

CAT
Critically Appraised Topic:
Sense or nonsense of (red blood cell) folate testing ?

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CLINICAL BOTTOM LINE

Serum folate is considered an indicator of recent folate intake. Red blood cell folate concentrations respond slowly to changes in folate intake because the erythrocytes, which have a 120-day lifespan, accumulate folate only during erythropoiesis. For that reason, the red blood cell folate assay has historically been recommended as a more reliable indicator of long-term tissue folate stores compared to the serum folate assay. However the red blood cell folate assay is more complex to perform, requires specific sample handling and has a lower precision when compared with serum folate measurements.

CLINICAL/DIAGNOSTIC SCENARIO

Folate deficiency can be assessed by serum and red blood cell folate measurements. In most laboratories, both analyses are performed daily, and doctors often request both tests at the same time to screen for folate deficiency.

Serum folate is easily measured with various immunoassays but levels seem to be more influenced by recent food intake. Red blood cell analysis gives a more long-term idea of folate storage but requires time-consuming preliminary sample preparation with possible problems of imprecision and accuracy. The aim of this critically appraised topic is to determine the most appropriate strategy for diagnosing folate deficiency and to give a critical view on the impact of different cut-off values and methods on the prevalence of folate deficiency.

QUESTION(S)

1. *Serum and red blood cell folate are often requested at the same time to screen for folate deficiency. We searched literature for evidence of added value of red blood cell folate testing and did a retrospective analysis of the serum and red blood cell folate testing in our own center on a one-year period.*
2. *During our search, we were confronted with different cut-off values for folate and its impact on prevalence of folate deficiency. Is there a consensus in defining folate deficiency?*

SEARCH TERMS

- 1) *MeSH Database (PubMed): MeSH term:*
"folate erythrocyte and analysis", "folate and analysis"
- 2) *PubMed Clinical Queries (from 1966; <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>): Systematic Reviews; Clinical Queries using Research Methodology Filters :*
"folate erythrocyte imprecision", "folate erythrocyte analysis", "folate analysis"
- 3) *Guidelines :*
Centers for Disease Control and Prevention
FDA Food and Drug Administration
Mayo Medical laboratories
World health organization (WHO)
Centers for Disease Control and Prevention CDC / National Health and Nutrition Examination Survey (NHANES)
- 4) *UpToDate Online version*
Schrier S, Mentzer W, Tirnauer J. Clinical manifestations and diagnosis of vitamin B12 and folate deficiency.
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RELEVANT EVIDENCE/REFERENCES

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- 2) *Original Articles*
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14. Koury MJ, Ponka P. New insights into erythropoiesis: the roles of folate, vitamin B12 and iron. *Annu Rev Nutr* 2004; 24:105
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16. Galloway M, Rushworth L. Red cell or serum folate? Results from the National Pathology Alliance benchmarking review. *J Clin Pathol* 2003; 56- 924-926

3) *Books*

17. Hoffman R, Benz E, Silberstein L et al. *Hematology Basic Principles and Practice*, 6nd ed. Churchill Livingston, 2012.
18. Löwenberg B, Ossenkoppele GJ, de Witte T, et al. *Handboek hematologie*. De Tijdstroom, 2008.

4) *Manuals and laboratory guides*

19. Roche : Elecsys® Folate RBC (2014) en Elecsys folate III (2015)
20. Mayo : <https://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/9198>
21. Mayo : <https://www.mayoclinic.org/drugs-supplements-folate/art-20364625>

APPRAISAL

I/ INTRODUCTION FOLATE

I.1. Folate source

Naturally occurring folate is found in dark green leafy vegetables, beans, peas, nuts, liver and tropical fruits.

The synthetic form of folate is folic acid which is present in dietary supplements and fortified foods.

The recommended daily amount of folate for adults is 400 micrograms (mcg). Adult women who are planning to become pregnant should be supplemented with folic acid. Many countries have already instituted supplementation of cereals, flours, and grains. [21]

I.2. 'Pharmacokinetics'

- Absorption

Folate (vitamin B11, pteroylglutamic acid) is a water-soluble B vitamin which can be easily destroyed by cooking. It is composed of polyglutamates, which first have to be cleaved to monoglutamates to get absorbed in the jejunum. Folic acid at the other hand is a monoglutamate. The bioavailability of naturally occurring folate is therefore less than that of folic acid. [2]

Folate must be enzymatically reduced to be effective in its biological role to dihydrofolate and then to tetrahydrofolate (THF). THF is subsequently converted to 5,10-methylene THF, which is converted to L-5-methylene-THF by the enzyme methylene-THF-reductase. [20]

The predominant folate in plasma (82 tot 93% of the total folate) is L-5-methylene-THF. [6] Folates other than L-5-methylene-THF and folic acid antagonists (such as methotrexate) may, under some circumstances, be present in serum and will also be measured in the laboratory).

A peak concentration is seen 1 hour after ingestion and is followed by a fast distribution to peripheral tissues with normalization of serum concentrations after a few hours. Once transported into the cells, folate is again polyglutamated to a biologically active form that cannot diffuse back out of the cell. [16]

- Distribution

More than 95 % of folate occurs in the red blood cells (RBC). The folate concentration in erythrocytes reflects a more stable tissue concentration (for 3-4 months), while the serum level significantly fluctuates with the diet. Folate is also found in serum and stored in liver. [17]

- Metabolisation

THF is rapidly cleared by hepatocytes and other cells (enterohepatic circle). [17]

- Excretion

Folates are eliminated from the liver via bile. They exit the body via urine and feces.

Important remark:

In vitamin B12 deficiency, folate molecules remain trapped in circulation because methylfolate cannot be demethylated and polyglutamated to form its active intracellular counterpart. The treatment of a misdiagnosed vitamin B12 deficiency with folate alone is dangerous, as it may cause a temporary clinical improvement but may mask induce or aggravate the irreversible neurological consequences associated with cobalamin deficiency. [5,9]

I.3. Causes of folate deficiency

- Inadequate dietary intake

The most common cause of deficiency is nutritional. It is rare in countries where there is a routine folic acid fortification but still exists in individuals who are unable to consume a varied diet or in chronic alcohol use. Cooking foods destroys folates, so individuals who consume exclusively cooked foods may also be at risk.

- Intestinal malabsorption

This can result from surgery (gastric bypass) or inflammatory disorders such as celiac disease.

- Increased requirements

Certain settings associated with rapid cell proliferation (pregnancy and lactation, chronic hemolytic anemias, hemodialysis) may require more folate. Folic acid supplementation should be given in this population.

- Medications

Several medications can interfere with folate metabolism and may cause folate deficiency and/or megaloblastic anemia due to effects on DNA synthesis:

- Methotrexate: inhibits dihydrofolate reductase (DHFR)
- Antibiotics : certain antibiotics (trimethoprim) inhibit DHFR
- Antiseizure agents : may affect folate absorption/utilization (phenytoin, valproate, carbamazepine) [5]

I.4. Consequences and symptoms of deficiency

Polyglutamated tetrahydrofolate acts as a carbon donor for biologic processes. It is a necessary cofactor for

- synthesizing purine and thymidine nucleotides used in DNA synthesis
- as well as converting homocysteine to methionine.

Folate is so essential for normal metabolism, DNA synthesis and red blood cell regeneration. [17]

Disturbed DNA synthesis

a/ Effect on hematopoiesis

Hematopoietic precursor cells are among the most rapidly dividing cells and are so most sensitive to abnormal DNA synthesis. [14]

- Ineffective erythropoiesis: nuclear division is slower than cytoplasmic maturation (nuclear-cytoplasmic dyssynchrony) which results in megaloblastic changes (giant erythroblasts, metamyelocytes and megakaryocytes)
→Peripheral blood smear shows macro(oval)ocytic red blood cells, hypersegmented neutrophils and giant thrombocytes.
- Intramedullary hemolysis : the abnormal bone marrow precursor cells are more sensitive to premature death (phagocytosis or apoptosis). There may be hypercellularity of the bone marrow.
→A mild anemia and other cytopenia can be seen. The reticulocyte count is low.

→Laboratory findings of hemolysis are indirect bilirubin and lactate

dehydrogenase (LDH) and low haptoglobin.

b/ Effect on other rapidly dividing cells

- Effect on pregnancy : abruptio placentae, preeclampsia, spontaneous abortion, stillbirth, preterm delivery, low birth weight and neural tube defects. [2]
- Neuronal defects are less likely than seen in vitamin B12 deficiency.

Disturbed aminoacid synthesis

Low folate status also increases plasma homocysteine levels, a potential risk factor for cardiovascular disease.

I.5. Diagnosis

- Anamnesis

There should be questioned for intestinal malabsorption, lack of intake of fresh vegetables, alcohol use and neurologic symptoms.

- Total blood count and peripheral blood smear

Deficiencies of vitamin B12 and folate can both cause megaloblastic anemia. The findings on the complete blood count (CBC) and the blood cell morphology on the peripheral blood smear are identical with both deficiencies and may include one or more of the following:

- Anemia, mild leukopenia and/or thrombocytopenia, low reticulocyte count
- Macrocytic RBCs (MCV >100 fL) or macro-ovalocytosis, hypersegmentation of neutrophils
- Increased indirect bilirubin, increased lactate dehydrogenase and low haptoglobin

- Serum vitamin B12 and folate levels

The investigation of macrocytic anemia could be done by measuring both folate and vitamin B12 concentrations or could start with vitamin B12 measuring only, given the higher prevalence of vitamin B12 and the low pretest probability of folate deficiency, even in those with macrocytic anemia. Vitamin B12 diagnosis may not be missed cause of the risks of untreated deficiency ! [8]

- Homocysteine and methylmalonic acid (MMA)

Determinations could help distinguish between vitamin B12 and folate deficiency states. In folate deficiency, homocysteine levels are elevated and MMA levels are normal. But testing is more expensive, and the results can be affected by poor renal function and other vitamins.

- Bone marrow aspirate and biopsy

It is not used to evaluate for folate deficiencies. However, if a bone marrow evaluation is performed in an individual with folate deficiency a hypercellular marrow with megaloblastic erythroid hyperplasia, giant metamyelocytes, and frequent mitosis will be seen.

I.6. Treatment

If substitution is started, a complete blood count should be performed after two weeks to evaluate for an appropriate increase in hemoglobin and decrease in MCV. Once a full hematologic response is seen, treatment is not further necessary unless the cause persists. In that case, sometimes lifelong substitution is necessary. [8]

2/ SENSE OR NONSENSE OF (RED BLOOD CELL) FOLATE TESTING

2.1 General concerns

1. Folate is highly requested. [6]
2. Folate determination is a relatively nonspecific test.

The laboratory measurement of folate is complicated because not all measured forms of folate are characterized by biological activity and various inactive degradation products may interfere. [6]

Low serum folate levels may be seen in the absence of deficiency and normal levels may be seen in patients with macrocytic anemia, dementia, neuropsychiatric- disorders, and pregnancy disorders. [20]

3. The pretest probability of folate deficiency is low.

Evaluation of macrocytic anemias should start with vitamin B12, given the higher prevalence and the risks of untreated deficiency. SF and RCF tests should not be routinely ordered. Even in those with macrocytic anemia, the pretest probability of folate deficiency remains low. [8, 20]

4. Its results do not affect the clinical management of patients. [6]

2.2 Determination of serum folate in the laboratory

At the Imelda hospital, folate assays are performed on a Cobas Analyzer (Roche) with an electrochemiluminescence competitive assay. (Figure 1). Folate pretreatment reagent first releases the bound folate from his endogenous folate binding proteins. Ruthenium labeled folate binding proteins are then added to form a complex. Finally streptavidin-coated microparticles and folate labeled with biotin are associated to form a sandwich complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. [19]

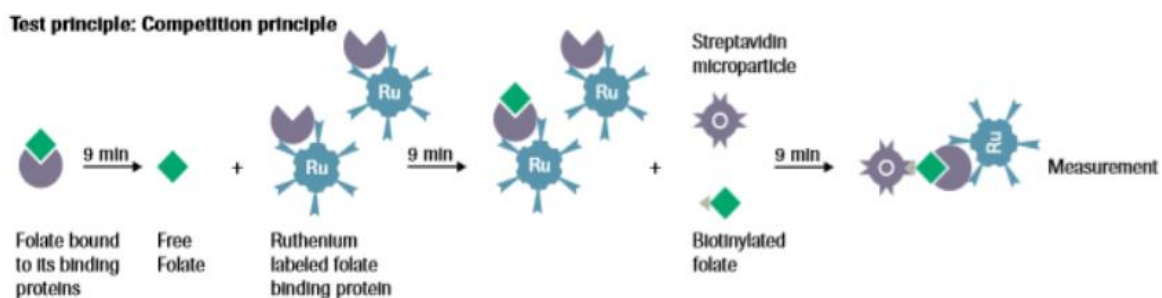


Figure 1: Electrochemiluminescence competitive assay of folate

The reference value for serum folate is 3.89-26.8 ng/mL.

2.3 Determination of red blood cell folate in the laboratory

Whole blood is mixed with ascorbic acid solution (Folate RBC hemolyzing reagent) and incubated for approximately 90 minutes. Lysis of the erythrocytes takes place, with liberation and stabilization of the intracellular folate. After this manual preparation, the same method is performed on the Cobas analyzer. Results are automatically sent to the LIS. The system then corrects the result with a dilution factor 2 and takes into account the hematocrit level with the following formula : folate concentration x100/ hematocrit. [19]

The reference value for red blood cell folate is > 523 ng/mL.

2.4 Pro and contra RCF testing

1. Pro RCF

- It more accurately reflects tissue stores than serum folate and
- is not influenced by recent folate intake and less by alcohol, pregnancy and antiseizure drugs

2. Contra RCF

A higher variability for whole blood results compared with results for serum samples is seen and can be due to

- a poorer method comparison (inter-laboratorium)

Whole blood samples showed poorer agreement than was seen with serum samples. [15]

- a larger analytic imprecision (intra-laboratorium) as a consequence of
 - differences in manual hemolysate pretreatment
 - incomplete lysis of RBC which can contribute to underrecovery of folate
 - folate trapping with oxyhemoglobin (< denaturation of hemoglobin) or in the case of vitamin B12 deficiency [15]

We analyzed the analytic imprecision for folate on the basis of the internal and external quality controls (QC) used in the Imelda Hospital.

- Internal QC

In our hospital Bio-Rad quality controls are used and two different levels are runned each day : Liquicheck Immunoassay plus L1, L2 en L3 for SF and RCF and Lypocheck Immunoassay whole blood L3 for RCF (table 1)

| | SF | | | RCF | | | |
|------------|--------|-------|-------|--------|--------|--------|----------------|
| Levels | L1 | L2 | L3 | L1 | L2 | L3 | Lyophilized L3 |
| Target | 2,39 | 7,14 | 10,48 | 185,35 | 340,2 | 443,17 | 417,32 |
| Gemiddelde | 2,42 | 6,92 | 10,20 | 185,81 | 340,54 | 443,91 | 441,37 |
| SD | 0,28 | 0,62 | 0,94 | 8,81 | 13,94 | 25,60 | 25,87 |
| CV | 11,62% | 8,93% | 9,21% | 4,74% | 4,09% | 5,78% | 5,86% |
| Bias | 0,17% | 4,74% | 9,40% | 0,25% | 0,10% | 0,17% | 10,34% |

Table 1: Target, mean, SD, CV, Bias for SF and RCF for the Bio-Rad Liquicheck and Lypocheck control levels (Lot 40933). Values are expressed in ng/mL.

Higher CV's were seen in SF when compared with RCF, due to the lower measured values in SF.

A higher CV for SF is seen in the lower level (due to lower measured values) compared with the higher level.

Comparable CV for RCF L3 liquid and lyophilized, but bias was larger for the lyophilized QC.

- External quality controls

Z-scores for external quality controls for SF are good. An external quality control for RCF doesn't exist.

3/ RETROSPECTIVE STUDY MAYO CLINIC

Few studies have looked at the utility of the RCF versus SF.

The Mayo Medical Laboratories undertook a 10-year retrospective analysis of 152,166 SF and 15,708 RCF results to determine the clinical utility of RCF beyond that of the SF. [10] They decided to discontinue their folate tests on red blood cells and to look for the benefit of ending RCF determining.

A competitive-binding receptor assay was used to measure folate. Abnormal values were defined by the National Health and Nutrition Examination Surveys (NHANES)/Center for Disease Control (CDC) criteria for folate deficiency (SF < 3,0 ng/mL, RSF < 140 ng/mL).

FINDINGS:

1. Folate deficiency is rare

The prevalence of folate deficiency using only SF values was 0.39% and using only RCF 0.27%. There were 1082 patients in which SF and RCF were ordered concurrently (Table 2). Simultaneous deficiency was only detected in 0.09% of the patients.

| 1082 simultaneous measurements | | Serum folate (3 ng/mL cut-off) | |
|--|----------|-----------------------------------|--------------|
| | | Abnormal | Normal |
| Red blood cell folate (140 ng/mL cut-off) | Abnormal | 1 (0,09%) | 4 (0,4%) |
| | Normal | 8 (0,7%) | 1069 (98,8%) |

Table 2: Analysis of patients with paired SF and RCF using NHANES/CDC definition of folate deficiency

2. Good correlation between SF and RCF

Most of the patients had simultaneous abnormal SF and RCF tests or simultaneous normal SF and RCF tests.

3. Possible 'misdiagnosis' of folate deficiency when aborting the RCF test is low

There were only 4 patients who had normal SF but low RCF. In only 1 of those 4 patients folic acid supplementation was started.

Those were the 4 clinical cases:

- 1) a 6 year old male - known folic acid transporter deficiency R/ Leucovorin
- 2) a 58 year old male - gout, hypertension, psoriasis, and hyperlipidemia - normal MCV and hemoglobin (Hb)
- 3) a year old 65male - chronic diarrhea, suspected alcohol abuse -MCV 100.3 fL and normal Hb
- 4) a 51 year old male - multifactorial gait disorder, alcohol abuse - previous history of vitamin B12 deficiency (B12 levels normal at this time) - MCV=115.1 fL and normal Hb

CONCLUSIONS:

They concluded that folate deficiency is exceedingly rare and that both results gave the same interpretation. They also concluded that RSF quantitation didn't serve a unique purpose anymore in the continuum of care and they preferred the SF measurement cause of better analytic variability. The RSF test was aborted on September 20th, 2010. [10]

4/ RETROSPECTIVE STUDY IMELDA HOSPITAL BONHEIDEN

4.1. Results

In the Imelda Hospital we did a similar retrospective study. Patient samples analyzed between 1th July 2016 and the 30th June 2017 were collected. We checked for SF and/or RCF results. If assayed at the same time of sampling, the results of hemoglobin (Hb), mean corpuscular volume (MCV) and serum vitamin B12 were also extracted. A total of 5932 SF and 3844 RCF were performed over that year. The prevalence of folate deficiency using only SF values was 17.1% and 4.1% using only RCF. There were 1025 patients in which SF and RCF were ordered concurrently (Table 3).

1. Prevalence folate deficiency (table 3)

| | Total folate measurements | # folate deficiency | % folate deficiency | Mayo Clinic |
|-----------------------|---------------------------|---------------------|-----------------------|-----------------------|
| Serum folate | 5932 | 1014 | 17,1% (<3,9 ng/mL) | 0,39% (<3 ng/mL) |
| Red blood cell folate | 3844 | 156 | 4,1% (<523ng/mL) | 0,22% (<140 ng/mL) |

Table 3: Prevalence folate deficiency Imelda Hospital Bonheiden (1th July 2016 and the 30th June 2017)

The prevalence of folate deficiency was higher than seen in the Mayo Clinic study.

2. Indication not always correct (table 4)

| | Total folate Measurements with determined Hb and MCV | Anemia | Macrocytic | Macrocytic anemia |
|-------|--|-----------------|----------------|-------------------|
| Men | 3790 | 1757 (46,3%) | 464 (12,2%) | 328 (8,6%) |
| Women | 4737 | 1601 (33,8%) | 474 (10%) | 249 (5,2%) |
| Total | 8527 | 3358 (39,3%) | 938 (11%) | 577 (6,7%) |

Table 4: Prevalence of macrocytic anemia in patients in which folate was examined in Imelda Hospital Bonheiden (1th July 2016 and the 30th June 2017)

Macrocytic anemia was noticed in only 6,7% of patients.

3. Prevalence of folate deficiency in the group with macrocytic anemia (table 5)

| | Macrocytic anemia | # SF deficiencies | # RCF deficiencies | # folate deficiencies in serum and/or RBC | % folate deficiencies in serum and/or RBC |
|-------|-------------------|-------------------|--------------------|---|---|
| Men | 328 | 55 | 2 | 56 (1 simultaneous) | 17% |
| Women | 249 | 41 | 5 | 44 (2 simultaneous) | 17,6% |
| Total | 577 | | | 100 | 17,3% |

Table 5: Prevalence of folate deficiency in the group with macrocytic anemia in Imelda Hospital Bonheiden (1th July 2016 and the 30th June 2017)

Rates of folate deficiency are only slightly higher in the group of patients with macrocytic anemia (17,3%) compared with the general population (17,1%). Folate deficiency is not effected by gender.

4. Prevalence of vitamin B12 deficiency in the group with macrocytic anemia (table 6)

| | Macrocytic anemia | # patients in which vit B12 is determined | # vit B12 deficiencies | % vit B12 deficiencies |
|-------|-------------------|---|------------------------|------------------------|
| Men | 328 | 316 | 27 | 8,5% |
| Women | 249 | 241 | 140 | 58% |
| Total | 577 | 557 | 167 | 30% |

Table 6: Prevalence of folate deficiency in the group with macrocytic anemia anemia in Imelda Hospital Bonheiden (1th July 2016 and the 30th June 2017)

Vitamin B12 deficiency is more frequently present in the female population and has a higher prevalence than folate deficiency.

5. Simultaneous determinations of both assays (table 7)

| 1025 simultaneous determinations | | Serum folate (3,9 ng/mL cut-off) | |
|--|----------|-------------------------------------|-------------|
| | | Abnormal | Normaal |
| Red blood cell folate (523 ng/mL cut-off) | Abnormal | 33 (3,2%) | 7 (0,7%) |
| | Normaal | 142 (13,8%) | 843 (82,2%) |

Table 7: Analysis of patients with paired SF and RCF in Imelda Hospital Bonheiden (1th July 2016 and the 30th June 2017)

CONCLUSIONS:

- The prevalence of simultaneous folate deficiency is low (3,2%)
- A possible 'misdiagnosis' of folate deficiency when aborting the RCF test is low. From the 7 patients with a normal SF but low RCF, there was only 1 patient who had a macrocytic anemia (as a result of total gastrectomy) and who was already treated with vitamin B12.

4.2. Critical remarks about differences in 'folate deficiency'

An estimate of folate deficiency in the European countries is lacking. [6] We try to give an overview of possible explanations for the difference in number of patients labeled as having 'folate deficiency' in our study compared to that of the Mayo Clinic.

- **Folic acid fortification**

- In 1997, the US government started a folate fortification program (FDA) in all grain products. This program resulted in a dramatic decrease in the prevalence of very low serum folate levels <3 µg/L from 18.3% to 0.2%. [9]
- Belgium as many other European countries has no fortification program.

Is there a need to start fortification in Europe and Belgium?

The safety of this fortification for the general population is being questioned. Folic acid supplementation may increase the concentration of unmetabolized folic acid. The accumulation may result in various adverse outcomes, such as epileptic and neurotoxic effects. It may reduce the response to antifolate drugs used against malaria, rheumatoid arthritis and psoriasis. Folate protects against cancer initiation but could also facilitate progression and growth of subclinical cancers. Thus, a high folic acid intake may be harmful for some people. At the other site, a cost-benefit analysis in the US has shown that folic acid fortification is associated with health gains such as the enhanced prevention of myocardial infarction, colorectal cancer, annual burden of disease... There are conflicting opinions about starting fortification in Europe. [6]

SF reflects recent intake and may be more appropriate for the clinical detection of deficiency in non-fortified countries. In fortified countries the determination of RCF is preferable, being a sensitive and long-term indicator of folate status and stores. [6]

- **Different methods and reference intervals**

Folate measurement can differ depending on the method used for assessment, including various radioimmunoassays, microbiological assays and competitive binding protein assays (CPB). The currently available CPB assays are built on the use of highly specific folate binding proteins (FBP). Various assays bind different folate species in the sample with different affinities. [6]

Differences in results between methods may result in practical problems when patients move between healthcare institutions. Different reference intervals may affect the interpretation of analysis results and have implications for the clinical treatment of patients. [7]

Kristensen et al. [7] evaluated immunoassay results for 5 components (cobalamin, folate, ferritin, free T4 and TSH) obtained by 5 different procedures (Roche Cobas and Modular, Abbott Architect, Beckman Coulter Unicel and Siemens Advia Centaur) at 38 different clinical-chemistry laboratories.

They based their quality specifications on biological variation data. [3] (table 8)

| | CVi | CVg | I (%) - CVa | B (%) | TE (%) |
|-----------------------|--|---|--|---|--|
| | within-subject biologic variation | between-subject biologic variation | desirable specification for imprecision | desirable specification for inaccuracy | desirable specification for allowable total error |
| Serum folate | 24 | 73 | 12% | 19.2% | 39% |
| Red blood cell Folate | 12 | 66 | 6% | 16.8% | 26.7% |

Table 8 : Ricos databases on biological variation for SF and RCF

Their conclusions:

- I. Bias was acceptable

This could be due to the large biological variation of folate and so the large acceptance criterion (Ricos database). The Roche methods didn't show very similar results. (Table 9)

The target value of their examined sample (a serum pool) was defined as the total mean from 3 single mean values for the method groups Roche, Abbott and Siemens (Unicel wasn't included for the mean due to the small number of responders). This target value was divided by the individual laboratory's results.

| | | | Bias from the target value | | | | |
|-----------------|----------------------|---|----------------------------|--------|-------|---------|-------|
| | Target value serum X | Quality goal for desirable bias (19,2%) | Architect | Unicel | Cobas | Modular | Advia |
| Folate (nmol/L) | 14 | ± 2.7 | -1.4 | -1.5 | 2.1 | 0.6 | 0.1 |

Table 9: Bias from the target value for folate.

2. Low method repeatability

Within-method analytical coefficient of variation (CVa) was low for Unicef (but only 2 laboratories included), acceptable for Roche and Abbott but high for Advia, with values above the desirable analytical quality goal. Quite large variations were seen indicating low method repeatability.

3. Method-dependent reference intervals may not compensate for method-dependent differences

The adjusted reference interval (RI) was obtained by multiplying the target with upper and lower reference limits in order to 'eliminate' differences in reference intervals because of method differences. But large variations were seen in the adjusted reference intervals, especially regarding the upper limits.

Laboratories are not able to eliminate method differences by adjusting their reference intervals according to the method they use. This could be explained by the use of different sources of information when determining their reference intervals and thus their lower reference limit (LRL). [7]

During the years, the WHO hold different 'technical consultations' during which they discussed standardizing of cut-off values. [13] They revised the reference values and defined the cut-off value for folate deficiency as the folate concentration below which homocysteine concentrations starts to rise, which is 4 ng/mL.

Some commercial folate methods have recently undergone recalibration to this international standard in order to improve harmonization of the assays, as did the MAYO clinic and the Imelda Hospital.

In January 2016 there was a WHO-recalibration of the Roche Diagnostics Folate III assay which was introduced in our laboratory in June 2016. Despite a change into a lower LRL of 3.9 µg/L -compared to 4.6 µg/L with the previous assay- an increase in prevalence of SF deficiency was noticed when compared with the prevalence of SF deficiency a year before harmonization (17 % respectively 7.8%). This important increase in folate deficiency was also remarked by the hematologist in our hospital.

To rule out an analytical error, the WHO internal standard was tested several times on the Cobas. The target value of the international standard was 5,33 µg/L and the mean of five measurements in our hospital was 5,1 µg/L, with an acceptable bias. Samples with results lower than the LRL were also confirmed in a neighboring hospital laboratory 'Heilig Hart Lier' by another method (Vitros analyzer from Ortho) with different reference intervals (2.8-20 ng/mL).

The higher deficiency prevalence was also confirmed by an external quality assessment program which cumulates data generated by participants using the Roche system. Roche confirmed that this change in standardization resulted in a mean shift of values of +/- 20% over the total measuring range and up to 60% in the lower measuring range. Our higher SF prevalence could partly be explained by the implementation of the new assay and maybe also partly by a small population change caused by a new hematologist in our hospital.

LRL were defined by Roche on the basis of a population study. They collected 404 sera of patients who had a normal homocysteine value and were not pregnant or giving breastfeeding. A LRL of 3,9 ng/mL was found. When the new cohort was tested with the old Folate III assay, a higher cut-off of 5.41 ng/mL was found. This confirmed that a higher percentage of patients was now classified as deficient. Roche suggested that each hospital should examine his own patient population and should so determine his own reference values. They also emphasize that their cut-off value (3.9 ng/mL) correlated well with the cut-off value enunciated by the WHO (4,0 ng mL) which is based on metabolic indicators (homocysteine).

As a reaction on this situation, Ferraro et al. [4] collected serum samples of 322 Italian individuals who had a normal MCV and no signs of hemolysis and were not supplemented with folic acid. The mean folate value in this population was 4,1 µg/L and the reference interval was between 2,9 and 5,6 µg/L. Their LRL was markedly lower than that reported by Roche (3,9 µg/L), likely due to the inclusion of fortified patients in the Roche study.

A misleading overestimate of the prevalence of folate deficiency is expected if the recalibrated Roche assay will be used together with the manufacturer's newly recommended LRL. New experimental data from healthy blood donors have to be quickly obtained with the recalibrated assay in order to accurately define the reference distribution in the proper population and derive strategies for folic acid supplementation. [6]

- **EQA materials**

Kristensen et al. [7] demonstrated that none of the commercial EQA samples (EQA1 liquid pooled serum -EQA2 human-based lyophilized serum) were commutable for folate measurements performed by the Architect and Advia. EQA1 was noncommutable on the Unicel and EQA2 was noncommutable on the Cobas and Modular. The EQA results for SF exhibit a poor inter-method agreement, particularly at clinically significant lower concentrations of the analyte. Commercial EQA samples are therefore a useless tool for harmonization.

Further efforts should be made to produce commutable reference materials and to ensure that their use has an effect on the harmonization of patient results. A lot of organizations such as the Joint Committee for Traceability in Laboratory Medicine, the WHO and the International Federation of Clinical Chemistry are doing an effort lately. [7]

5/ BENCHMARK

We did a benchmark in several hospitals in Belgium. We first checked if the RCF assay was still performed in the questioned hospitals. Four of the seven responding hospitals didn't. In table 10 we present an overview of the analyzers, methods and reference values used for determining folate in the questioned hospitals. (table 10).

| | Hospital | Analyzer | Method | Ref. value SF (µg/L) | Ref. value RCF (µg/L) |
|------------------|------------|-----------------------------|--------|----------------------|-----------------------|
| RCF examined | Hospital A | Cobas (Roche®) | ECLIA | >3,9 | >523 |
| | Hospital B | Modular (Roche®) E | ECLIA | >4 | >523 |
| | Hospital C | Dimension Vista (Siemens®) | CLIA | 3,1 – 17,5 | 140-628 |
| | Hospital D | Dxl (BC®) | CLIA | >3,1 | >140 |
| RCF not examined | Hospital E | Architect (Abbott®) | CMIA | >2,3 | |
| | Hospital F | Architect i1000SR (Abbott®) | CMIA | >3,5 | |
| | Hospital G | Modular (Roche®) E | ECLIA | 3,89 – 26,8 | |
| | Hospital H | Cobas (Roche®) | ECLIA | 3,89 – 26,8 | |

Table 10 : Devices, methods and reference values used for determining folate in several hospitals in Belgium

In table II we give an overview of the participating laboratories with the numbers of performed SF and RCF tests and their rate of deficiency.

| | Ref. value SF (µg/L) | # SF folate/year | # SF def./year | # RCF/year | # RCF def./year | Simult. determ. | Simul. Def. | Def SF - Normal RCF | Norm. SF Def. RCF | Macro. anem |
|--------------------------|----------------------|------------------|---|------------|-----------------|-----------------|-------------|---------------------|-------------------|--|
| Imelda Bonheiden (Cobas) | >3,9 | 5932 | 1014 17% | 3844 | 156 4% | 1025 | 33 3,2% | 142 13,8% | 7 0,7% | 1 (vit B12 started, no Folic acid) |
| Hospital A (Cobas) | >3,9 | | 17,5% | | 4,1% | | 2,3% | 13,6% | 1,5% | No |
| Hospital B (Modular) | >4 | 26686 | 5964 22% (11% zo < 3 µg/L) (0,03% zo < 2 µg/L) | 17117 | 345 2% | 13536 | 207 1,5% | 2721 20% | 54 0,4% | 1 (vit B12 nl, no folic acid) SF: 5,5 µg/L RCF : 512 µg/L (nauwelijks te laag) MCV : 105 fl Hb 12,7 g/dL (renal factor) |
| Hospital C (Dimension) | >3,1 | 17010 | 994 5,8% | 1155 | 3 0,2% | 181 | 0 0% | 15 8,2% | 1 0,5% | No |
| Hospital D (DXL) | >3,1 | 5493 | 257 4,6% | 951 | 10 10,5% | 951 | 1 0,1% | 47 4,9% | 10 1% | 6 (1 vit B12 started, no Folic acid) |

Table II: Number of folate test and deficiencies in several hospitals in Belgium

Folate deficiency was more prevalent in Bonheiden, hospital A and B where analyses were performed on Roche (Cobas and Modular) analyzers. Those hospitals already adapted their reference value to the International Standard. RCF was most frequently determined in Bonheiden and hospital B, compared with the other hospitals. In Hospital D, RCF was always analyzed simultaneously with SF.

6/ FINANCIAL FACTOR

- The reagent cost of SF and RCF is similar. For the RCF test an extra manual handling is necessary and thus results in an overall higher cost. Performing both RCF and SF assays doubles the cost when trying to answer the question of whether or not a patient is folic acid deficient.
- The cost-effectiveness of the test is maximal when reserved for patients suggestive / at risk for deficiency, but becoming low if the test is used as a screening tool. [6]
- On the other hand, it has been shown that the direct costs of folate testing overcome those of supplementation. [6]

CONCLUSION

1. The “Things We Do for No Reason” (TWDFNR) series reviews practices which have become common parts of hospital care but which may provide little value to the patients.
We conclude that there is no added value for using RCF testing as compared to SF testing because SF and RCF levels correlate well with an absence of clinical impact. [8]
2. More representative national surveys are recommended, with standardization of the methods. There is a need for international reference materials (commutable EQA material) and more interaction and communication among laboratories so that population prevalences of deficiency can be correctly determined and compared. Application of universal cutoffs is recommended. [13]
Roche adapted his cut-off value (3.9 ng/mL) which now correlate well with the cut-off value enunciated by the WHO (4,0 ng mL) which is based on metabolic indicators (homocysteine). Though we have to be aware of proved differences in prevalence depending on populations (general fortification programs, individual substitution).
3. Further research about folate substitution is necessary to identify good and bad effects. Only then decisions for an entire population can be made. [11]

TO DO/ ACTIONS

1. The added value of RCF testing could not be demonstrated in this CAT. This was also confirmed by the results of the benchmark, showing that half of the participating laboratories do not perform RCF testing. The results of our analysis will be discussed with our clinicians in view of removing the test in our laboratory.
2. We need to get a better insight/understanding of the (appropriateness of) indications for serum folate testing from our clinicians. This CAT will therefore be presented as a topic on the weekly seminar at the Imelda hospital.
3. We consider adding a note to the folate result on our laboratory report regarding the impact of the ‘new’ reference values on the prevalence of folate deficiency.
4. Follow-up of evolutions in folate substitution, population prevalence, international standards...