

Review

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Red cell or serum folate: what to do in clinical practice?

Abstract

Folate deficiency has been linked to diverse clinical manifestations and despite the importance of accurate assessment of folate status, the best test for routine use is uncertain. Both serum and red cell folate assays are widely available in clinical laboratories; however, red cell folate is the more time-consuming and costly test. This review sought to evaluate whether the red cell assay demonstrated superior performance characteristics to justify these disadvantages. Red cell folate, but not serum folate, measurements demonstrated analytical variation due to sample pre-treatment parameters, oxygen saturation of haemoglobin and haematocrit. Neither marker was clearly superior in characterising deficiency but serum folate more frequently showed the higher correlation with homocysteine, a sensitive marker of deficiency. Similarly, both serum and red cell folate were shown to increase in response to folic acid supplementation. However, serum folate generally gave the greater response and was able to distinguish different supplementation doses. The C677T polymorphism of methylenetetrahydrofolate reductase alters the distribution of folate forms in red cells and may thereby cause further analytical variability in routine red cell folate assays. Overall, serum folate is cheaper and faster to perform than red cell folate, is influenced by fewer analytical variables and provides an assessment of folate status that may be superior to red cell folate.

Keywords: competitive protein binding assays; folate status; red cell folate; serum folate.

Introduction

Folate is a water-soluble vitamin critical for health as a cofactor in a multitude of single-carbon transfer reactions. Different forms of folate participate in different reactions and the term ‘folate’ refers to a family of substances containing a pteridine ring joined to *p*-aminobenzoic acid and one or more glutamate residues. Forms of folate may differ in the single-carbon moieties on the nitrogens present at position 5 of the pteridine ring or position 10 of the *p*-aminobenzoic acid, or cross-linked between the two positions: methyl, methenyl, methylene, formyl and formimino. There are also variations depending on the presence or absence of double bonds at specific sites on the pteridine ring, giving rise to dihydrofolate and tetrahydrofolate (THF) forms. 5-MethylTHF is the most abundant form of folate in serum and red cells. Nevertheless, each form of folate has specific metabolic roles [1, 2].

Severe folate deficiency manifests as megaloblastic anaemia due to impairment of DNA synthesis. Additionally, low folate status during early pregnancy is a risk factor for neural tube defects (NTDs) in offspring. Folate deficiency has also been linked to a wide range of neurological conditions [3] as well as conditions as diverse as cancer [4], cardiovascular disease [5] and osteoporosis [6]. Nowadays, clinical laboratories almost exclusively use automated competitive protein binding (CPB) assays to measure folate. These assays use folate binding protein (FBP) for analyte capture, which is typically a β -lactoglobulin isolated from cow's milk. These β -lactoglobulins will capture 5-methylTHF, as well as other forms of folate depending on pH [7]. These assays can be used to quantify folate both in serum and red blood cells.

Despite the clinical importance of being able to accurately determine patients' folate status, the best test for routine use is uncertain. Recommendations and practice varies as to the preferred matrix. For instance, the National Pathology Alliance benchmarking review in the UK recommends the measurement of serum folate [8]. In contrast, the pathology testing manual of Royal Pathologists of

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Australasia states that serum folate alone is of little diagnostic value [9] and, unlike red cell folate, the measurement of serum folate alone does not receive public funding in Australia [10]. Red cell folate measurement is somewhat more technically demanding than serum folate measurement. It requires collection of a whole blood sample with measurement of haematocrit, followed by red cell lysis and dilution of the folate released. Due to the requirement for increased staff time and reagent costs, red cell folate is therefore the more expensive assay. This review sought to evaluate whether there is evidence of superiority for red cell folate analysis to justify the higher cost.

Methods

We searched Medline electronic database using the terms 'red cell folate' and 'serum folate' over the period 1987 to present, limiting the search to human studies and those written in English. Three hundred and twenty-eight articles were thus identified. Papers assessing the genetic determinants of serum and/or red cell folate concentrations and studies performed on specific patient groups were included in the review. Articles using these markers as the comparator or assessing only one marker were excluded. The reference lists of relevant articles were scanned for additional studies. In comparing the performance of serum and red cell folate, we considered analytical and kinetic differences as well as their ability to identify patients with deficiency and respond to folic acid supplementation and food fortification. Differences in the performance of the markers in specific clinical contexts as well as genetic influences on marker levels were addressed. We also considered the limitations of measuring total folate levels, and the advantages of using methods able to individually quantify all major folate species.

Analytical issues

The analytical issues involved in the measurement of red cell and serum folate have been comprehensively reviewed previously [11]. Importantly, the between-laboratory variability of red cell folate analysis is typically greater than that for serum folate. This is related to the influence of a number of variables that specifically affect red cell folate measurements. Clinical laboratories will usually measure red cell folate with the same assay used for serum folate. However, pre-treatment of the sample is required. The pre-treatment serves to lyse the red cells in the sample,

deconjugate the polyglutamate folate forms present and dilute the high concentration of released intracellular folate down into a range that may be measured by the assay. The pre-analytical treatment of samples introduces a number of variables into the measurement process. These include the type and dilution factor of lysing agent as well as the duration of the lysis step. Ascorbic acid solutions are commonly used as lysing agents, yet variations in the strength of these solutions has been found to give differences in assayable folate concentrations [12]. The dilution factor may influence results because non-linear dilution curves have been demonstrated for red cell folate assays [13]. The time of lysis influences the degree to which polyglutamate folate species will be deconjugated by endogenous glutamylcarboxypeptidases in the patient sample. FBPs have a higher affinity for polyglutamate folate species than monoglutamate species, therefore increasing lysis time decreases folate results [14]. Sample pre-treatment parameters vary between laboratories. In a survey of Australian laboratories it was found that ascorbic acid was used in the lysis reagent with a concentration varying from 0.15% to 1.0%, with some assays additionally using guanidine hydrochloride; dilution factors ranged from 21% to 31% and lysis times varied from 60 to 180 min [15].

After measurement of the amount of folate present in the whole blood lysate, the red cell folate concentration is calculated using the patient's haematocrit. The measurement uncertainty of haematocrit is an additional variable specific to red cell folate results. Some laboratories may use an equation which also accounts for the presence of serum folate in the whole blood sample. In Australia, for instance, 26% of laboratories correct for serum folate [15]. This may introduce further between-laboratory differences in red cell folate results.

An additional variable in the measurement of red cell folate is the binding of folate to deoxyhaemoglobin [16]. It has been found that red cell folate measurements increase as oxygenation of haemoglobin is reduced [17]. It is thought that the binding of folate to deoxyhaemoglobin maintains folate in a stable state for deconjugation and measurement [18]. This is an important variable for red cell folate analysis because venous blood has a significant concentration of deoxyhaemoglobin and the haemoglobin saturation in peripheral veins can vary in healthy individuals from 40% to 85% [19].

A further confounding factor in the analysis of red cell folate is that results appear to vary with haematocrit. This effect has been observed by both the data-mining of large pathology databases and in vitro studies of varying mixtures of whole blood and autologous plasma [13, 20].

The effect has been shown to persist after correction for plasma folate [20] and does not appear to be due to differences in folate deconjugase concentration differences or pH of the lysate solution [13]. Whole blood folate measured in the lysate varies linearly with haemocrit. However, the red cell folate concentration is expected to be independent of haemocrit because it is calculated by dividing the whole blood result by haemocrit and is expressed per litre of packed red cells. The interaction between haematocrit and red cell folate results appears to be mediated by a curvilinear relationship between whole blood folate results and haemocrit [13].

The difficulties with achieving standardisation among folate assays have been discussed previously [21]. However, differences among numerous parameters specific for red cell folate measurement have translated into greater between-laboratory variation for red cell folate results. For example, in a comparison of five automated serum and red cell folate assays with the Bio-Rad Quantaphase II radioassay, differences of up to 40% were observed for serum folate and up to 250% for red cell folate were found [22]. Similarly, Gunter et al. analysed folate results from six serum and whole blood samples obtained from 20 laboratories using a range of methods. The red cell folate results showed greater variation between laboratories, with an overall inter-laboratory coefficient of variation (CV) of 35.7% compared to the overall CV for serum folate results of 27.6% [23].

Quantification of specific folate species

The quantification of the various folate species, or ‘vitamers’, in serum and red blood cells is challenging. Reduced folate forms are known to be sensitive to heat, pH, oxidation, pressure, and ultraviolet light [24–26] and therefore rapidly undergo interconversion or degradation processes. Therefore, sample preparation and measurements for serum and red blood cell folate must be performed under strictly controlled conditions.

In the 1970s, the first chromatographic methods able to distinguish folate vitamers were developed. Numerous high performance liquid chromatography [27, 28] and gas chromatography [29] methods have been described for the detection of folate forms in serum and red blood cells. Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) methods have been developed to quantify folate forms in biological fluids and food [30–32]. Modern LC-MS/MS methods use more or less complex sample extraction and clean-up procedures, from protein precipitation to solid phase extraction, with or without concentration of the analytes. They are capable of quantifying

several folate forms at nanomolar concentrations and provide high levels of selectivity and sensitivity. Monoglutamate or polyglutamate folate standards and stable isotope-labelled internal standards for most monoglutamate forms are available. However, at present it is not possible to detect all forms of folate with a single method. Nevertheless, an ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the determination of key folate forms (5-methylTHF, 5-formylTHF, 5,10-methenylTHF, THF, and folic acid) in serum [33] and of 5-methylTHF and non-methylTHF (sum of formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, dihydrofolate, and folic acid) in whole blood haemolysates [34] was recently described. It was shown to deliver fast, sensitive, and reliable results with a good correlation to a CPB method in serum ($r=0.939$, $p<0.001$). Other groups have also described methods for the measurement of folate vitamers in serum [30, 35] and whole blood [36].

There are a number of potential advantages of measuring folate vitamers in serum and/or red cells. These include less susceptibility to the variation seen in CPB assays, specific measurement of 5-methylTHF and detection of unmetabolised folic acid. CPB assays for total folate show significant variation [22]. One aspect of this variation may be differences in the detection of particular folate species by CPB methods [21, 37]. This is particularly relevant for subjects with MTHFR polymorphisms [38]. Measurement of all relevant folate vitamers limits this variation. Vitamer analysis also allows specific determination of 5-methylTHF, which has been shown to have a stronger correlation with plasma homocysteine than total folate [33]. In addition, (UP)LC-MS/MS methods can be used to monitor the metabolism of folic acid or detect unmetabolised folic acid after consumption of supplements and fortified foods [39, 40]. Measurement of unmetabolised folic acid may be of interest because of concern over negative effects on immune function, impaired cognition in those with unrecognised vitamin B₁₂ deficiency and epigenetic regulation [41]. Thus, (UP)LC-MS/MS methods are preferred over CPB methods when accurate information about the distribution of folate forms is required, while automated CPB assays are acceptable for routine applications.

Kinetic differences

Serum and red cell folate concentrations respond differently to changes in folate status, with serum folate responding more rapidly [42]. Red blood cells obtain virtually all their intracellular folate during erythropoiesis. Red cell progenitor cells readily take up folate through

membrane-associated folate receptors. However, these folate receptors are not significantly expressed on mature red cells [43]. Consequently, mature red cells take up a negligible amount of folate: even when serum folate concentrations exceed 10 nmol/L, only femtomol/L (i.e., 10^{-6} nmol/L) amounts of folate are taken up by mature red cells [44]. Kinetic studies have confirmed that the half-life of red cell folate closely matches the half-life of red blood cells, 60 days [45]. Kinetic principles dictate that following a change in intake it will take four half-lives for blood concentrations to reach 94% of the new steady state levels [46]. Thus, red cell folate will change relatively slowly after an alteration of intake and may not reach steady-state levels until after 35 weeks.

In contrast, serum folate is considered to reflect recent dietary intake [47]. However, it has been demonstrated that serum folate provides information about folate status over a significant time-frame. Studies using controlled diets in which folate intake has been reduced to inadequate levels have shown that serum folate concentrations continue to decrease over 7 weeks and stabilise after 8 weeks. Similarly, when folate intake is increased with a high-folate diet it can take 9 weeks for levels to plateau [48].

There have been concerns that post-prandial increases in serum folate may mask underlying deficiency [49]. Significant increases in serum folate have been observed 1 to 3 h after consumption of a 400 µg folic acid supplement [50]. Similarly, when blood was collected 2½ h after 200 µg folic acid given in fortified milk, serum folate concentrations were on average 7.7 nmol/L greater than fasting concentrations and the classification of a number of patients was altered from deficient to sufficient [51]. However, this degree of fortification is significantly higher than that used in currently available fortified foods. For example, in the US there is approximately 43 µg folic acid in a slice of fortified bread [52]. Thus, post-prandial increases may be less marked in subjects consuming a normal diet.

Identifying folate deficiency

Serum and red cell folate results may provide differing information about a patient's folate status. Although studies consistently demonstrate a significant correlation between serum and red cell folate, the correlations are not particularly high. Studies in both healthy and patient populations have shown correlation coefficients between the tests of 0.52–0.68 [53–57]. Therefore, the question is whether one test is able to better identify patients with folate deficiency.

There is no readily available gold standard for the diagnosis of folate deficiency. Thus, studies have taken a number of different approaches to evaluating serum and red cell folate as markers of deficiency. These include assessing correlation with plasma homocysteine concentration, ability to predict a haematological response to folic acid supplementation and evaluating correlation with tissue folate concentrations.

Correlation with homocysteine

Pearson correlation coefficients

The plasma homocysteine concentration provides an indication of whether folate is present in sufficient intracellular quantities to fulfil its role as a cofactor in the conversion of homocysteine to methionine. An elevated homocysteine concentration is therefore a sensitive marker of folate deficiency [58]. Numerous studies have assessed the correlation of serum and red cell folate with homocysteine in different populations (Table 1). Most studies have shown serum and red cell folate to correlate to a similar degree with homocysteine. Unfortunately, any differences in the correlations with homocysteine between these two markers have not been statistically evaluated. Studies that did appear to show differences in correlation favoured serum folate as the better test. One study (not shown in Table 1), performed on 79 unselected inpatients and 26 controls, did demonstrate a better correlation for red cell folate ($r=-0.41$) than serum ($r=-0.10$) [67]. However, this study excluded subjects with serum folate concentrations <12 nmol/L and red cell folate concentration below 600 nmol/L and therefore did not evaluate the ability of the tests to diagnose deficient patients. Data from 320 in- and out-patients referred to our own hospital laboratory for testing, with normal vitamin B₁₂ and estimated glomerular filtration rate (eGFR) >90 mL/min/1.73 m², showed correlation coefficients similar to those reported in the literature: serum folate -0.30, red cell folate -0.20.

Prediction of hyperhomocysteinaemia

A variation of the assessment of markers against homocysteine was used by Cheng et al. [53]. The study examined the ability of the markers to predict borderline increased homocysteine (≥ 10.2 µmol/L) or overtly increased homocysteine (≥ 14.9 µmol/L). The two markers appeared to perform similarly, with a trend toward better performance of serum folate (Table 1).

Study	Population	n	Serum folate	Red cell folate
Correlation with homocysteine				
Drogan [54]	General population	363	−0.30	−0.17
Raeder [59]	Geriatric inpatients	114	−0.32	Not significant
Marouf et al. [60]	Subjects with venous thromboembolism	201	−0.31	Not significant
Dierkes et al. [61]	General population	336	−0.32	−0.20
Dickey et al. [62]	Outpatients with coeliac disease	100	−0.46	−0.42
Gonzalez-Gross et al. [63]	Institutionalised elderly	218	−0.29 ^a	−0.26
Golbahar et al. [56]	Coronary artery disease patients and controls	400	−0.35 ^b	−0.42 ^b
Silberberg et al. [64]	General population	165	−0.30	−0.25
McMullin et al. [65]	Pregnant women	263	−0.30 ^c	−0.39 ^c
McGregor et al. [66]	CKD patients, not on dialysis and matched controls	99	−0.35	Not significant
Chadefaux et al. [67]	Unselected inpatients	194	−0.1	−0.41
De Vecchi et al. [68]	CKD patients, on dialysis	152	−0.54	−0.38
Current authors	Patients with normal vitamin B ₁₂ and eGFR	320	−0.30	−0.20
Prediction of increased homocysteine, AUC (95% CI)				
Cheng et al. [53]	General population	130	0.81 (0.67–0.95) ^d	0.76 (0.65–0.87) ^d
Cheng et al. [53]	General population	130	0.76 (0.68–0.85) ^e	0.66 (0.56–0.76) ^e
Current authors	Inpatients and outpatients with normal vitamin B ₁₂ and eGFR	320	0.70 (0.61–0.79)	0.56 (0.45–0.66)
Regression against homocysteine				
Marouf et al. [60]	Men with venous thromboembolism	91	Significant	Not significant
Marouf et al. [60]	Women with venous thromboembolism	110	Not significant	Not significant
Cheng et al. [53]	General population	130	Significant	Not significant
Dierkes et al. [61]	General population	336	Significant	Significant
Golbahar et al. [56]	Subjects with coronary artery disease and matched controls	400	Not significant ^b	Significant ^b

Table 1 Studies reporting correlation coefficients between serum folate and plasma homocysteine as well as red cell folate and plasma homocysteine concentration.

CKD, chronic kidney disease; Hcy, homocysteine. ^aCorrelation coefficient for log serum folate versus homocysteine. ^b5-methylTHF specifically measured in serum and red cells by radioassay. ^cCorrelation coefficient for log serum folate versus log homocysteine, similarly for red cell folate. ^dPrediction of overt hyperhomocysteinaemia (plasma homocysteine ≥ 14.9 $\mu\text{mol/L}$). ^ePrediction of borderline or overt hyperhomocysteinaemia (plasma homocysteine ≥ 10.2 $\mu\text{mol/L}$).

In our own analysis, we investigated the ability of serum and red cell folate to predict elevated plasma homocysteine levels (≥ 15 $\mu\text{mol/L}$) in 320 randomly selected patient samples sent to our hospital laboratory. Among patients with a normal serum vitamin B₁₂ concentration and eGFR, serum folate demonstrated a significantly higher area under the receiver operating characteristic curve (AUC) for predicting increased plasma homocysteine concentration. The difference between the AUCs for the tests was 0.14 (95% CI 0.06–0.23), $p=0.001$ (Figure 1).

Regression analysis

A few studies have also used multivariate analysis to evaluate the predictors of homocysteine concentrations (Table 1). Again, there is no clearly superior marker, although serum

folate was more frequently found to be a significant predictor of homocysteine. The study by Dierkes et al. [61] noted that in multiple regression analysis the contribution of red cell folate was small compared to that of plasma folate.

Prediction of haematological response to folic acid supplementation

One study has evaluated the ability of markers to predict a significant haematological response to folic acid supplementation. Jaffe and Schilling reviewed 1355 simultaneously obtained red cell and serum folate results from in- and out-patients of a teaching hospital [55]. Sixty-two patients were identified with a low red cell folate and 57 of these had adequate clinical notes for review as to whether they received folic acid supplementation and whether

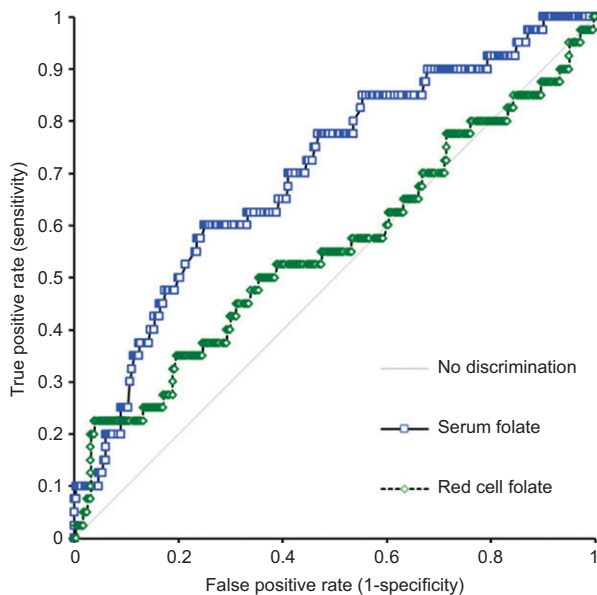


Figure 1 ROC curve analysis of the ability of serum and red cell folate levels to predict hyperhomocysteinaemia ($\geq 15 \mu\text{mol/L}$) for 320 randomly selected patient samples with normal serum vitamin B12 concentration and estimated glomerular filtration rate $>90 \text{ mL/min/1.73 m}^2$.

supplementation produced a haematological response, defined as an increase in haemoglobin concentration $\geq 10 \text{ g/L}$ and/or a decrease in mean cell volume from elevated to normal. Among those who responded to prescribed supplementation ($n=11$), there were only three patients who did not have a concurrent low serum folate. Therefore, it may be concluded that only approximately 0.2% (3/1355) of all patients from this population had their clinical outcome affected by the measurement of red cell rather than serum folate. The prevalence of low red cell folate concentrations in this study population was 4.6%; however, in populations in which folic acid fortification is mandatory the prevalence is $<1\%$, even among requests to hospital-based laboratories [69, 70]. In these populations, therefore, the proportion of patients who would have their outcome altered by red cell folate measurement in addition to serum folate would be expected to be significantly lower than 0.2%. Unfortunately, the frequency of a haematological response in patients with normal red cell folate but low serum folate was not evaluated because this combination of results was considered to reflect negative folate balance without depletion of tissue stores [42].

Correlation with liver folate

Red cell folate is frequently believed to be a better indicator of tissue stores of folate [47]. However, there is a dearth

of data correlating blood markers with tissue levels. One study [71] compared serum and red cell folate results to the levels of folate in the subject's liver, the principal storage site of folate [1]. The study compared marker levels with the folate concentration in liver biopsy specimens in 45 alcoholic patients. Both markers showed significant correlations with liver folate, with the correlation between serum and liver folate ($r=0.53$) marginally higher than that between red cell and liver folate ($r=0.47$). It is possible to calculate the performance parameters of the markers for identifying reduced liver folate, using the reference intervals established by the authors. On this basis, red cell folate demonstrated the marginally better performance. Red cell folate: sensitivity 64%, specificity 81%, positive-predictive value 60%, negative-predictive values 84%. Serum folate: sensitivity 47%, specificity 78%, positive-predictive value 50%, negative-predictive value 76%. However, these calculations were limited by the very small number of biopsies used to establish the liver folate reference interval ($n=27$). Overall, there was no firm indication of either marker being the superior indicator of tissue folate levels.

Response to supplementation and fortification

Multiple studies have reported the response of serum and red cell folate to various supplementation regimens. Both markers increase in response to supplementation with doses ranging from 76 to 6400 $\mu\text{g/day}$ (Table 2). In 21 of the 23 experiments, serum folate demonstrated a greater proportional increase than red cell folate. However, seven of these experiments were of only 2–4 weeks duration, over which time the red cell folate would not be expected to increase greatly. Nevertheless, in five of the six experiments conducted over 6 months, the proportional increase in serum folate was still greater. In particular, it was in the studies using lower supplementation dosages that serum folate consistently showed the greater increase. It was only once doses reached 2000 $\mu\text{g/day}$ that there was a study reporting a greater increase in red cell folate.

Serum folate may be better able to discriminate between different levels of folate intake. For instance, in a study using controlled daily intake of 450 μg versus 850 μg folic acid over 12 weeks, the 850 μg group had higher serum folate concentrations but the same red cell folate levels as the 450 μg group [86]. A meta-analysis of supplementation studies has shown a 2.13 nmol/L increase in serum folate concentration for every 0.1 mg/day increase in folic acid intake in women 20–25 years of

Study	Population	Folic acid dosage, µg/day	Follow-up period	Hcy response	Baseline serum folate, nmol/L	Response to supplementation		
						Serum folate response	Baseline red cell folate, nmol/L	Red cell folate response
Keane et al. [72]	Elderly	76 ^a	6 months	Not measured	4.9	169% ^b	717	61% ^b
Hao et al. [73]	Healthy women	100	6 months	−4%	9.7	108%	595	28%
Vahteristo et al. [74]	General population	180	4 weeks	No change	11.9	31%	606	15%
de Jong et al. [75]	General population	200	4 weeks	−7%	8.6	64%	348	4%
Schorah et al. [76]	General population	200	4 weeks	−6%	19.7	47%	1022	7%
Schorah et al. [76]	General population	200	8 weeks	−8%	19.7	32%	1022	−11%
Schorah et al. [76]	General population	200	24 weeks	−11%	19.7	59%	1022	22%
Hao et al. [73]	Healthy women	400	6 months	−17%	9.6	259%	603	72%
Hao et al. [73]	Healthy women	571 ^c	6 months	−9%	9.8	141%	611	45%
Rosenthal et al. [77]	General population	714 ^d	12 weeks	Not measured	15.6	46%	455	25%
West et al. [78]	Non-pregnant women	750 ^e	12 weeks	Not measured	44.9 ^f	46%	1161	10%
West et al. [78]	Pregnant women	750 ^e	12 weeks	Not measured	50.6 ^f	22%	2076	10%
West et al. [78]	Lactating women	750 ^e	10 weeks	Not measured	52.2 ^f	24%	2936	No change
Araki et al. [79]	Healthy men	800	2 weeks	−12%	10.7	169%	386	36%
Truswell and Kounnavong [80]	Healthy females	1000	3 weeks	Not measured	25.0	25%	886	5%
Rosenthal et al. [77]	General population	1000	12 weeks	Not measured	14.3	136%	452	48%
Von der Porten et al. [81]	Healthy men	1600	4 weeks	Not measured	18.0	150%	501	50%
Truswell and Kounnavong [80]	Healthy females	2000	3 weeks	Not measured	25.0	68%	886	16%
Hankey et al. [82]	Stroke patients	2000	6 months	−33% ^b	20.9	43% ^b	896	113%
Hao et al. [73]	Healthy women	4000	6 months	−22%	9.7	460%	599	137%
Pena et al. [83]	Children with type 1 DM	5000	8 weeks	−12%	29.0	37%	855	65%
Thambyrajah et al. [84]	Predialysis CKD	5000	12 weeks	−33% ^b	17.4	406% ^b	498	236% ^b
Bennett-Richards et al. [85]	Children with CKD	6400 ^g	8 weeks	−16%	26.5	5327%	824	694%

Table 2 Studies reporting the response of serum and red cell folate to various folic acid supplementation regimes. CKD, chronic kidney disease; DM, diabetes mellitus; Hcy, homocysteine. ^aAverage minimum^h supplementation intake. ^bComparison made to control group. ^cEquivalent daily dosage: supplementation given as 4000 µg/week. ^dEquivalent daily dosage: supplementation given as 5000 µg/week. ^eSubjects additionally consumed an average of 404 µg/day of folate from natural food. ^f5-methylTHF measured. ^gBased on average patient characteristics: dosages individualised to 5 mg/m².

age and 5.76 nmol/L for people 40–65 years of age [87]. There is currently insufficient data to conclude whether red cell folate is similarly able to detect graded increases in supplementation.

There may be a modest influence of baseline folate levels on the degree of marker increase. For instance, in different studies of healthy populations using the same follow-up interval (de Jong et al. [75] and Schorah et al. [76]), the population with the lower baseline folate concentration (8.6 vs. 19.7 nmol/L) demonstrated the greater increase (64% vs. 47%). Analysing data from 13 studies, Wald et al. found a greater proportional increase in serum folate for those with lower baseline concentrations [87]. It is not possible to evaluate the influence of baseline levels on red cell folate increase because of the small number of studies with sufficient duration of follow-up.

Serum folate also appears to demonstrate the greater response to the introduction of food fortification. This is consistent with the finding of the greater increase in serum folate in supplementation studies employing the lower folic acid doses. As shown in Table 3, all studies evaluating marker levels following the introduction of folate fortification showed a greater increase in the serum folate, although in one study the increase in red cell folate approached that of serum folate. In these studies, the baseline folate status appeared to influence the increase in both markers. Increases were greatest in the population with the lowest baseline concentrations and least in the population with the highest baseline levels. This relationship also held for the different populations in the two reports from Australia. At the highest baseline concentrations, serum folate still demonstrated a reasonable increase (24%), while there was no change in red cell folate concentrations.

Specific patient groups

There are a limited number of clinical contexts in which one marker may provide a superior assessment of folate

status. These include vitamin B₁₂ deficiency, haemodialysis and assessment of NTD risk. Serum folate appears to be the superior marker of folate status in vitamin B₁₂ deficiency. Vitamin B₁₂ is required for the normal uptake of 5-methylTHF into developing red blood cells [90]. Therefore, vitamin B₁₂ deficiency may falsely lower red cell folate results in the absence of folate deficiency [91, 92]. This is supported by one clinical study showing a high prevalence of vitamin B₁₂ deficiency in patients with low red cell folate but normal serum folate [93]. Moreover, vitamin B₁₂ supplementation in patients with vitamin B₁₂ deficiency has been shown to increase red cell folate levels but have no effect on serum folate [94]. Serum folate also appears to be a better marker of risk of toxicity from capecitabine, a 5-fluorouracil prodrug [95].

By providing an assessment of long-term folate status, red cell folate may be the preferable marker in a limited number of specific circumstances. In patients on haemodialysis, red cell folate may be the preferable marker if blood is collected soon after a dialysis session because serum folate results are decreased in the immediate post-dialysis period [96]. However when blood is collected pre-dialysis, serum folate may be a better marker of folate status, as indicated by a higher correlation coefficient with homocysteine for serum folate than red cell folate in one study [68]. As a marker of long-term folate status, red cell folate appears better able to predict risk of NTDs when blood is collected at times the woman is not pregnant. Studies have found lower red cell folate concentrations in non-pregnant women with a history of one or more prior pregnancies affected by NTDs [97]. There was a linear association between red cell folate concentrations and number of NTD pregnancies. These studies showed no significant differences in serum folate concentrations. However, when blood is collected during pregnancy, serum folate appears to predict NTD risk as well as red cell folate. In a prospective study using blood samples collected at the first antenatal visit, the women carrying pregnancies affected by NTDs had similar reductions in red cell (–22%) and serum (–32%) folate compared to controls [98].

Study	Population	n	Baseline serum folate, nmol/L	Serum folate response	Baseline red cell folate, nmol/L	Red cell folate response
Brown et al. [69]	Australia	20,592	17.7	31%	881	22%
Hertrampf and Cortes [88]	Women in Chile	605	9.7	284%	290	144%
Farrell [70]	Australia	12,713	31.3	24%	2564	No change
McDowell et al. [89]	USA	~5000	12.5	158%	394	102%

Table 3 Studies reporting the response of various populations to the introduction of folic acid fortification of food.

Genetic influences on serum and red cell folate

Methylenetetrahydrofolate reductase polymorphisms

Methylenetetrahydrofolate reductase (MTHFR) is a flavoprotein that catalyses the conversion of 5,10-methylenethf to 5-methylthf. A single nucleotide substitution (C677T) is a common polymorphism of the *MTHFR* gene with a prevalence of 7.2%–15% in Caucasian populations [99]. The polymorphism causes MTHFR to readily dissociate from its normal dimer structure into monomers and lose its FAD-binding capacity [100]. Consequently, individuals heterozygous for the polymorphism have MTHFR activity reduced by approximately 29% and homozygotes have enzyme activity reduced by 64% [101].

Methylenetetrahydrofolate reductase polymorphisms: effect on folate concentrations

The MTHFR C677T polymorphism may decrease both serum folate and red cell folate concentrations. The interaction between *MTHFR* genotype and circulating folate concentrations appears to be mediated by the folate status of the population. This was illustrated by a depletion study in a population with high baseline folate status [102]. In this study there was no significant difference in serum folate between the different genotypes at baseline. However, after being restricted to a low folate diet (116 µg/day) for 7 weeks, those with the *TT* genotype had significantly lower concentrations. Subjects were then given a diet with the recommended daily allowance (400 µg/day) for 7 weeks, at which point the difference between the genotypes had again disappeared. Red cell folate was unable to respond as rapidly to the changes in folate intake in this study. However, it did show a similar response: there was no difference in levels between the genotypes at baseline, yet at the end of the study period those with the *TT* genotype had significantly lower concentrations. The effect of folate status may be one reason for mixed results in studies investigating the relationship between *MTHFR* genotype and folate concentrations. Although many studies have shown homozygotes to have significantly lower serum and red cell folate concentrations [103–108], some have shown no difference [86, 109].

The difference in serum and red cell folate results across the genotypes appear to reflect real changes in folate metabolism. The decreased levels in the *TT* genotype are accompanied by increased plasma homocysteine [103–108]. Additionally, the homocysteine variation between the genotypes

also shows interaction with folate status. *TT* individuals have higher homocysteine concentrations when folate intake is reduced but no difference with high folate intake [107, 110]. DNA methylation, another marker of folate function, also demonstrates this gene-nutrient interaction [107]. This suggests that the observed differences in serum and red cell folate between *MTHFR* genotypes indicate clinically important differences. Both parameters provide this information, with neither being clearly superior in this context.

Methylenetetrahydrofolate reductase polymorphisms: effect on assays

MTHFR polymorphisms may affect the measurement of total folate by CPB assays. Genetic alterations in the activity of MTHFR have been demonstrated to alter the mix of circulating folate forms; this has principally been investigated in red cells. Median 5-methylthf concentrations in those with *CC* genotype are 88%–100% of the total red cell folate. In contrast, levels in those with the *TT* genotype are 58%–70% [38, 111, 112]. In individuals with the *TT* genotype formylthf forms have been observed up to 59% of the total [38]. Initial reports have suggested that there is less variation in folate forms in serum. A study analysing serum samples from 96 blood donors found no difference in serum folate pattern by genotype [111]. A smaller study (n=32), showed the proportion of 5-methylthf to be marginally increased in those with the *CT* or *TT* genotype (88%) compared to the *CC* genotype (83%, $p=0.04$) [33]. While 5-formylthf itself has no known metabolic function, it is readily converted to 5,10-methylenethf by the enzyme methylenetetrahydrofolate synthetase [113]. It therefore represents an important component of the total available folate pool.

CPB assays rely on the binding properties of FBP for the detection of the various folate forms. Depending on their source, FBPs may have different binding affinities for different folate species. For instance, FBP has a low binding capacity for 5-formylthf [114] and this is thought to underlie a mean negative proportional bias (29%) in the Bio-Rad FBP radioassay compared to stable-isotope-dilution tandem mass spectrometry when random serum samples are analysed [115]. In subjects with the *MTHFR* 677 *TT* genotype the effect of under-recovery of 5-formylthf could result in a considerable negative bias in total folate measurements. Indeed, significantly different analytical performance has been demonstrated for a CPB assay when compared to a microbiological assay for subjects with the *TT* genotype versus *CC* or *CT* subjects [116]. This remains to be

examined in current automated CPB assays against higher-order measurement procedures such as LC-MS/MS but it is a potential source of variation among red cell folate assays that may have a lesser effect on serum folate measurements.

Other genetic influences on marker levels

Common polymorphisms of a number of other genes involved in folate metabolism have been investigated for their influence on serum and red cell folate concentrations. Genes which have demonstrated influence of marker levels, in some reports, include those coding for dihydrofolate reductase [117], the reduced folate carrier 1 [118, 119], glutamate carboxypeptidase [120–123] and thymidylate synthase [124–126]. However, the magnitude of any reported effects are not clinically significant, appear to reflect physiological differences in folate status rather than artefactual changes and influence both serum and red cell folate levels to similar extents.

Conclusions

Assessment of a patient's folate status is a clinically important evaluation. Clinical laboratories are readily able to provide measurement of serum or red cell folate using automated CPB assays. After assessing many different aspects of the performance of serum and red cell folate assays, this review did not find evidence to justify the higher cost of routinely measuring red cell folate. Before analysis, red cell folate tests require sample pre-treatment, which increases the cost of this test and introduces additional variation into results from different laboratories. There are also a number of analytical issues which influence red cell folate, but not serum folate, results. For instance, red cell folate results are increased by reductions in the oxygen saturation of haemoglobin. Decreased haematocrit has also been shown to increase measured red cell folate results.

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In the evaluation of serum and red cell folate against homocysteine, there was no evidence for the better performance of red cell folate and, of the two, serum folate appeared superior. Studies have also demonstrated that very few patients would have their clinical outcome altered by the measurement of red cell folate in addition to serum folate. Serum and red cell folate both respond to folic acid supplementation and fortification. However, serum folate appears to give the greater response at both short- and long-term follow-up. Serum and red cell folate perform similarly in most patient groups. However, serum folate is the superior marker in patients with vitamin B₁₂ deficiency. In contrast, after haemodialysis sessions red cell folate gives the better assessment of folate status. The markers equally assess risk of a current pregnancy being affected by a NTD. However, between pregnancies red cell folate appears to be the better risk indicator. MTHFR polymorphisms may alter folate concentrations in populations with low folate status. Both serum and red cell folate are able to identify these changes. The polymorphisms also alter the distribution of folate species in red cells and cause analytical variation in measured folate concentrations in some CPB assays. Serum folate measurements appear less affected by these changes. Overall then, as a routine test of folate status, serum folate appears to offer the best combination of test cost and clinical information.

Conflict of interest statement

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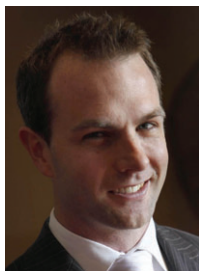
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