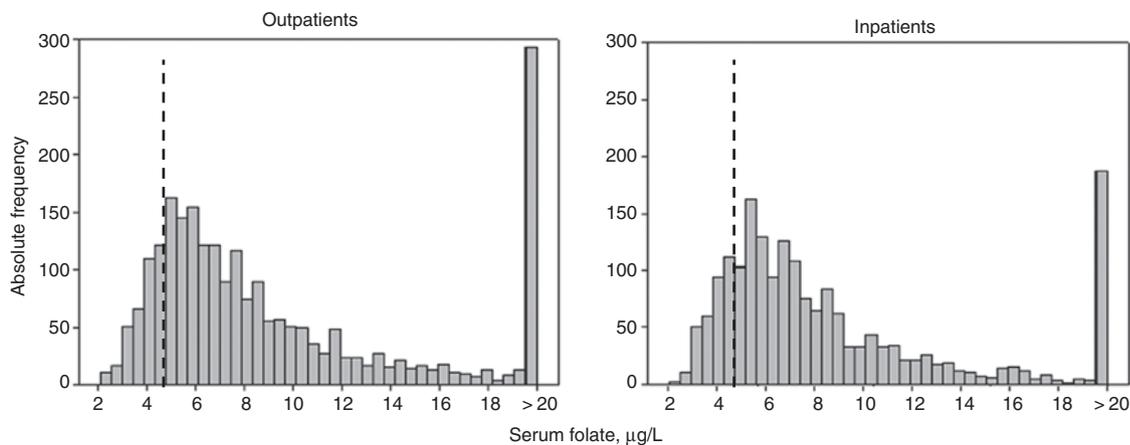
Figure 1: Frequency distributions of serum folate results in outpatients (n = 2318) and inpatients (n = 1907) retrieved from 1-year consecutive case series in the population served by the authors' laboratory.
Vertical dashed lines indicate the lower reference limit (4.6 µg/L) recommended by the manufacturer of folate assay (Roche Diagnostics Folate III, code 04476433190).
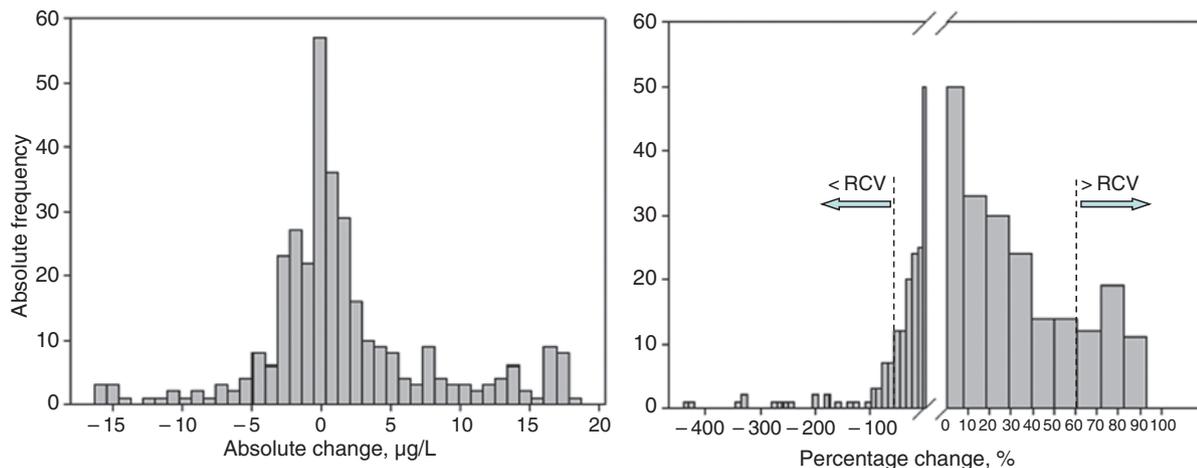
Figure 2: Frequency distribution of absolute and percentage changes between the first two serial measurements of serum folate in retested subjects (n = 337) from the surveyed population.
RCV, reference change value.

different, beyond the measurement error and the biological variability of the analyte [74]. In particular, at the average analytical CV for our assay of 4.0% (data from 2015 fiscal year internal quality control), the derived RCV for serum folate is 60.6%. By applying it to our population, we can identify a subgroups of 43 subjects (12.8% of retested patients) with a positive change > 60.6% between the two serial tests, likely related to a recently started folic acid supplementation, and another group of 32 individuals (10.4%) with a decrease in serum folate > RCV, in whom an actual folate deficit was conceivable (Figure 2). Interestingly, only 12 subjects in the latter group had the second value < 4.6 µg/L, suggesting a possible underestimation of vitamin deficiency if the evaluation is resorting

to a fixed cut-off. Furthermore, 14 individuals from this subgroup showed a baseline value > 14 µg/L indicating a recently interrupted supplementation. It is noteworthy that folate body stores generally represent a 2–3 months supply, and after stopping supplementation, folate deficiency may suddenly occur for an inadequate intake or increased requirement for the vitamin [75]. The remaining 262 retested subjects displaying a change < 60.6% between two measurements (i.e. a stable trend of folate values) could be in turn divided into three subsets, the first with folate values stably ≥ 14 µg/L, indicating an ongoing supplementation, the second with baseline values inside the reference interval and the third with baseline folate concentrations < 4.6 µg/L. In the first and the third subsets,
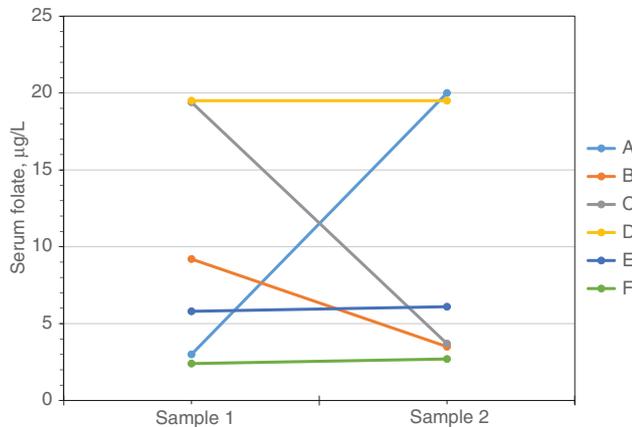
Figure 3: Typical patterns of serum folate change in retested subjects. The average (SD) time of retesting was 3.4 (2.5) months. (A) Subject with a positive change higher than the reference change value (RCV) (60.6%), likely related to a recently started folic acid supplementation; (B) subject with a decrease in serum folate > RCV, in whom an actual folate deficit is conceivable; (C) subject with a decrease in serum folate > RCV and a baseline value > 14 µg/L, indicating a recently interrupted supplementation; (D) subject with folate values stably ≥ 14 µg/L, indicating an ongoing supplementation; (E) subject with baseline value inside the reference interval and change lower than RCV, indicating a unchanged lifestyle; (F) subject with folate concentrations lower than the lower reference limit, indicating a possible folate deficiency.

the retesting was not justified. Figure 3 displays typical patterns of folate change found in retested subjects.

Healthy population

We also retrieved data from a continuous case series of 538 blood donors [men, 58.4%; median (25th–75th percentile) age, 36 (25–45) years, 11.3% non-Italian] routinely tested for serum folate (Roche Folate III assay, code 04476433190). Among these subjects, three had concentrations > 20 µg/L, and the remaining 535, a median concentration of 6.0 µg/L, with 2.5th and 97.5th percentile limits at 3.3 and 13.9 µg/L, respectively. Figure 4 reports the frequency distribution. There was no correlation between serum folate and blood Hb concentrations and between folate and the mean corpuscular volume of erythrocytes in these subjects. Interestingly, 100 (18.6%) of these apparently healthy individuals were below the assay manufacturer's declared lower reference limit (4.6 µg/L) that was apparently too high for our population. On the other hand, by defining the adequate status of folate according to Dhonukshe-Rutten et al. [28] as serum concentrations > 6.6 µg/L, we observed that only 40.7% of subjects seemed to have an adequate folate intake from diet. A significant
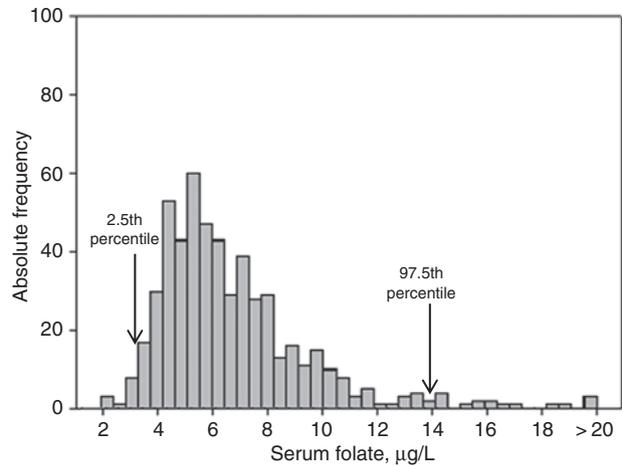
Figure 4: Frequency distribution of folate concentrations in serum of 538 healthy blood donors.
Arrows indicate the 2.5th (3.3 µg/L) and the 97.5th (13.9 µg/L) percentile of value distribution.

increase (p < 0.01) of folate concentrations in serum was shown with age increase, in female gender, and in non-Italian individuals.

Appropriateness of test request

In agreement with previous reporting claiming an overuse/misuse of serum folate testing [1, 4], we confirmed an excess of test requests in our institution quite equally distributed between different population subsets and often consistent with screening purposes. Furthermore, according to our data, the proportion of subjects undergoing folate testing during or just after vitamin supplementation seems to be quite relevant, even if testing folate in these patients is probably useless for some consolidated reasons:

(a) except for patients with pernicious anemia, an intake of synthetic folic acid is unlikely to be associated with adverse events [21];

(b) there are no data assuring that the available assays can reliably detect serum folate absolute concentrations related to the maximum tolerable intake level of folic acid (i.e. 5 mg/day in the general population or 1 mg/day in the elderly and in patients with pernicious anemia) [21];

(c) serum folate concentrations may rapidly fall down after discontinuing the assumption [76].

The data by Hao et al. [76] support the doubts on the effectiveness of monitoring folic acid supplementation. These authors showed that in healthy women, the

supplementation with 0.4 mg/day corresponds to circulating folate concentrations of approximately 16 µg/L, measured 3 months after starting ('plateau level'). No further increase is recorded at 6 months, whereas a rapid decrease (– 55% in average) occurs 3 months after discontinuing supplementation. For higher folic acid doses (i.e. 4 mg/day, which is close to the tolerable upper intake level), serum folate concentrations amounted to 24 µg/L after the first 3 months and showed a far sudden decrease (– 66%) after cessation [76].

In agreement with these experimental data, we can argue that there is no suitable cost-effectiveness in monitoring the vitamin supplementation by folate testing. Furthermore, considering that the folic acid intake is no harmful in the general population and only patients with pernicious anemia may be potentially affected if supplemented with > 1 mg/day (corresponding to a serum plateau level of ~ 20 µg/L), the dilution of samples with folate concentrations > 20 µg/L (i.e. the upper calibration limit for several assays), once the test is executed, to provide definite concentrations within 40 µg/L (corresponding to the highest folic acid intake) is also not cost-effective. On the other hand, it is proved that, by discontinuing intake, folate retesting is useless to evaluate the actual correction of the deficit [77].

In summary, the serum folate test is inappropriately ordered when

(a) there is no specific risk condition/suspicion for folate deficiency/inadequate status (Table 1);
(b) the subject is undergoing or has recently undergone folic acid supplementation;
(c) retesting at 3–6 months to evaluate the actual correction of the deficit after intake discontinuation.

By applying these recommendations on the current folate testing strategy applied in our institution, we can estimate a ~ 50% saving in laboratory costs.

Estimating folate deficiency and need to predict the effect of assay recalibration

In the general population, serum folate testing is frequently used to screen for vitamin deficiency. In our experience, ~ 94% of requests are indeed associated to those for vitamin B_{12} testing. According to our data, the prevalence of subjects exhibiting folate concentrations below the lower reference limit is actually remarkable in any evaluated setting (inpatients, ~ 25%; outpatients, ~ 17%; healthy subjects, 18.6%), always quite far from estimates in fortified countries [6]. How this frequency is directly connected with the prevalence of folate deficiency and how much it is conversely dependent from the correctness of cut-off establishment and its usefulness and from accuracy of folate assays is, however, unknown.

It has long been known that the actual prevalence of folate deficiency in the population may significantly shift with the reformulation of assays [78]. This is troublesome when monitoring over time trends in folate concentration distributions to promote public health intervention and policies on the population [26, 63, 64]. To improve harmonization of assays, some commercial folate methods have recently undergone recalibration to the NIBSC 03/178 International Standard in order to fulfil the European Union Directive on in vitro diagnostic medical devices that asks diagnostic manufacturers to ensure traceability of their analytical systems to recognized higher-order references [79]. However, tracing back the calibration of commercial assays to a new reference material can modify results and actually alter their relation to existing reference intervals and decision limits, invalidating the clinical decision-making criteria currently used [80–82]. In fact, we have recently experienced this problem when Roche Diagnostics has replaced the Folate III assay code 04476433190 (which was home-made calibrated) by the Folate III assay code 07559992190 (traceable to NIBSC 03/178 material). After the kit replacement, we immediately recorded a significant change in the average folate value measured on a fresh-frozen serum pool used daily to check the long-term assay imprecision, with a mean underestimation of 28.8% of total folate when measured by the new version of the assay (6.6 vs. 4.7 µg/L, respectively). We confirmed the effect of assay recalibration in providing lower folate results by a preliminary comparison study on 15 clinical samples, aliquoted and stored at – 80 °C, measured by each assay. Comparing the previous with the new assay, the mean (±SD) folate concentrations resulted 6.6 (±0.5) vs. 4.7 (±0.4) µg/L, respectively (average bias, – 28.8%). A stronger proof was finally derived from the regional EQA program where, by cumulating interlaboratory data generated by participants using the Roche assay, a dichotomous distribution of total folate results became evident. After partitioning data for assay code, the obtained mean (±SD) for laboratories using code 04476433190 assay (n = 6) was 4.0 (±0.4) µg/L vs. 2.0 (±0.5) µg/L for code 07559992190 laboratories (n = 30) [data from EQA exercise no. 4/2016]. Thus, we can speculate that at a folate concentration around the lower reference limit of the old assay, a positive bias of 50% vs. the new assay can be observed.

Given this large variation in folate results, it is very relevant to predict the impact due to the introduction of the recalibrated assay on the prevalence of folate deficiency with respect to the previous generation assay. In the modified package insert, the assay manufacturer is recommending a new lower reference limit (3.9 μg/L) for the interpretation of serum folate results in European populations. Taking into account the ~50% between-assay difference experimentally found at this concentration level, the shift from 4.6 μg/L (old calibration) to 3.9 μg/L (new calibration assay) appears, however, to be inconsistent. Consequently, a misleading overestimate of the prevalence of folate deficiency is expected if the recalibrated Roche assay will be used together the manufacturer's newly recommended lower reference limit. New experimental data from healthy blood donors (similar to those presented in Figure 4 for the former assay) have, therefore, to be quickly obtained with the recalibrated assay in order to accurately define the reference distribution in the proper population and derive decisional strategies for folic acid supplementation. This might also require the (re)formulation of safe and effective health policies that are patient-focused and seek to achieve the best outcome.

Concluding remarks

Today, the evaluation of folate status and intake still represents an important aspect that healthcare systems of several European countries have to deal with. Several surveys have reported that the folate intake and status in most European countries are suboptimal to ensure physiologic growth and fetal development and maintenance of optimal health [28]. Considering the poor stability and incomplete bioavailability of natural food folates when compared to the synthetic form, approaches to optimize vitamin intake and improve health population outcomes, in particular focusing on the NTD risk reduction, have to be considered [41, 42, 72]. Some authors agree with the introduction of mandatory fortification following the US and Canada policies, while others boost for the supplementation with folic acid capsules of groups at risk (i.e. elderly and women of childbearing age) [83, 84]. In general, in policy decisions, Europe shows a greater caution than North America region, dictated by the safety issues of folic acid supplementation/fortification [32, 85]. Concerns have been raised about the potential adverse effects of high circulating concentrations of folic acid and of traces of UMFA, the latter not reliably detectable by clinical assays [86–88]. Finally, the information derived

from the recent medical literature showing no decrease in the incidence of NTD in Europe [89] should be considered in promoting or not supplementation strategies.

The ability to develop regional or national consensus strategies on populations at risk and to promote evidence-based health policy-making are contingent on having reliable data and this cannot disregard the knowledge of biomarkers of folate status and of related assays [25]. Reviews by multidisciplinary panel of authoritative experts have focused on the pre-analytical, analytical, and post-analytical issues related to the biomarkers of folate status, clarifying that the choice of the method (serum or RBC folate) depends on the features of the investigated population (prevalence of deficiency, spread of fortified staple food) and on the outcome (assessment of the individual folate status/risk for deficiency or characterization of the population intake/status) [6, 26, 28, 90]. For surveys on the general population, the measurement of serum folate may not be a reliable method, but it becomes the test of choice for the clinical use and the assessment of individual deficiency [26]. Fulfilling recommendations on the pre-analytical management of samples is mandatory to obtain reliable results, but it is their interpretation that is central for identifying individual risk for folate deficiency. In addition to the limited usefulness of a cut-off value (i.e. the lower reference limit) due to the wide inter-individual biological variability of folate, we can doubt on the reliability of the reference intervals reported by assay manufacturers and recommended for European populations [28, 73]. As across European countries, the voluntary and liberal fortification policies are greatly heterogeneous, the estimation of reference intervals is likely to be conditioned by the possible inclusion in the reference group of subjects consuming fortified staple foodstuffs [28, 37]. Furthermore, results obtained by investigating and comparing folate intake across populations have suggested shifting the outcome from detecting folate deficiency to assessing folate status at the individual level. As reported by Bailey et al. [91], meeting a criterion for assessing individual folate deficiency (or its prevalence in a population) is not synonymous of optimal folate status also in the "not deficient" portion of the population [91]. In several settings, although an out-and-out folate deficiency may be rare, a suboptimal folate status impairing health conditions may be more common and, for this reason, the test ordering should be appropriately addressed.

A cost-effective use of folate testing in the clinical setting implies the knowledge of (a) what biomarker order, (b) what population investigate, and (c) when or if retest. All main documents agree on the use of serum folate testing without adding homocysteine test when the

outcome is to evaluate the individual risk of folate deficiency/status [6, 26]. In countries undergoing mandatory fortification, the population exhibits a distribution of circulating folate vitamers different from European countries and the prevalence of deficiency in the general population is far lower [52]. Accordingly, in fortified populations the test request should be restricted to some groups at risk, but not recommended in both inpatients and outpatients [5]. In European countries, particularly in those not adopting national fortification policies, these recommendations should be cautionary considered, since there is a sounded evidence of a suboptimal folate intake in the general population. Our local data further show a considerable prevalence of risk for deficiency in all tested populations (outpatients, inpatients, and blood donors) and suggest that in all these subsets folate testing may be ordered according to the individual risk and to the suspect for an inadequate intake, possibly requiring correction with folic acid supplementation or simply a change in dietary habits. We should remind that several European National Institutes of Health are working to promote the implementation of healthcare policies to enhance the general availability of fortified foods with the final aim to prevent in the population the increase of clinical conditions and genetic defects linked to an impaired folate status [42, 92–94]. Starting from 2005, the National Health System in Italy enables healthcare providers to prescribe folic acid supplements without any charge to all women of childbearing age (the cost to the National Health System of a box containing 28 pills of 400 μg folic acid is ~2 €) [41]. Facing on data on the prevalence of folate deficiency, the need for a possible extension of this policy to other groups at risk (e.g. the elderly) may be considered. Finally, we should remind that it is useless to measure and monitor by retesting serum folate in individuals undergoing supplementation, since this cannot warrant a long-term supply (>3 months) (examples C and D in Figure 3) [75, 76]. Furthermore, repeating folate measurement is also useless in individuals with strongly suspected folate deficiency (example F in Figure 3) [77].

In conclusion, with respect to fortified countries, in European populations, there is evidence on the need and relevance to perform serum folate testing without specific restrictions. According to the data analysis, it is, however, mandatory to boost for more appropriate requests to curb the excess of costs linked to the spreading of folate testing. We should emphasize that the effectiveness of the test is maximized when oriented to subjects suggestive or at risk for deficiency and low or absent when used as tool for screening or serial monitoring of vitamin intake or supplementation. In the latter case, to prevent retesting,

a computer-based-limiting management strategy may be considered [95]. Finally, we should be aware that the recent recalibration of several commercial assays in view of harmonization policies is likely significantly shifting results and, if not correctly managed, potentially affecting the detection of folate deficiency in individuals at risk.

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