Narrative Review

Managing folate deficiency implies filling the gap between laboratory and clinical assessment

Simona Ferraro [a], Giacomo Biganzoli [b, *], Michele Gringeri [c], Sonia Radice [c], Alessandra Stefania Rizzuto [d], Carla Carnovale [c], Elia Mario Biganzoli [b, 1], Emilio Clementi [c, e, 1]

[a] *Endocrinology Laboratory Unit, "Luigi Sacco" University Hospital, Università degli Studi di Milano, ASST Fatebenefratelli-Sacco, Milan, Italy*
[b] *Medical Statistics Unit, Department of Biomedical and Clinical Sciences L. Sacco, "Luigi Sacco" University Hospital, Università degli Studi di Milano, Milan, Italy*
[c] *Clinical Pharmacology Unit, Department of Biomedical and Clinical Sciences L. Sacco, "Luigi Sacco" University Hospital, Università degli Studi di Milano, Milan, Italy*
[d] *Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy*
[e] *Scientific Institute, IRCCS E. Medea, Bosisio Parini, LC, Italy*

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SUMMARY

The characterization of folate status in subjects at risk of deficiency and with altered vitamin homeostasis is crucial to endorse preventive intervention health policies, especially in developed countries. Several physiological changes (*i.e.* pregnancy), clinical situations and diseases have been associated to increased requirement, impaired intake and absorption of folate. However clinical practice guidelines (CPG) endorse folic acid supplementation generally discarding the use of its determination in serum to assess the risk of deficiency and/or its concentration at baseline. Poor confidence on the diagnostic accuracy of serum folate assays still persists in the current CPGs although recent standardization efforts have greatly improved inter-method variability and precision. In this review we critically appraise the methodological issues concerning laboratory folate determination and the evidence on the potential adverse effects of folic acid exposure. The final aim is to build a sound background to promote serum folate-based cost-effective health care policies by optimizing folic acid supplementation in subjects at risk of deficiency and with altered folate homeostasis. Our first result was to adjust in relation to current serum folate assays the thresholds reported by CPGs as index of folate status, defined on the association with metabolic and hematologic indicators. We identify a statistically significant difference between the estimated thresholds and accordingly show that the assessment of folate status actually changes in relation to the assay employed. The use of the method-dependent thresholds here reported may pragmatically endorse the stewardship of folic acid supplementation in clinical practice and increase the cost-effectiveness of health care policies.

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1. Introduction

Folate deficiency has been a primary focus of research over the past few years leading to a more refined definition of optimal status and intake from a clinical and epidemiological perspective, [1–3]. Survey programs on the assessment of folate status in the population by key stakeholders in healthcare policy and decision-making have sparked an intriguing debate regarding the laboratory determination of folate concentrations, the assessment of optimal risk thresholds for its overt and possible deficiency, and the need to implement specific food fortification policies with the ultimate goal of improving health care outcomes [1,2,4,5].

The current recommendations released by the World Health Organization (WHO) still state that the determination of folate in serum through the employment of competitive protein binding (CPB) assays (*i.e.* commercially available for clinical routine) is not effective to assess the deficiency state compared to other technologies (*i.e.* red blood cell (RBC) and microbiological assays (MBA)) [6]. Only serum folate re-testing performed over the course of one month was endorsed, when used to confirm low vitamin status or

Abbreviations

WHO	World Health Organization
CPB	competitive protein binding
RBC	red blood cell
MBA	microbiological assays
FA	folic acid
5MeTHF	5-methyltetrahydrofolic acid
UK NEQAS	United Kingdom National External Quality Assessment Service
CV	coefficient of variation
NHANES	National Health and Nutrition Examination Survey
QP II	Quantaphase II
NIST	National Institute of Standards and Technology
SRM	standard reference material
CI	confidence intervals
IS	International Standard
UMFA	Unmetabolized FA
UL	Upper intake Level
ICSRs	Individual Case Safety Reports
FDA	Food and Drug Administration
FAERS	Food and Drug Administration Adverse Event Reporting System
ADR	Adverse drug reaction reports

depletion [6,7]. Furthermore, some clinical practice guidelines (CPG) do not require serum folate testing prior to initiating supplementation [8].

The MBA is still considered a reference method as it is used to establish folate risk thresholds for neural tube defects, being erroneously considered unbiased and sufficiently precise [9—12]. Accordingly, the current MBAs are reported to be eligible for assessing the population folate status to endorse intervention strategies, despite being considered unsuitable for the clinical laboratory setting, which preferably should require RBC and serum determinations which have indeed replaced MBAs [1,9—13]. A sound consensus has been recently achieved on the exclusive use of serum folate assays as test of choice for the clinical use and for the assessment of the individual deficiency in the population at risk [14,15]. This choice has been partly supported by the introduction of fully automated, high-throughput assays and fast turn-around times since both timely and accurate laboratory test results are a cornerstone of effective diagnosis and treatment of patients [11,16]. The standardization efforts recently required to harmonize different CPB assays have actually improved method accuracy and results' consistency and have strongly encouraged their exclusive use in laboratory practice [14,15,17]. Notably, baseline data retrieved from clinical-laboratory databases now represent valuable and cost-effective research resources for examining large cohorts of individuals, even taking into account that historical population-based studies were affected by several drawbacks [18,19].

Further steps have been made to maximize the cost-effectiveness of serum folate determination and folic acid (FA) supplementation by endorsing appropriate test request (*i.e.*, directed at subjects whom are at risk of deficiency), pre-analytical phase (*i.e.*, sample drawing and management) and post-analytical management of the results finalized to a correct risk assessment [1,20—22].

Indeed, applying serum folate assays may help decision making on the need to start, discontinue or modulate FA supplementation and thus limit excessive FA exposure [23,24].

Lastly, the heterogeneity of the measurand (*i.e.* total folate) should be acknowledged to predict the diagnostic performance and the clinical application of CPB assays, since different circulating forms may occur in patients undergoing supplementation or not [25].

Here, we critically evaluate all these issues providing a discussion focused on filling the gap between clinical and laboratory assessment of folate deficiency to promote cost-effective health care policies. (see Fig. 1)

2. Laboratory folate methods: issues and clarifications

2.1. Appropriateness of test ordering

In European countries that do not fulfill FA fortification policies, the evaluation of folate status and intake still represents a critical aspect that healthcare systems must deal with, to ensure physiologic growth, fetal development and maintenance of good health [1,26]. In hospitalized patients a high frequency of folate test ordering has been observed, partly related to anemia workup and mainly associated to other comorbidities not causally linked to folate deficiency [27]. Twenty-five percent of inpatients have been identified to be at high risk of folate deficiency and a suboptimal folate intake may occasionally characterize 20% of general population and ~50% of hospitalized patients [1,15].

In any case, folate assaying is not to be ordered for screening, as the cost-effectiveness is maximized only if directed at subjects at risk of deficiency. Several conditions potentially increase folate requirement (*i.e.* pregnancy, lactation), impaired folate intake (*i.e.* malnutrition), and folate absorption (*i.e.* gastrointestinal disease/surgery, genetic factors, alcohol abuse, use of several drugs such as metformin, proton pump inhibitors, anticonvulsants). Several clinical situations further imply folate testing as evidence of anemia and/or macrocytosis, neuropsychiatric symptoms, cognitive disorders, peripheral neuropathy, chronic kidney disease and renal dialysis, malignancy, myeloid and non-myeloid malignancies [1].

Recent CPGs have restricted folate testing to individuals with abnormal hematological profiles and suspected gastrointestinal disorders associated with malabsorption and dementia, whereas it is disregarded in pregnant women prior to initiating supplementation [28—30].

2.2. Folate assays in clinical laboratories

It is now evident that the majority of CPGs do not include recommendations concerning folate testing informed by high quality and updated evidence, since many recommendations are based on clinical consensus or expert opinion alone [31]. Yet, there is great disagreement among experts regarding the optimal folate assay to use and thus some documents still recommend the use of MBAs [3]. The National Pathology Alliance supports the use of serum methods considered of scant diagnostic value if compared to RBC assays by the Royal Pathologist of Australia and the WHO [6,32,33]. It has also been made clear that MBAs and liquid chromatography—tandem mass spectrometry are to be confined to research projects for scientific purposes only [11,13]. RBC and serum folate CPB assays have been set up for clinical routine applications and currently the majority of European laboratories (*i.e.* twelve out of one) exclusively perform the determination on serum, although the same reagents may be applied to both matrices [34].

CPB folate assaying is *per se* an inexpensive diagnostic tool and the laboratory costs are substantially equal for serum and RBC methods, but it might trigger higher healthcare costs and patients risks (*i.e.*, addition of second level tests, unnecessary supplementation) if the concentrations are not accurately detected and the results are not appropriately interpreted and managed [29].

Accordingly, the pre-analytical and analytical variables affecting both folate methods have been comprehensively reviewed and

compared, and now a general consensus has been achieved on the greater reliability of serum vs RBC methods [14]. The measurement of total folate, including all vitamers, is undoubtedly challenging for both serum and RBC methods, considering that pH, temperature and photodegradation are critical parameters in order to obtain reliable folate results [14]. A mandatory prerequisite is to prevent the loss of 5-methyltetrahydrofolic acid (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). For serum folate determination, fasting is required as well as the rejection of hemolyzed samples [free hemoglobin (Hb) ≥ 0.25 g/L] [1]. For RBC folate, the pre-analytical treatment of the samples, the *in vitro* conversion of 5MeTHF polyglutamates to monoglutamate, the effects of the hematocrit, the hemoglobin saturation and the C677T polymorphism of MTHFR, represent further and greater limitations with respect to serum determination [1,14]. Recent clinical evidence has defined that fasting serum folate measurement overcomes RBC determination in: a) identifying folate deficiency even in particular conditions (*i.e.* vitamin B12 deficiency, patients on hemodialysis in pre-dialysis samples), b) providing information about folate status over a long time frame, c) reflecting hematological and metabolic response to FA supplementation and fortification, d) discriminating between different levels of FA exposure, e) predicting hyperhomocysteinemia and toxicity from capecitabine [1,14,15]. There is, however, no firm indication as to whether either marker is a good index of (or proxy for) liver FA storage [14].

Undoubtedly, over the past decades the high inter-assay and inter-laboratory variability reported for folate CPB assays has caused drawbacks among pathology authorities and key stakeholders in the setting of healthcare policy [35]. This is reasonable when considering that imprecision and bias exceeding the goals established on biological variability likely affect the estimation of the risk of deficiency and the clinical outcome [17,22,35].

Recent data by the United Kingdom National External Quality Assessment Service (UK NEQAS) survey show, for serum CPB assays, fivefold lower inter-methods coefficient of variations (CV) than for RBC assays [34]. Furthermore, over the past five years the standardization efforts have greatly improved the harmonization of folate results obtained from serum CPB assays, allowing to achieve a mean inter-assay variability (CV ~12%) fairly equal to the one reported for other tests covering a crucial role in decision making (*i.e.* prostate specific antigen) [36].

3. Overcoming the impact of inter-method variability on decision thresholds

3.1. Tracking a traceability chain

Some studies have tried to predict the impact of the inter-method bias on the assessment of the individual risk of deficiency. In other words, they have tried to assess if the shift to a different CPB method may change the recommendation on FA supplementation. These studies have endorsed the use of method-dependent decision thresholds further adjusted to the recalibration changes [17,20−22]. Indeed some marketed assays might underestimate the risk of folate deficiency in the clinical practice (*i.e.* over-recovery of ~+5−9% of the WHO International Standard (IS) 03/178 with assigned value of 5.3 μg/L) and in general the imprecision of all assays is noticeable around the decision cut-off point (~10%), although within the minimum goal for the clinical application of folate results (*i.e.* <1/2 of the intra-individual variability [CVw = 24%]) [1,17,22].

Some clinical studies further foster the importance of including multiple thresholds as index of folate status to identify who may gain a beneficial effect from supplementation and to individualize

treatment, bringing into question the cost-effectiveness of the extensive supplementation of unselected subjects [37,38].

Current risk thresholds of folate deficiency have been defined on the basis of: a) the association with the evidence of megaloblastic anemia, or b) the correlation with the measurement of RBC folate or of a metabolic indicator (*i.e.* homocysteine) [6,15,38].

By using macrocytic anemia as a hematological indicator, the WHO CPGs recommend that subjects with serum concentrations below 3 and 6 μg/L may be classified with having an overt and possible deficiency, respectively, whereas those with values falling within the range of 6−20 and >20 μg/L may be considered to have normal and elevated values [6]. By using homocysteine concentrations as a metabolic indicator, the cut-off recommended by WHO for folate deficiency is 4 μg/L [6]. Notably, the folate thresholds associated to a hematological indicator were first proposed ~50 years ago by using MBAs; the folate threshold associated to homocysteine increase was retrieved by the National Health and Nutrition Examination Survey (NHANES) III and estimated by Bio-Rad Quantaphase II (QP II) serum isotopic assay [4,39−41].

It is no easy task to reliably predict the serum folate thresholds from estimates obtained by the traditional MBAs and isotopic assays, since a real traceability chain has been only recently implemented; however, the recoveries of the different assays vs the international reference materials may be of aid [4]. The traditional MBA overestimated the folate concentration of 2.65 μg/L (National Institute of Standards and Technology (NIST) standard reference material (SRM) 1955) by ~25% with respect to the Bio-Rad QP II [4]. The latter has been further replaced by subsequent generations of automated assays, the current one being calibrated against WHO IS 03/178 for most marketed methods (*i.e.* Roche, Abbott, Beckman). For Roche (Folate III) this implied an average underestimation of folate concentrations by 28.8% with respect to the previous generation assay (Roche Folate II) [1]. Bio-Rad QP II and current Folate III Roche assays are reported to underestimate WHO IS 03/178 by ~10 and 8.3% respectively [4,17]. Taking into account the recoveries previously reported, we are able to convert the serum folate thresholds declared by the WHO and those validated by recent studies into the corresponding concentrations detected by the current Folate III Roche assays (Table 1) [6,15,38].

3.2. Method-dependent serum folate thresholds for recommending supplementation

The investigation of the state of harmonization of currently marketed serum folate assays (*i.e.* Abbott Diagnostic Alinity, Beckman Coulter DxI Access, Siemens Healthcare Diagnostic Advia Centaur) has previously shown a constant relative bias of the results obtained from the 4 analytical platforms when compared to their median values [17]. This was a basic prerequisite to allow a reliable conversion of the results obtained from Roche Folate III assays into the corresponding concentrations detected by the other methods which was performed in the present work by applying a Passing-Bablok regression analysis on the same data set of serum pool results obtained from the 3 above mentioned analytical platforms compared to the Roche Folate III reference (Fig. 2). The extended results from such a recalibration assessment are reported in the online Supplementary.

Table 2 reports the method-dependent folate thresholds as index of folate status together with the 95% confidence intervals (CI) estimated by the use of bootstrap method. For the rapid conversion a nomogram plot is also reported, in Fig. 3.

Observing these results, some critical issues emerge.

Firstly, we may appraise that there is statistical evidence of a difference between the thresholds on different assays and that the assessment of folate status actually changes in relation to the assay

Table 1
Estimation of folate thresholds as index of folate status according to running Roche assay (Folate III).

Established thresholds (μg/L)	Reference	Assay	Indication	Roche Folate III thresholds (μg/L)
<3*	WHO CPG [6]	MBA	Overt deficiency	2.3
<4**	WHO CPG [6]	Bio-Rad QP II	Folate depletion concentrations impairing metabolic indices	4.1
3–<6	WHO CPG [6]	MBA	Possible deficiency	2.3–<4.5
6	De Bruyn et al. [15]	Roche Folate II	Folate depletion concentrations associated to hematological abnormalities	4.3
8	De Bruyn et al. [15]	Roche Folate II	Folate depletion concentrations impairing metabolic indices	5.7
6–20	WHO CPG [6]	MBA	Normal balance	4.3–20.0
>20	WHO CPG	MBA	Elevated	>20
11.3[a]	Chen et al. [38]	MBA	Preventing neural tube defects in pregnancy	8.7

Acronyms: MBA, microbiological assay; QP II, Bio-Rad Quantaphase II; WHO, World Health Organization; CPG, clinical practice guidelines.
Conversion factors:
Bio-Rad QP II vs MAB = −25%.
Roche folate III vs Bio-Rad QP II = +1.7%.
Roche folate III vs Roche Folate II = −28.8%.
  [a] Originally expressed as nmol/L: 1 ng/mL = 2.265 nmol/L.
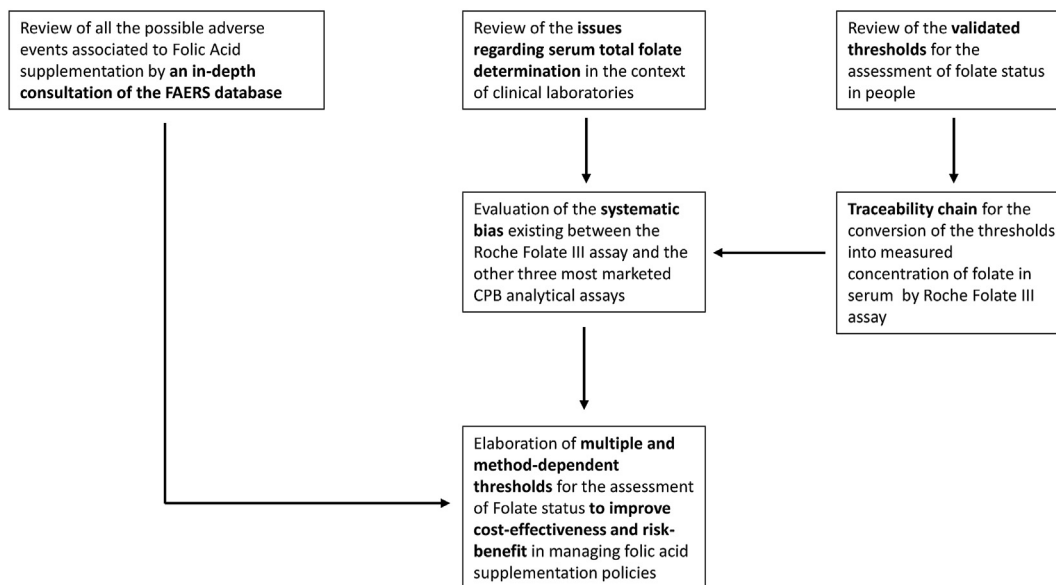
General Workflow

Fig. 1. General workflow to assist the reader in understanding the key thrust of the presented review.

employed. Accordingly, the use of the method-dependent thresholds here reported may pragmatically endorse the stewardship of FA supplementation in the clinical practice and increase the cost-effectiveness of health care policies.

Secondly, in these tables the estimated concentrations of serum total folate are exclusively reported as μg/L and we discourage the use of nmol/L when folate determination is based on CPB assays. Indeed, the measurand is heterogeneous and the recognition of various vitamers in serum samples depends on the assay design (*i.e.* CPB used and vitamers extracted are not declared in the method sheet by the manufacturers) and on the distribution of folate species which widely varies according to individual conditions (*i.e.* dietary habits, use of fortified foods or FA consumption) [13].

An additional issue is that the upper limit of the calibration curve (20 μg/L) of the assays has been generally assumed by the CPGs as index of folate elevation, although all current methods may characterize concentrations up to 40 μg/L after applying the recommended dilution [6].

According to our data, subjects undergoing FA supplementation are characterized by serum folate concentrations >12 μg/L, and in ~10% of all tested patients serum concentrations increase above 20 μg/L [1,20]. To our knowledge, no studies have been performed so far to assess the impact of serum folate concentrations ≥20 μg/L on patients' outcome or to investigate the association and possible causal relationship between these high serum concentrations and adverse effects possibly linked to FA supplementation.

4. Adverse health outcomes associated to high FA exposure

4.1. Evidence from literature

The investigation of adverse effects possibly caused by excessive FA intake has been discouraged due to lack of confidence in the diagnostic value of folate assays and in the defined cut-off points for the assessment of folate status. However, protective effects of supplementation have been rigorously demonstrated [6,24,42–45].
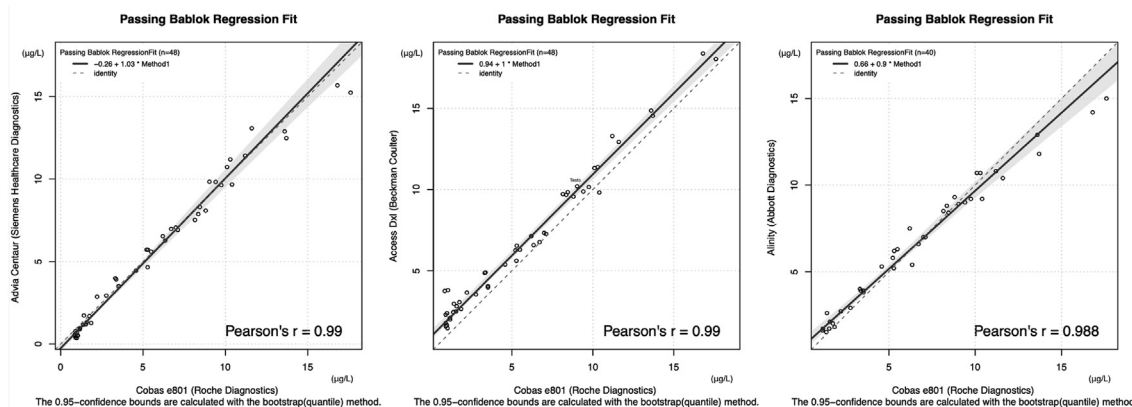
Fig. 2. Passing-Bablok regression analysis performed on the data set of serum pool results obtained from Abbott Diagnostic Alinity, Beckman Coulter DxI Access, Siemens Healthcare Diagnostic Advia Centaur compared to the Roche Folate III reference.

Table 2
Estimation of folate thresholds as index of folate status according to running assays.

| Roche Diagnostics Cobas e801 (Folate III) μg/L	Abbott Diagnostics Alinity i μg/L (95% CI)	Beckman Coulter Dxl Access μg/L (95% CI)	Siemens Healthcare Diagnostics Advia Centaur μg/L (95% CI)	Indication
≤2.3	<2.73 (2.53, 3.06)	<3.24 (3.08, 3.57)	<2.11 (1.99–2.30)	Overt deficiency
2.4–4.5	2.82 (2.62, 3.14)–4.72 (4.57, 4.94)	3.34 (3.18, 3.67)–5.43 (5.29, 5.74)	2.21 (2.10, 2.40)–4.38 (4.14, 4.55)	Impaired folate balance
4.6–5.7	4.72 (4.54, 4.94)–5.80 (5.58, 6.07)	5.53 (5.39, 5.84)–6.63 (6.47, 6.93)	4.48 (4.24, 4.66)–5.61 (5.31, 5.86)	Suboptimal folate status
5.8–12	5.89 (5.67–6.16)–11.47 (10.88–12.11)	6.73 (6.57, 7.02)–12.93 (12.55, 13.29)	5.72 (5.40, 5.97)–12.10 (11.36, 12.75)	Normal
>12–20	11.47 (10.88–12.11)–18.68 (17.50, 19.82)	12.93 (12.55, 13.29)–20.93 (20.22, 21.61)	>12.10 (11.36, 12.75)–20.34 (19.04, 21.51)	Increased folate intake likely associated to supplementation
>20	18.68 (17.50, 19.82)	20.93 (20.22, 21.61)	20.34 (19.04, 21.51)	Far increased folate intake due to supplementation
8.7	8.50 (8.12, 8.94)	9.63 (9.38, 9.93)	8.70 (8.20–9.14)	Minimal folate concentration to prevent neural tube defects in pregnancy
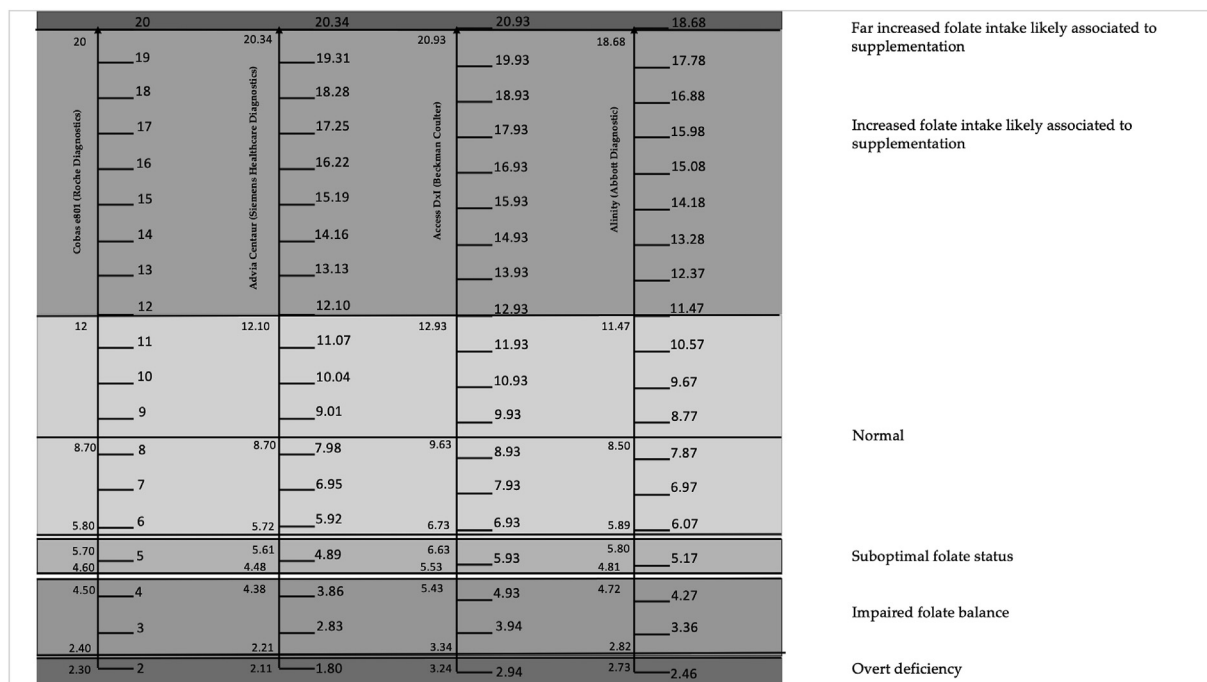
Fig. 3. Nomogram plot reporting the indication of the folate status according to folate results (μg/L) obtained from the four assays.

FA has greater chemical stability and bioavailability compared to folate deriving from natural sources (85% vs 50%), thus being more effective in inducing an early elevation of blood concentrations and of tissue stores [46]. A supplementation of 200 μg/day implies the saturation of the absorptive ability of the intestine and the presence in the blood of Unmetabolized FA (UMFA) has not been yet associated to functional and health consequences [47]. In the 1940s, two case reports showed that FA supplementation failed to adequately treat pernicious anemias and was associated to an exacerbation of neurological complications [48,49]. Accordingly, in 1998 the American Institute of Medicine set a FA tolerable Upper intake Level (UL) from fortified foods or supplements of 1 mg/day for adults, based on the observed lowest adverse effect level [50]. A recent review however has questioned the scientific basis for setting an UL, reporting no evidence of association between the exacerbation of the neurological progression and the administered FA dose [51].

Some studies have suggested a possible association between high FA intake and increased risk of cancer progression in individuals with preexisting neoplasms considering the crucial role of folate in cell proliferation and the relationship between excess of folate and impairment of immune function [52—54] However, several meta-analyses and systematic reviews have not demonstrated a causal relationship between FA intake and cancer risk [55—57].

No evidence has further supported the relationship between FA consumption in pregnancy and the onset of asthma and wheeze, diabetes-related disorders and thyroid diseases in the offspring [45,58—60]. Conversely, children aged 8—9 years, born from mothers who received daily supplementation of 400 μg of FA during pregnancy, were reported to exhibit a significantly lower risk of metabolic syndrome and of kidney dysfunction [61].

The effect of FA on offspring health seems to be linked to its critical role in fetal development and the presence of a homozygous 19bp deletion mutation in DHFR gene, encoding for an enzyme responsible for the conversion of UMFA to intracellular folate. Patients with such a mutation seem to be more susceptible to the adverse effects of high folate status (*i.e.* retinoblastoma, cognitive impairment) [62—64].

Of note, literature only describes long term effects associated to high FA intake and in 2015 the US National Toxicology Program thoroughly assessed that there is not enough evidence to support any specific toxic association [65].

4.2. Pharmacovigilance analysis

We investigated the risk of FA toxicity through the analysis of Individual Case Safety Reports (ICSRs) retrieved from the Food and Drug Administration Adverse Event Reporting System (FAERS) database, which is the largest pharmacovigilance database available, since it collects adverse drug reaction (ADR) reports regarding drugs approved by FDA submitted from all countries attending the WHO Program for International Drug Monitoring [66]: if occurring within the USA, both serious and non-serious ADRs are reported, while if occurring in a third country, only serious ADRs are recorded in FAERS.

In order to shed light on FA use in a real life setting, all ICSRs reporting FA (*i.e.*, WHO Anatomical Therapeutic Chemical classification code B03BB001) as primary or secondary suspect (*i.e.* held responsible of the ADR occurrence by the reporter) submitted from January 1st 2010 to December 31st 2020 were retrieved [67]. After a systematic process of data cleaning to remove all duplicates and incomplete records, we selected all ICSRs recording the administration of FA as dietary integration in: a) pregnancy, b) macrocytosis due to folate deficiency, c) rheumatoid arthritis, while excluding those cases reporting an association with antitumoral therapy

(Fig. 4). We thus identified 2038 reports indicating FA as primary or secondary suspect, 49.7% with complete information on the administered dose, 80% were serious and occurred in patients with a median (25th—75th percentile) age of 49 [29—70] years (65.9% female). The mean dose of FA *per* administration and *per* day were 18.2 mg (2.9 dosage units) and 15.9 mg (1.7 dose units) respectively, and in 635 cases (62.7%) the daily intake exceeded the UL.

Out of this group of reports, we selected all 232 ICSRs (11.4%) reporting FA intake as primary suspect of adverse events in a population with a median (25th—75th percentile) age of 43 [26—62] years (73.7% female). The most frequent ADRs reported were rash, urticaria, erythema (27% total), nausea, pruritus, hypersensitivity, dyspnea, flushing (6—10% each), fatigue, abdominal pain, drug ineffective (joint swelling, chest pain, dizziness (3—5% each)). About 81% of these 232 ADRs were classified as serious by the reporter. The outcomes included congenital anomaly (1.3%), death (2.6%), life-threatening conditions (3.0%), and disability (5.2%). We identified 139 cases with complete information on the administered dose: the mean dose *per* administration and *per* day were respectively 18.9 mg (5.2 dosage units) and 13.3 mg (3.2 dosage units). In 67 cases (48.2%) a FA consumption exceeding the therapeutic dose was recorded. Thirty-two IRCS were recorded in pregnant women and all the ADRs were rated as serious, and generally included all those described previously. Cases of spontaneous abortions, premature delivery, vaginal hemorrhage and neonatal drug withdrawal syndrome (~15% each) were also reported.

According to this analysis, the rate of serious ADRs related to FA consumption reported by healthcare practitioners over the past 10 years should not be overlooked.

These data should stimulate the prescribers to exert a close monitoring of FA exposure and of the related effects in pregnant women, considering their high risk of hypertension and insulin-resistance and the potential effects on offspring [68,69]. FA is generally perceived as a harmless dietary supplement, but our data revealed that over 200 patients experienced adverse effects ascribed to FA by the reporter and that most of them were exposed to a daily FA dose above the recommended UL. This evidence is confirmed by a clinical trial investigating the dosage of FA intake for, revealing that ~89% of enrolled patients were exceeding the UL of 1 mg/day [70]. This was partially associated to the additional exposure to dietary FA ($\geq$520 μg/day in 43.4% of participants), since multiple prenatal supplements contained 1000 μg FA.

5. Discussion

The ongoing research allows to ascribe serum folate to the list of circulating markers covering a crucial role in decision making at the individual level, and this now calls into question the need to fill the gap between clinical and laboratory practice [71—74]. Accordingly, the clinical use of this marker requires "statements that include recommendations, intended to optimize patient care, that are informed by a systematic review of evidence and by an assessment of the benefits and harms of alternative care options" [74,75]. Concerning folate measurement, what has surely been lost in the preparation of CPGs is the knowledge of the analytical issues whose impact on the assessment of folate status had been well characterized in the NHANES surveys [4,5].

Our review sheds light on the clinical impact of the analytical performances of CPB assays performed for serum folate evaluation. These currently represent the customary method for the clinical assessment of folate status and have gained relevance thanks to the standardization efforts which have boosted their widespread availability [1,17,20]. We have further considered the potential impact of the analytical performance on the definition of the population at risk of deficiency speculating on the cost-
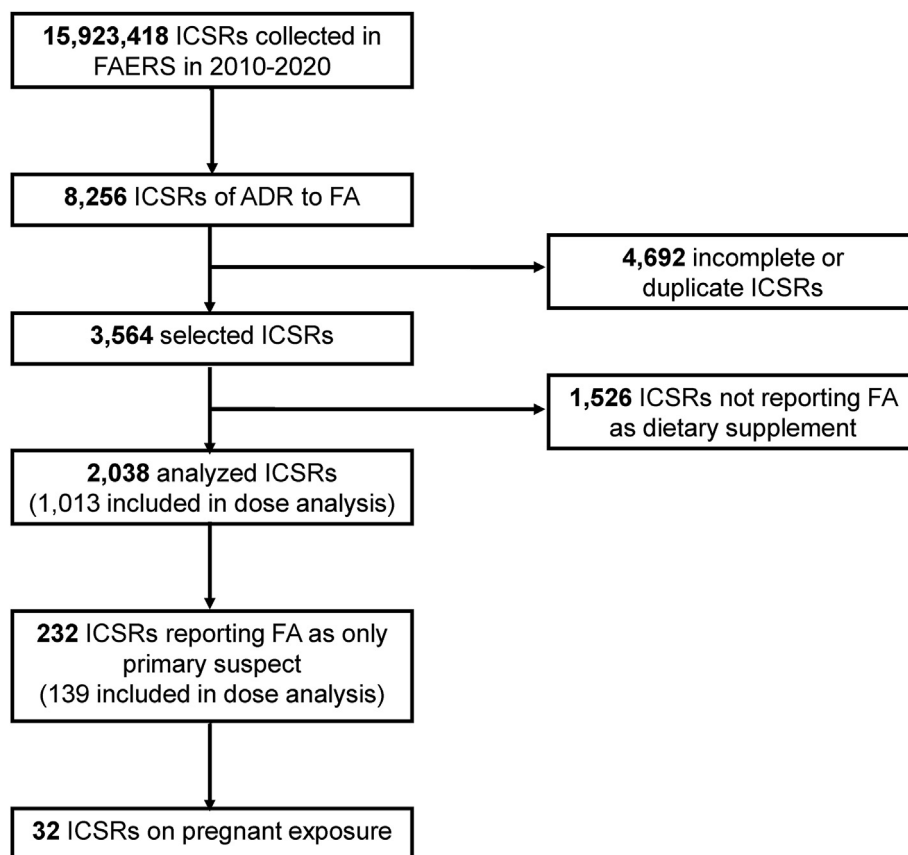
Fig. 4. Flowchart of the selected Individual Case Safety Reports. **Acronyms:** ICSRs, Individual Case Safety Reports; FAERS, Food and Drug Administration Adverse Event Reporting System; ADR, adverse drug reaction; FA, folic acid.

effectiveness of individualizing supplementation based on folate results at baseline.

There is a large number of CPGs (*i.e.* those applying to pregnant women) that recommend FA supplementation without mentioning folate testing, since this is wrongly reported not to be cost-effective [8]. The implication is that supplementation is prescribed to individuals without performing prior measurement of folate levels or assessment of previous FA exposure. Serum folate testing at baseline would actually be crucial for ensuring safety and avoiding the occurrence of side effects, even more so in populations who are at higher risk of prior FA exposure, such as pregnant women who have undergone or are undergoing treatment with prenatal supplements [70].

This review supports changing the recommendations by contributing evidence on the favorable cost-effectiveness ratio of serum folate testing performed by current assays, according to an appropriate test request (*i.e.* high pre-test probability), to ensure a more effective, safe and personalized FA supplementation.

Economic analyses relevant in defining the cost-effectiveness of folate testing have been performed by considering its request for screening purposes (*i.e.* associated to a lowest pre-test probability) and the costs of the folate determination performed by previous generation assays resulting higher compared to those carried out on automated analytical platforms [23]. This had wrongly suggested that direct costs of folate testing might overcome those of FA supplementation, which had not yet been suspected to be associated to relevant side effects. Our reporting on adverse effects following excessive FA exposure based on updated clinical evidence and pharmacovigilance data further endorses the use of folate testing prior to initiating supplementation, to address treatment

length and intensity. Folate retesting during or after having interrupted supplementation should be strongly discouraged. Endogenous folate generally consists of a 3 months' supply. However, when this timeframe has passed, deficiency may occur for an inadequate intake or an increased requirement for the vitamin (*i.e.* pregnancy, lactation) [1]. In these clinical situations serum folate retesting at 3–6 months should be recommended in order to monitor the actual correction of the deficiency and the maintenance of vitamin homeostasis [1].

In doing so, the use of folate determination may greatly increase the cost-effectiveness of health care policies by addressing preventive FA supplementation in developing countries and by optimizing intervention programs in advanced countries [76,77]. Indeed, a serum folate-based characterization of the subset suitable to apply to the intervention program may pragmatically enhance sustainability, compliance and coverage, which are all important criteria in the choice of supplementation regimens in terms of a public health intervention [76,77].

In this review we also underline that the crucial role of serum folate determination in health care depends on the analytical performances of the assays employed (*i.e.* bias and imprecision must be taken into account). These assays should fulfill the goals for the clinical application of folate results based on biological variability data, rather than according to the clinical outcome, since it is commonly thought that the clinical consequences of false positives folate results are relatively small [29].

In the currently available CPGs, poor confidence on the diagnostic accuracy of folate assays and on the thresholds reported for assessing folate status at the individual level still persists. Fundamentally, current CPGs ignore the analytical performances of

available serum folate assays, and further disregard that these methods have changed over the past two decades with a consistent improvement toward harmonization of the results, thanks to the recalibration of the assays to WHO IS. Now we are aware that at a serum folate concentration of ~3.0 μg/L (*i.e.* cut-off point for the diagnosis of deficiency established by the WHO), an imprecision <12% and a bias <11% are allowable for the clinical application of folate results. Anyhow, the poor interchangeability of serum folate results obtained from different assays should be critically appraised by CPGs and a recommendation against the use of harmonized thresholds to address the need of FA supplementation should be released.

Thus, additional relevant evidence generated within this review is the definition of the method-dependent cut off points to characterize folate status and individualize FA supplementation.

Our data demonstrate that folate thresholds as index of overt deficiency may vary by ~50% across the different assays and/or by using the WHO recommended value as baseline. In the case of underestimation, (*i.e.* Roche and Siemens) the clinical consequences might be relatively small if FA is taken in amounts that are not higher than the recommended dietary allowance. In cases in which folate is overestimated, patients would be harmed by being given a risk that is too low as it may inappropriately change decision-making to no further supplementation. In this case the impact on healthcare outcomes may become far more severe in those patients with increased requirement for vitamins, such as pregnancy and lactation. Therefore, the adoption of method-dependent thresholds is crucial in order to personalize FA supplementation and improve the risk-benefit ratio, also taking into account pharmacovigilance data.

Undoubtedly, the use of a spontaneous reporting system database has several intrinsic limitations. FAERS database does not provide the total number of patients exposed to the drug of interest and this prevents from the estimation of the: a) causal relationship between the administered drug and the occurred event, b) incidence of ADRs c) absolute risk of adverse events. Furthermore, the quality of reporting is poor due to its wide heterogeneity, since ICSRs may be either submitted by consumers, or healthcare professionals, or marketing authorization holders. This may imply the introduction of potential biases likely associated to under-reporting (*i.e.* concerning preferentially non-serious ADRs), over-reporting (*i.e.* stimulated reporting) and misreporting (*i.e.* due to the presence of confounders) of ADRs. Nevertheless, all mentioned limits are well characterized and may be reduced through a series of adjustments and selection of higher quality reports as it has been performed in our analysis. Therefore, this comprehensive database may ultimately offer precious insights on FA use in actual clinical practice and on FA exposure of a wide variety of patients (e.g., comorbid, pregnant, elderly patients).

In conclusion, the evidence we obtained could harness the use of folate testing and result interpretation to shift the balance between costs and benefits of FA supplementation, which may significantly contribute to pharmaceutical and health expenditure. Data are lacking on this issue, however, by simply considering the costs and sustainability associated to pregnancy and lactation, we may speculate that FA supplementation needs for better management across growing economies.

CPG writing groups should focus more on laboratory items that can influence healthcare outcomes to help clinicians to effectively interpret folate results in order to optimize FA supplementation in the population at risk of deficiency or in subjects with altered folate homeostasis. Notably, folate-mediated one carbon metabolism is a major target of many therapies in human diseases [78].

Author contributions

Conceptualization, S.F., S.R., E.M.B., G.B.; software, M.G., E.M.B., G.B.; validation, S.F., E.M.B., G.B., M.G.; investigation, S.F., M.G., E.M.B., G.B.; resources, C.C.; A.S.R.; data curation, S.F., G.B, MG.; writing—original draft preparation, S.F., G.B M.G.; writing—review and editing, S.R., E.C., E.M.B., G.B., A.S.R.; supervision, E.C., S.R., E.M.B. All authors have read and agreed to the present version of the manuscript.

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Conflict of interest

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Appendix A. Supplementary data

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