

Guidelines for the diagnosis and treatment of Cobalamin and Folate disorders

Vinod Devalia¹, Malcolm S Hamilton², and Anne M Molloy³ on behalf of the British Committee for Standards in Haematology

¹Princess of Wales Hospital, Bridgend, ²Royal Devon and Exeter Hospital, Exeter,

³School of Medicine, Trinity College Dublin, Ireland,

Keywords: cobalamin, folate, holotranscobalamin, homocysteine, methylmalonic acid, intrinsic factor antibody

Address for correspondence:

BCSH Secretary
British Society for Haematology
100 White Lion Street
London
N1 9PF
e-mail bcs@b-s-h.org.uk

Disclaimer

While the advice and information in these guidelines is believed to be true and accurate at the time of going to press, neither the authors, the British Society for Haematology nor the publishers accept any legal responsibility for the content of these guidelines. The British Committee for Standards in Haematology (BCSH) will review this guideline at suitable periods to ensure it remains up-to-date. Please consult the website at www.bcsguidelines.com for updates. The website will also show the date the guideline was last reviewed.

Source of funding: None

Summary of key recommendations

- The clinical picture is the most important factor in assessing the significance of test results assessing cobalamin status since there is no 'gold standard' test to define deficiency.
- Serum cobalamin remains the first line test currently, with additional second line plasma methylmalonic acid to help clarify uncertainties of underlying biochemical/functional deficiencies. Serum holotranscobalamin has the potential as a first line test, but an indeterminate 'grey area' may still exist. Plasma homocysteine may be helpful as a second line test, but is less specific than methylmalonic acid. The availability of these second-line tests is currently limited.
- Definitive cut-off points to define clinical and subclinical deficiency states are not possible, given the variety of methodologies used and technical issues, and local reference ranges should be established.
- In the presence of discordance between the test result and strong clinical features of deficiency, treatment should not be delayed to avoid neurological impairment.
- Treatment of cobalamin deficiency is recommended in line with the British National Formulary. Oral therapy may be suitable and acceptable provided appropriate doses are taken and compliance is not an issue.
- Serum folate offers equivalent diagnostic capability to red cell folate and is the first line test of choice to assess folate status.

Introduction

Measurement of serum cobalamin and folate forms a considerable proportion of the volume of testing in routine haematology and clinical chemistry laboratories, performed for a wide spectrum of clinical conditions (outlined in Table I). The previous British Society for Haematology guidelines on investigation and diagnosis of cobalamin and folate deficiencies (British Committee for Standards in Haematology 1994) were published twenty years ago and this update reflects changes in diagnostic and clinical practice.

These guidelines aim to provide an evidence based approach to diagnosis and management. However, such evidence, particularly in the form of randomised controlled trials, is lacking. As a result, these guidelines provide a pragmatic approach to testing and treatment of cobalamin and folate disorders, with recommendations based, as far as possible, on the GRADE system (Appendix 1: <http://www.gradeworkinggroup.org/index.htm>). In the majority of situations, the recommendations inevitably rely more on clinical judgement and consensus than

objective laboratory data. Table II provides a clinical guide to patient evaluation in assessing cobalamin or folate deficiency.

The Guidelines Writing Group (GWG) reviewed publications upto 2013 including Pubmed and Cochrane databases using index terms including cobalamin, vitamin B12, folate, methylmalonic acid, homocysteine, holotranscobalamin, and combined with deficiency, treatment, pregnancy, oral contraceptive, metformin, bariatric surgery and infancy. Initial review of the manuscript was performed by members of the General Haematology Task Force of the British Committee for Standards in Haematology (BCSH), the executive committee, and a sounding board drawn from UK haematologists.

Cobalamin deficiency

In patients with classical megaloblastic anaemia, the presence of a low serum cobalamin level and objective assessment of response in terms of the rise in haemoglobin concentration clearly outlines the treatment pathway. However, the majority of patients do not have such a clear cut picture. Neurological presentation (peripheral neuropathy, sub-acute combined degeneration of the cord) may occur in the absence of haematological changes, and early treatment is essential to avoid permanent neurological disability. Low cobalamin levels of uncertain significance may occur with non-specific symptoms and no anaemia. Furthermore, patients with strong clinical features of cobalamin deficiency may have serum cobalamin levels which lie within the reference range (false normal cobalamin level). As a result, other tests may be used to try and determine an underlying functional or biochemical deficiency [reviewed in (Quadros 2010) and (Fedosov 2012)]. These tests, plasma homocysteine, plasma methylmalonic acid, and serum holotranscobalamin may help but are not widely available currently and the cut-off points to indicate deficiency vary between different laboratories (Carmel 2011, Heil, *et al* 2012) . In addition, their role is not clearly defined in the routine diagnostic setting. Therefore, there is currently no ‘gold standard’ test for the diagnosis of cobalamin deficiency (Herrmann and Obeid 2012, Solomon 2005).

Since the biochemical pathways of cobalamin and folate are closely intertwined, with patients showing similar clinical features for both deficiencies, assessment of cobalamin and folate status is usually performed concurrently. In the presence of true cobalamin deficiency, the serum folate is often normal or can be elevated. However, a low serum cobalamin level may be found in the presence of folate deficiency.

A. Tests to confirm/diagnose cobalamin deficiency

(a) Mean cell volume and blood film examination

Identification of hypersegmented neutrophils, defined as >5% of neutrophils with 5 or more lobes and the presence of oval macrocytes, may suggest either cobalamin or folate deficiency, but they are not sensitive in early cobalamin deficiency (Carmel, *et al* 1996a) and are not specific for it (Westerman, *et al* 1999) . Oval macrocytes, hypersegmented neutrophils, and circulating megaloblasts in the blood film and megaloblastic change in the bone marrow are the typical features of clinical cobalamin deficiency. However, an elevated mean cell volume (MCV) is not a specific indicator of cobalamin deficiency (Galloway and Hamilton 2007) and the

possibility of underlying myelodysplastic syndrome has to be considered (having excluded alcohol excess, drugs and other causes of an elevated MCV).

The absence of a raised MCV cannot be used to exclude the need for cobalamin testing since neurological impairment occurs with a normal MCV in 25% of cases(Lindenbaum, *et al* 1988) (Healton, *et al* 1991).

(b) Serum Cobalamin

A serum cobalamin assay is currently the standard initial routine diagnostic test. It quantitates both the inactive forms (transcobalamin I- and transcobalamin III- bound, now referred to as holohaptocorrin) and the active form (transcobalamin II-bound, now referred to as holotranscobalamin) of cobalamin in serum. It is widely available, low cost, automated and based on intrinsic factor binding of cobalamin and immune-chemiluminescence based assays. However, it lacks the specificity and sensitivity required of a robust diagnostic test (see appendix 2).

A number of assays are registered on the UK NEQAS scheme, and the overall coefficient of variation of performance between different assays is 5-15%, with intermethod biases of plus 10% or minus 20% from the all-laboratory trimmed mean. Some assays may give false normal results in sera with high titre anti-intrinsic factor antibodies(Carmel and Agrawal 2012, Hamilton, *et al* 2006, Hamilton, *et al* 2010). It is not entirely clear what should be regarded as a clinically normal serum cobalamin level, although it has been proposed that a serum cobalamin of less than 200 ng/L (148 pmol/L) would have a sensitivity of diagnosing 97% of true cobalamin deficiency(Carmel and Sarrai 2006, Snow 1999). It is even less clear what levels of serum cobalamin represent 'subclinical' deficiency i.e. a low serum cobalamin in the absence of clinical symptoms. An additional problem when comparing different assays, and results from different laboratories, is the units for reporting of serum cobalamin levels. Some report in ng/L and some in pmol/L (1 pmol/L = 1.355 ng/L). Despite the absence of an international reference method of measurement, an international standard is available(Thorpe, *et al* 2007) which can facilitate harmonisation of reporting in the future.

Establishment of reference ranges by individual laboratories can be challenging since the serum cobalamin level can be affected by many variables i.e. diet, pregnancy, vitamin supplements, contraceptive pill, metformin etc. They are best conducted by robust studies, accounting for such variables, by the manufacturer. The laboratories may then report their results adjusted in relation to assay bias of the consensus mean in UKNEQAS surveys. Exploration of assay sensitivity and specificity by a laboratory may be done by ROC curve analysis, using methyl malonic acid (MMA) or an alternative appropriate metabolic marker, to define evidence of metabolic disturbance due to cobalamin deficiency [as in (Valente, *et al* 2011)].

(c) Plasma Total Homocysteine (tHcy)

Deficiency of cobalamin results in elevation of plasma homocysteine (tHcy). Plasma tHcy is a sensitive biomarker of cobalamin deficiency and increases early in the course of deficiency, sometimes preceding symptoms and progresses as the deficiency worsens. However, tHcy is not specific to cobalamin deficiency as concentrations of tHcy are also elevated in folate deficiency, B6 deficiency and in patients with renal failure, hypothyroidism and as a result of certain genetic polymorphisms.

In the clinical laboratory, plasma tHcy is measured by a variety of techniques. There is no consensus on the reference range although most laboratories regard a concentration above 15 $\mu\text{mol/L}$ as indicative of hyperhomocysteinemia. Nevertheless, individual laboratories should determine their own reference ranges.

A major drawback to the clinical utility of tHcy is that sample collection and processing is critical as the plasma sample must be kept cool and then centrifuged and removed from the red cells within 2 hour of collection.

(d) Plasma Methylmalonic Acid (MMA)

Plasma MMA is raised in cobalamin deficiency. However, plasma MMA may be falsely elevated in subjects with renal disease, small bowel bacterial overgrowth and haemoconcentration. Despite these limitations, exceptionally high levels of plasma MMA ($>0.75 \mu\text{mol/L}$) almost invariably indicate cobalamin deficiency.

Plasma MMA is quantified using gas-chromatography-mass spectrometry. Hence, this is a high cost test, which has prevented its widespread use. There is no routine national quality assessment scheme for plasma MMA assays in Britain.

High cut off normal reference intervals for plasma MMA vary from 0.27 to 0.75 $\mu\text{mol/L}$ (Heil, *et al* 2012). The GWG recommends collaborative national studies to determine reference ranges.

(e) Holotranscobalamin

Holotranscobalamin (HoloTC), the 'active' fraction of plasma cobalamin, may be more specific than serum cobalamin levels, and an immunoassay for this fraction is now available. In clinical research studies, the HoloTC assay performs better than serum cobalamin assay in assessing deficiency based on MMA levels (Miller, *et al* 2006, Nexo and Hoffmann-Lucke 2011) and red cell cobalamin levels (Valente, *et al* 2011) as reference assays. However, arguments have been raised against accepting this (Carmel 2012a, Schrempf, *et al* 2011) since even MMA or red cell cobalamin may not be regarded as gold standard tests for determining deficiency. Despite this, the assay has a smaller 'grey zone' (uncertainty range) than serum cobalamin assays and better sensitivity and specificity characteristics.

The expected values for HoloTC in healthy individuals are 35-171 pmol/L. Lower and upper reference intervals for plasma HoloTC range from 19-42 pmol/L and 134-157 pmol/L respectively (Brady, *et al* 2008, Refsum, *et al* 2006, Sobczynska-Malefora, *et al* 2014). A recent multicentre study suggested a cut-off of 32 pmol/L of HoloTC for screening for cobalamin deficiency based on MMA level of greater than 0.45 $\mu\text{mol/L}$ (Heil, *et al* 2012). The GWG recommends that individual laboratories should either determine their own reference ranges dependent upon the particular HoloTC assay used or implement the manufacturer's reference range where a suitable study has been conducted.

Further studies are needed to evaluate the clinical utility of HoloTC in assessing cobalamin deficiency in a routine high output laboratory testing. It may cut down the percentage of indeterminate results, particularly in patients over the age of 65 years. There is the added advantage of use in pregnancy and in patients on oral contraceptives since the HoloTC fraction of cobalamin does not seem to be subject to the physiological drop seen in total serum cobalamin over the course of pregnancy (Greibe, *et al* 2011).

Since samples for HoloTC analysis do not need any special pre-analytical preparation, and can be stored for batch analysis, it appears to be a strong candidate for

future routine first line assessment of cobalamin deficiency, particularly if costs of the test become favourable.

(f) Bone marrow examination

Bone marrow examination was historically recommended in situations where the clinical picture is unclear based on laboratory tests alone (BCSH guidelines 1994). However, some cobalamin deficient patients have no overt haematological abnormalities and the value of a marrow examination is unknown.

Recommendations

- **A blood film showing oval macrocytes and hypersegmented neutrophils in the presence of an elevated MCV may alert the clinician to the presence of underlying cobalamin or folate deficiency (Grade 2B)**
- **Cobalamin and folate assays should be assessed concurrently due to the close relationship in metabolism (Grade 1A)**
- **The writing group recommends adoption of reporting for cobalamin assay results in pmol/L (Grade 2C)**
- **A serum cobalamin cut-off level of either 148 pmol/L (200 ng/L) or one derived from a local reference range should be used as evidence of cobalamin deficiency in the presence of a strong clinical suspicion (Grade 2B)**
- **The report providing the result of a serum cobalamin assay should include the following**
 - **The interpretation of the result should be considered in relation to the clinical circumstances**
 - **Falsely low serum cobalamin levels may be seen in the presence of folate deficiency or technical issues**
 - **Neurological symptoms due to cobalamin deficiency may occur in the presence of a normal MCV (Grade 1B)**
- **Plasma tHcy and/or plasma MMA, depending on availability, may be considered as supplementary tests to determine biochemical cobalamin deficiency in the presence of clinical suspicion of deficiency but an indeterminate serum cobalamin level (Grade 2B)**
 - **Although plasma tHcy is a sensitive marker of cobalamin deficiency, plasma MMA is more specific**
 - **Both assays have to be interpreted in relation to renal function**
- **Holotranscobalamin is suggested as a suitable assay for assessment of cobalamin status in a routine diagnostic laboratory in the future (Grade 1B)**

B. Tests to determine the aetiology of cobalamin deficiency

Pernicious anaemia is one of a number of autoimmune diseases, including Hashimoto's disease, type 1 diabetes, vitiligo and hypoadrenalism, which may coexist together (Chanarin 1972, Dittmar and Kahaly 2003, Perros, *et al* 2000, Toh, *et al* 1997). Antibodies against specific tissue antigens can help to diagnose specific conditions.

Pernicious anaemia is characteristically diagnosed by the presence of anti-intrinsic factor antibodies (Annibale, *et al* 2011, Bizzaro and Antico 2014). However,

autoimmune profiles done in patients as part of overall assessments of various endocrinopathies and other autoimmune disorders can reveal antibodies which may be associated with pernicious anaemia (anti-intrinsic factor antibody, anti-parietal cell antibody), raising the possibility of co-existent pernicious anaemia. During investigation of pernicious anaemia, other autoimmune disorders may be found to co-exist, particularly thyroid disease and type 1 diabetes, and it has been suggested that investigation for these should be considered (Chanarin 1972, De Block, *et al* 2008, Ottesen, *et al* 1995). There are no guidelines on screening for pernicious anaemia in other autoimmune disorders and each case has to be judged on individual clinical features.

(a) Anti-intrinsic factor antibody

The finding of a low total serum cobalamin level may be further evaluated by testing for anti-intrinsic factor antibody (IFAB). If positive, the test has a high positive predictive value (95%) for the presence of pernicious anaemia (Toh, *et al* 1997), with a concurrent low false positive rate (1-2%) i.e. a high specificity. It identifies those patients with a need for lifelong cobalamin replacement therapy. IFAB is positive in 40-60% of cases (Ungar 1967) i.e. low sensitivity, and the finding of a negative intrinsic factor antibody assay does not therefore rule out pernicious anaemia (hereafter referred to as AbNegPA). In addition, the positivity rate increases with age (Davidson 1989) and in certain racial groups [Latino-Americans and African-Americans; (Carmel 1992)].

The incidence of pernicious anaemia in the UK population is estimated (extrapolated from NHANES surveys in United States) to lie between 1-5/100,000 per annum i.e. a rare disease. Reflex testing of all low cobalamin samples in a routine diagnostic laboratory is therefore expensive with a low detection rate. A history of other autoimmune disease e.g. hypothyroidism, and family history (Banka, *et al* 2011) increases the pre-test probability of pernicious anaemia.

High titre intrinsic factor antibody may interfere with assays of cobalamin, leading to a false normal serum cobalamin. Testing for intrinsic factor antibody is therefore advised in patients with strong clinical features of deficiency such as megaloblastic anaemia or sub-acute combined degeneration of the cord despite a normal serum cobalamin level. In these cases pre-treatment serum should be stored for investigation with an alternative assay (HoloTC or MMA) to confirm the presence of a severe deficiency.

Intrinsic factor antibody assays, based on automated chemiluminescence cobalamin binding, are vulnerable to give false positive intrinsic factor antibody results if the patient has had a recent cobalamin injection. Manufacturer product literature warns that these assays are only suitable for samples with a specified upper limit of serum cobalamin levels, and laboratories must comply with this advice. Results of assays using these methods should not be reported if the serum cobalamin is greater than 295 pmol/L (400ng/L) based on UK NEQAS survey data. True immunoassays for intrinsic factor antibody based on porcine or recombinant intrinsic factor binding can be used for post treatment samples.

(b) Gastric anti-parietal cell antibody

Gastric parietal cell antibodies (GPC antibodies) have a low specificity for the presence of pernicious anaemia since, despite being positive in 80% of pernicious anaemia subjects, they are also positive in 10% of normal individuals. Positive gastric parietal cell antibodies may cause gastric acid achlorhydria and progression to pernicious anaemia may occur. However, a positive GPC antibody test is not definitive for pernicious anaemia (Khan, *et al* 2009).

(c) Others

In the past a proportion of low cobalamin normal IFAB (AbNegPA) patients might have undergone a Schilling test, which is no longer available. Recently, another test to assess cobalamin absorption based on serum HoloTC levels (Hvas, *et al* 2011) has been described but requires further evaluation.

Pernicious anaemia patients who develop subsequent iron deficiency (indicating evidence of chronic atrophic gastritis) should be investigated with endoscopy due to the slight increased risk of gastric carcinoma, but surveillance endoscopy is not recommended.

Recommendations

- **All patients with anaemia, neuropathy or glossitis, and suspected of having pernicious anaemia, should be tested for anti-intrinsic factor antibody regardless of cobalamin levels (Grade 1A).**
- **Patients found to have a low serum cobalamin level in the absence of anaemia, and who do not have food malabsorption or other causes of deficiency, should be tested for IFAB to clarify whether they have an early/latent presentation of pernicious anaemia (Grade 2A).**
- **Anti-gastric parietal cell antibody testing for diagnosing pernicious anaemia is not recommended (Grade 1A).**

C. Treatment of cobalamin deficiency

Current clinical practice within the U.K is to treat cobalamin deficiency with hydroxocobalamin in the intramuscular form (outlined in the British National Formulary, BNF, <http://www.medicinescomplete.com/mc/bnf/current/PHP5867-drugs-used-in-megaloblastic-anaemias.htm>). Standard initial therapy for patients without neurological involvement is 1000 µg intramuscularly (i.m.) three times a week for two weeks. The BNF advises that patients presenting with neurological symptoms should receive 1000 µg i.m. on alternative days until there is no further improvement. However, the GWG recommends a pragmatic approach in patients with neurological symptoms by reviewing the need for continuation of alternative day therapy after three weeks of treatment.

Patients presenting with severe anaemia may develop a transient hypokalaemia following treatment, the clinical significance of which is unknown (Carmel 1988), and potassium replacement therapy may be considered. In patients presenting with anaemia, a reticulocyte response should be evident by 7-10 days provided the patient has adequate levels of iron and folate. If a haematological response is not achieved the initial diagnosis should be reviewed. A suboptimal response may indicate previously masked iron deficiency or presence of another co-existing cause of anaemia.

Maintenance treatment for patients presenting without neurological deficit is with hydroxocobalamin 1000 µg i.m. every three months. Those with initial neurological deficit should receive hydroxocobalamin 1000 µg i.m. every two months. No further testing for cobalamin levels is required. Although there is little evidence that more frequent dosing is harmful, specific objective studies demonstrating clinical benefit are absent, and the GWG cannot make specific recommendations.

Hydroxocobalamin is generally well tolerated though side effects include itching, exanthema, chills, fever, hot flushes, nausea, dizziness and exceptionally anaphylaxis(Hovding 1968, James and Warin 1971) may occur. This may be due to hypersensitivity to cobalt or any of the other components of the medication(Grant, *et al* 1982). Acneiform eruptions(Dupre, *et al* 1979) have been reported rarely. Due to cross-sensitivity of hydroxocobalamin and cyanocobalamin, treatment of patients may be a challenge. Skin patch testing may help to choose an appropriate product(Heyworth-Smith and Hogan 2002). If absolutely necessary, treatment may be considered under hydrocortisone cover in a hospital setting where severe hypersensitivity can be managed.

High dose oral cyanocobalamin (1000-2000 µg) is licensed for use in several countries outside of the U.K and is widely available via the internet. Passive, intrinsic factor-independent absorption of a small fraction of such large doses should suffice to meet daily requirements. A Cochrane review on the use of oral cobalamin suggests it is as effective as intramuscular B12, with benefit of fewer visits to health centres and reduced discomfort of injections(Vidal-Alaball, *et al* 2005). However the efficacy and cost-effectiveness of oral treatment in wider population based settings has yet to be established. There are arguments against the use of oral cobalamin in initiation of cobalamin therapy in severely deficient individuals who have poor absorption, especially due to pernicious anaemia. High dose oral cobalamin would be a reasonable alternative as maintenance in patients unable to tolerate i.m. injections provided there is good compliance with treatment(Stabler 2013). On the other hand, some patients may prefer i.m. injection therapy in order to assure effective treatment.

Low dose oral cyanocobalamin (BNF, 50 µg) is licensed within the U.K. and may improve serum cobalamin and biochemical markers in borderline cases. Their role in the treatment of subclinical deficiency is under active research. Care must be taken if low dose supplements are prescribed, as such an approach risks the suboptimal treatment of latent and emerging pernicious anaemia with possible inadequate treatment of neurological features.

Recommendations

- **Treatment of established cobalamin deficiency should follow the schedules in the British National Formulary (Grade 1A).**
- **Initial treatment with oral cobalamin may not be appropriate in pernicious anaemia, but may be considered in maintenance or correction of suboptimal levels in asymptomatic patients (Grade 2C).**

D. Recommendations on the clinical approach to investigation and treatment of cobalamin associated disorders

An approach to investigation and management of patients with suspected cobalamin deficiency can be found in algorithm 1 and 2 (figures 1, 2).

(a) Low serum cobalamin and anaemia or strong objective clinical features of glossitis or peripheral neuropathy [Algorithm 1]

These patients are likely to have pernicious anaemia.

Recommendations

- **Patients suspected of having pernicious anaemia should be tested for intrinsic factor antibody. Patients found to be positive should have lifelong therapy with cobalamin (Grade 1A).**
- **Patients negative for intrinsic factor antibody, with no other causes of deficiency, may still have pernicious anaemia and should be treated as anti-intrinsic factor antibody negative pernicious anaemia. Lifelong therapy should be continued in the presence of an objective clinical response. (Grade 2A)**

(b) Borderline or normal serum cobalamin, in the presence of anaemia or other symptoms (false normal cobalamin levels)

Serum cobalamin levels around the normal range have been reported in the presence of significant anaemia (Devalia 2006, Galloway and Hamilton 2007) or other problems (Mahood 1977). This may be a technical problem with the assay due to the presence of high levels of intrinsic factor antibodies (Hamilton, *et al* 2006). Further confirmation of tissue deficiency may be obtained by checking plasma methylmalonic acid and homocysteine, and empirical treatment (as for pernicious anaemia) initiated immediately pending results of second line tests. If possible, pre-treatment serum should be stored in case future further investigation is necessary by the laboratory or UKNEQAS. Confirmed false normal results should be reported to the Medicines and Healthcare products Regulatory Agency (MHRA).

Recommendation

- **Serum cobalamin level of greater than 148 pmol/L (200 ng/l) in the presence of a strong clinical suspicion of cobalamin deficiency should be evaluated further with MMA, tHcy or HoloTC and a trial of hydroxocobalamin given to ascertain any clinical improvement (Grade 1C).**

(c) Low serum cobalamin without anaemia or other significant objective parameters (low cobalamin of uncertain significance) [Algorithm 2]

This group arises from testing for non-specific symptoms (tiredness, neuropsychiatric, 'screening'), largely in the elderly population. This may be described as subclinical deficiency with the level of serum cobalamin possibly falling between 110-148 pmol/L (150-200 ng/L) i.e an 'indeterminate zone' within 25% below the lower reference range (Hvas and Nexø 2006). This highly heterogeneous group poses challenges in further investigation and management, with added patient anxiety of consequences of a low cobalamin level.

In general, this group may contain previously undiagnosed and 'latent' pernicious anaemia (Karnad and Krozser-Hamati 1992), patients with food malabsorption, patients on medications to reduce gastric acid production, and patients on metformin. These patients with a low serum cobalamin of unknown significance (this group is hereafter referred to as LCUS) lack specific symptoms, and even in this

group, there are two subgroups of patients, namely those with biochemical evidence of deficiency and those with none. Management should be based on clinical judgement, with second line tests to demonstrate deficiency in the small number of patients in whom a deficiency is strongly suspected (as above: section A. c, d, e).

In most patients, the serum cobalamin assay should be repeated after 1-2 months. In a proportion, this will return as normal and no further investigation is recommended in these. In those where repeat sampling still falls within the 'subclinical' range, a blood sample should be taken for anti-intrinsic factor antibody and a short trial of empirical therapy (oral cyanocobalamin 50 µg daily for four weeks) should be given (while awaiting results of intrinsic factor antibody), with instructions to the patient to report immediately if symptoms of neuropathy develop since this dose would be inadequate for a true pernicious anaemia. The short course of cobalamin may be of benefit since the elderly have a high incidence of food malabsorption, with some early studies suggesting possible cognitive improvement after cobalamin supplements with or without Folate/B vitamins (de Jager, *et al* 2012, Nilsson, *et al* 2001, Smith and Refsum 2009, Smith, *et al* 2010, van Asselt, *et al* 2001). On return of a positive anti-intrinsic factor, the future management is lifelong therapy. In those negative for this, a further serum cobalamin level is recommended after 3-4 months. If well within the reference range, food malabsorption is a strong possibility and it should be managed accordingly (see section D.h.2 below). If still within the 'subclinical' range, consider investigation (MMA or tHcy) to confirm biochemical deficiency and management as AbNegPA (see above).

In patients with a reduced serum cobalamin and normal tHcy and MMA no further action is required since a normal MMA and tHcy indicates an absence of a cobalamin deficient state. Some patients with paraproteinaemia may have a low serum cobalamin but normal MMA and tHcy, which on treatment of an underlying myeloma, will return to normal, suggesting the initial low value is due to assay interference (Vlasveld 2003).

Recommendation

- **In patients with serum cobalamin levels of 'subclinical deficiency' on two occasions, an empirical trial of treatment with oral cyanocobalamin (50 µg daily for four weeks) should be given. Strict instructions should be given to patients to seek immediate medical attention if symptoms of neuropathy develop. The cobalamin level should be rechecked after 3 months, and second line tests considered if there is no improvement (Grade 2c).**

(d) Low serum cobalamin on metformin

Use of metformin in type II diabetes is associated with reduced serum cobalamin levels (Leung, *et al* 2010, Tomkin, *et al* 1971), related to dose and duration of treatment (Ting, *et al* 2006). The mechanism is unknown but malabsorption may be one factor (Adams, *et al* 1983) and may be alleviated by increased intake of calcium (Bauman, *et al* 2000).

Biochemical evidence of cobalamin deficiency (increase in plasma tHcy) with metformin use has also been shown (Wulffele, *et al* 2003) (de Jager, *et al* 2010) with no resultant clinical impact. In contrast, precipitation and progression of peripheral

neuropathy has been reported (Bell 2010, Wile and Toth 2010). More recently, it has been suggested that despite a low serum cobalamin, the intracellular cobalamin metabolism is improved in patients taking metformin(Obeid, *et al* 2013).

The management dilemmas are similar to those of ‘subclinical’ deficiency, and short empirical treatment could be considered (Mazokopakis and Starakis 2012, Solomon 2011) in the presence of strong clinical suspicion of deficiency to prevent onset/progression of peripheral neuropathy which may prove irreversible otherwise.

Recommendations

- **No definitive advice can be given on the desirable frequency of monitoring of serum cobalamin in patients with type II diabetes mellitus on metformin therapy, but it is recommended that serum cobalamin is checked in the presence of strong clinical suspicion of deficiency (Grade 2B).**
- **If serum cobalamin levels are reduced, patients should have tests for anti-intrinsic factor antibody since the concurrence of pernicious anaemia with diabetes should be considered. If positive, the patient should have lifelong treatment with replacement cobalamin. If negative, the reduced level may be purely as a result of metformin, although underlying AbNegPA cannot be excluded. Treatment with oral cobalamin may be considered (50 µg for one month), with subsequent monitoring of serum cobalamin after six months and then at yearly intervals is suggested (Grade 2C).**
- **Currently no recommendations can be given on prophylactic administration with oral cobalamin in patients taking metformin.**

(e) Patients on hormone replacement therapy (HRT) and oral contraception

Oral contraceptive use causes a reduction in serum cobalamin levels(Lussana, *et al* 2003, Wortalik, *et al* 1972), but may not be significant when using ‘low dose’ oral contraception i.e. containing 20 µg of ethinyl estradiol (Sutterlin, *et al* 2003). The effect of hormone replacement therapy is not conclusive, since, both a reduction of cobalamin(Lacut, *et al* 2004), and no significant effect on cobalamin levels(Carmel, *et al* 1996b), have been reported. However, a cross-sectional study of young women taking oral contraceptives did not show any biochemical evidence of impaired cobalamin status (measuring MMA and tHcy) despite a reduction of serum cobalamin and HoloTC(Riedel, *et al* 2005) of up to 25%. The same study did not show any effect of HRT on serum cobalamin, holoTC, or metabolic markers.

Testing of serum cobalamin is suggested only in the presence of strong clinical suspicion of deficiency since interpretation of results can be challenging.

Recommendation

- **Asymptomatic women taking oral contraception or HRT with mildly reduced serum cobalamin (110-148 pmol/L; 150-200 ng/L) do not require further investigation but should be advised to review their dietary intake of cobalamin rich foods, and cobalamin supplements may be considered.**

Grade 1B)

(f) Pregnancy

Pregnancy causes a lowering of serum cobalamin (Baker, *et al* 2002, Chery, *et al* 2002, Koebnick, *et al* 2002). In normal pregnancy total serum cobalamin levels fall by 30% by the third trimester.

In a longitudinal study of healthy pregnant women, the biochemical marker MMA and tHcy showed a moderate increase in the third trimester indicating a functional intracellular depletion of cobalamin (Chery, *et al* 2002, Murphy, *et al* 2007) suggesting that pregnancy itself can cause a stress on the cobalamin status of the mother. However, the level of HoloTC has been shown to remain unchanged (Morkbak, *et al* 2007), suggesting that this is a better test to assess cobalamin status in pregnancy than serum cobalamin. In the absence of symptoms (parasthesiae, neuropathy or macrocytic anaemia) serum cobalamin levels are impossible to interpret.

Serum cobalamin levels of 110-148 pmol/L (150 to 200 ng/L) in pregnancy may be physiological, and other biochemical tests to determine tissue deficiency are unproven. However, in the presence of strong clinical suspicion, check anti-intrinsic factor antibodies and treat as pernicious anaemia if positive. If a low cobalamin result has been found in the presence of negative anti-intrinsic factor antibody, but with strong clinical suspicion of deficiency, in order to limit extensive investigation with resultant anxiety and to treat potential fetal deficiency, three injections of hydroxocobalamin are suggested to cover the pregnancy, with serum cobalamin levels being checked two months post-partum to ensure resolution to normal levels.

Recommendations

- **Serum cobalamin levels fall during pregnancy and are less reliable in determining underlying deficiency (Grade 1A).**
- **During pregnancy, in the presence of strong suspicion of underlying deficiency, a short course of empirical hydroxocobalamin should be given, with further investigations post-partum (Grade 2C)**
- **HoloTC may be more reliable than serum cobalamin in determining deficiency in pregnancy, and is recommended as the test of choice, if available (Grade 1B).**

(g) Vegetarians

Various studies show that vegetarians, especially vegans, have a lower cobalamin status than omnivores (Stabler and Allen 2004). Biochemical markers of cobalamin status may reach significantly abnormal levels, resulting particularly in hyperhomocysteinaemia, which may have cardiovascular health consequences, and therefore it has been suggested that cobalamin status should be monitored in vegetarians (Herrmann, *et al* 2003, Refsum, *et al* 2001). Strict vegans are at increased risk of deficiency, especially during pregnancy/breast feeding, and oral supplements during this period are recommended.

Recommendation

- **Vegetarians, particularly strict vegans, should be considered for monitoring of their cobalamin level according to clinical assessment (Grade 2C).**
- **Dietary alterations or oral supplementation may be considered according to the clinical situation (Grade 2C), particularly during pregnancy and breast-feeding.**

(h) Poor absorption due to gastrointestinal surgery or disease

(1) Patients who have had gastric surgery have a high prevalence of cobalamin deficiency (Sumner, *et al* 1996), and more recently, treatments for obesity including gastric banding and gastric bypass surgery also lead to cobalamin deficiency (Ledoux, *et al* 2006, Vargas-Ruiz, *et al* 2008). Recommendations for prevention and treatment of nutritional deficiencies have been published (Ziegler, *et al* 2009), which include monitoring of levels and prophylactic supplementation. Oral treatment may not be adequate (Donadelli, *et al* 2012) and lifelong compliance may be poor (Edholm, *et al* 2013). There are no studies of prospective intramuscular cobalamin supplementation in bariatric surgery.

(2) ‘Food-bound cobalamin malabsorption’ has been used to define a group of disorders characterised by gastric hypochlorhydria due to age-related gastric atrophy or secondary to drugs such as the proton pump inhibitors (Bradford and Taylor 1999, Carmel 1995, Lam, *et al* 2013).

In such conditions the cobalamin may not be separated from food protein and therefore is unavailable to the intrinsic factor (IF). This is associated with 30-40% of cases of sub-clinical cobalamin deficiency (Carmel 2012b) (see above).

The degree of this form of malabsorption can vary and the dose of oral cobalamin necessary to correct it cannot be prescriptive. Dose finding studies using 2.5 µg to 1000 µg of oral cyanocobalamin have shown good response in terms of serum cobalamin using lower doses but lesser response in terms of tHcy and MMA (Andres, *et al* 2009, Eussen, *et al* 2005, Rajan, *et al* 2002, Seal, *et al* 2002). A pragmatic approach would suggest starting with a low dose and increasing it as necessary. Future studies should help to clarify what parameters to use in assessing replacement therapy.

(3) Exocrine pancreatic disease results in failure of release of cobalamin from haptocorrin in the duodenum, resulting in cobalamin deficiency (Gueant, *et al* 1990). This is relatively rare and no specific recommendations can be given.

Recommendations

- **Patients who have had bariatric surgery should have their cobalamin status monitored and are likely to need cobalamin supplementation via a route depending upon the type of surgery (Grade 1B).**
- **Patients with food-bound cobalamin malabsorption may benefit from low dose oral replacement (Grade 2C).**

(i) Infancy

The typical symptoms in infancy are failure to thrive, movement disorders, developmental delay and megaloblastosis. Neurological symptoms and signs can

develop without haematological abnormalities. Early recognition and treatment of cobalamin deficiency is important since long-term consequences despite treatment may remain in the form of poor intellectual performance (Bjorke-Monsen and Ueland 2011, Graham, *et al* 1992).

True cobalamin deficiency, with significant clinical problems, is considered to be rare in infants in developed countries and limited data exists on its prevalence. It is largely due to maternal deficiency (Bjorke Monsen, *et al* 2001, Guez, *et al* 2012), particularly when mothers have adhered to a strict vegetarian diet (Rasmussen, *et al* 2001), (Allen 1994), (Specker, *et al* 1990)). Rare cases of occult maternal pernicious anaemia causing cobalamin deficiency in infancy have also been reported (Banka, *et al* 2010).

The cobalamin status undergoes changes during infancy and childhood and a biochemical profile of cobalamin deficiency is thought to be relatively common in breastfed infants (Bjorke-Monsen and Ueland 2011). Breastfeeding is associated with a low cobalamin status (Hay, *et al* 2008) in asymptomatic infants, particularly with prolonged exclusive breast feeding of 4 months or longer duration, in mothers who are replete of cobalamin levels (Greibe, *et al* 2013). This is thought to be due to reduced cobalamin levels in the breast milk with time. However, the clinical significance of this is unknown. It is not known if any replacement is necessary and future studies should hopefully clarify the issue.

Recently, a study in infants with feeding difficulties or development delay in association with impaired cobalamin status, showed benefit from treatment (Torsvik, *et al* 2013) and this too needs further clarification since varying degrees of such symptoms are common.

Various rare hereditary defects of cobalamin absorption and cellular uptake have been reported (Whitehead 2006) resulting in cobalamin deficiency, which may present as early as 1-3 months of age or later at 12-18 months when cobalamin stores acquired *in utero* are exhausted (Linnell and Bhatt 1995). Since the serum cobalamin level may be within reference limits in a number of the cases in this subgroup, further clarification of intracellular cobalamin deficiency has to rely on biochemical tests of MMA, tHcy and others. In addition, identifying the underlying abnormality requires specialist fibroblast culture and molecular biology techniques. Treatment of the inborn errors of cobalamin metabolism is with intramuscular cobalamin, although the response varies greatly in different disorders. In conditions where hyperhomocysteinemia is a feature, betaine can be added to cobalamin supplementation. In conditions with methylmalonic aciduria, total protein or valine, isoleucine, methionine and threonine restriction, carnitine supplementation, lincomycin and metronidazole may help to decrease methylmalonylCoA levels within cells.

Interestingly, asymptomatic newborns have been identified as having biochemical cobalamin deficiency by newborn screening programmes (Sarafoglou, *et al* 2011), mostly due to unsuspected maternal deficiency, but also due to known and recently discovered hereditary disorders (Quadros, *et al* 2010).

Recommendations

- **Reduced serum cobalamin levels in infancy in the presence of clinical features should be treated promptly to prevent long term neurological sequelae (Grade 1A)**
- **In the presence of clinical suspicion of underlying cobalamin deficiency, even in the presence of normal serum cobalamin levels, further biochemical**

tests including MMA and tHcy are recommended (Grade 1B). The role of HoloTC in this context is undefined. Further investigation to define any possible genetic abnormalities should be referred to a specialist centre. No specific recommendation can be made regarding treatment since each case has to be judged individually.

- **No specific recommendations can currently be made in relation to breastfeeding associated biochemical low cobalamin status in asymptomatic infants.**

Folate deficiency

Folate is the term encompassing all the different biologically active forms of the vitamin and folic acid is the synthetic form used in supplements, fortified foods, and for treatment. Both types are absorbed from the proximal small intestine and almost half of the body folate is found in the liver. The bioavailability of dietary folate is influenced by many factors within the intestinal lumen. Natural folates in food are also vulnerable to a variable degree of degradation by cooking processes (McKillop, *et al* 2002). These factors do not tend to affect folic acid and there is general consensus that the bioavailability of food folates is, on average, about 50% lower than folic acid (Gregory 1995).

A low intake or poor absorption leads to a low serum folate level, which then leads to low tissue levels. Since folate is required for DNA synthesis, the earliest signs of deficiency are seen in rapidly proliferating cells such as those of the bone marrow and gastrointestinal tract. Severe folate deficiency can cause pancytopenia and megaloblastic anaemia.

Since serum folate reflects recent dietary intake, concerns have been raised about any assay taken after any oral folate intake, and therefore, masking underlying deficiency and giving a 'false negative' result. In addition, a 'false positive' reduced serum folate may be found in patients with anorexia, acute alcohol consumption, normal pregnancy and patients on anticonvulsant therapy (Beck 1991). It is important to consider the result of the serum folate level within the context of the full clinical picture.

There is no clearly defined progression from the onset of inadequate tissue folate status to development of megaloblastic anaemia. Both biochemical and clinical evidence of folate deficiency can be observed in the absence of clinical symptoms. With increasing adoption of food fortification, the incidence of folate deficiency has declined significantly (Joelson, *et al* 2007), and interpretation of folate status in patients has to be made in relation to presence of known risk factors causing deficiency (Vinker, *et al* 2013).

A. Tests to diagnose folate deficiency

(a) Serum Folate

The serum folate concentration reflects recent folate status and intake. Most clinical laboratories today measure serum folate by competitive folate binding protein assays (FBP) using chemiluminescence or fluorescence detection systems. Despite considerable variation in performance between assays, using the isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) international reference methods, there was close correlation of the consensus mean in the United Kingdom NEQAS surveys (Blackmore, *et al* 2011). This offers the prospect of harmonization of reference ranges for serum folate assays.

There is no clear consensus on the level of serum folate that indicates deficiency. Conventionally, clinicians have used serum folate lower than 7 nmol/L (3 µg/L) as a guideline since the risk of megaloblastic anaemia greatly increases below this level. However, there is a sizeable 'indeterminate zone' (between approximately 7 and 10 nmol/L (3 and 4.5 µg/L)). Therefore a low serum folate should be taken as suggestive of deficiency rather than as a highly sensitive diagnostic test.

(b) Red cell Folate

The red cell folate gives an assessment of the tissue folate status over the lifetime of the red cells and is therefore regarded as an indicator of longer term folate status than the serum folate assay.

A red cell folate level below 340 nmol/L (150 µg/L) has been regarded as consistent with clinical folate deficiency (Joelson, *et al* 2007) in the absence of cobalamin deficiency. Whether serum folate or red cell folate is better for assessing body folate status has been extensively argued. A pathology benchmarking review concluded that serum folate measurement provides equivalent information to red cell measurement (Galloway and Rushworth 2003). However, it is suggested that in about 5% of patients the measurement of red cell folate may be useful in patients with macrocytosis who have a normal serum folate.

Current red cell folate assays are affected by pre-analytical and analytical variables which preclude them as robust assays. There is a lack of standardization of current commercial red cell folate assays, which show very poor inter-method agreement in UKNEQAS red cell folate surveys. A recent review also suggested serum folate measurement may be better than red cell folate since it is affected by fewer pre-analytical and analytical variables (Farrell, *et al* 2013).

(c) Homocysteine (in relation to folate disorders)

Elevated plasma tHcy is a sensitive indicator of folate status and is strongly correlated with serum folate levels in the low physiological range (i.e. serum folate levels below about 10 nmol/L (4.5 µg/L)). tHcy arises as a by-product of methionine metabolism and is normally present in plasma at concentrations below 12 µmol/L, depending on age, gender, renal function, genetic factors and the nutritional status of several other vitamins. It is not, therefore, a specific marker of folate status. Furthermore, it has

stringent requirements regarding sampling and technical analysis. As a result, it is not used for routine testing.

Recommendations

- **A serum folate level less than 7 nmol/L (3 µg/L) is indicative of folate deficiency (Grade 1B).**
- **Routine red cell folate testing is not necessary since serum folate alone is sufficient in most cases (Grade 1A).**
- **In the presence of strong clinical suspicion of folate deficiency, despite a normal serum level, a red cell folate may be undertaken, having ruled out cobalamin deficiency (Grade 2B).**
- **Plasma tHcy can be measured to confirm suspected folate deficiency only in special circumstances; a level above 15 µmol/L could be indicative of folate deficiency but must be assessed in relation to local reference ranges (Grade 2B).**

B. Clinical approach to investigation and treatment of folate associated disorders

There is usually a decrease in serum folate within several weeks of folate deprivation but it is not clear how low the serum folate must be in order to cause knock-on biological and haematological effects.

(a) Conditions mimicking cobalamin deficiency

In practice, cases of isolated clinical folate deficiency are extremely rare in the developed world and a diagnosis of folate deficiency should be made with consideration of a circumstance leading to shortage or malabsorption of multiple nutrients. Furthermore, the metabolic roles of folate and cobalamin are closely linked, and deficiency of either vitamin can result in the same clinical manifestations. In addition, a low serum folate may be associated with a low serum cobalamin, in which case treatment is initiated with cobalamin therapy before adding in folate therapy. Also, serum folate seems to be the preferred marker for folate status in cobalamin deficiency since the red cell folate may be falsely lower in the presence of cobalamin deficiency (Klee 2000).

(b) Anaemia due to folate deficiency

Anaemia due to folate deficiency is now most often seen in cases of poor diet, severe alcoholism, and in certain gastrointestinal diseases (Allen 2008). It may be a presenting feature of coeliac disease.

1) **Dietary deficiency:** The folate status is likely to be poorer in diets reliant on unfortified wheat or rice and consuming low amounts of legumes and green leafy vegetables. A usual dietary folate intake of less than 100 µg per day is regarded as low, and adding 300 µg per day of folic acid is adequate to maintain folate status even during pregnancy (Institute of Medicine 2000).

Goat's milk has a much lower folate concentration than cow's milk (Gregory 1975), and infants fed exclusively on this may become deficient, unless commercial folic acid fortified goat's milk is used.

2) Alcoholism: The risk of developing deficiency is increased when greater than 80 g of ethanol is consumed daily (Gloria, *et al* 1997), with presence of liver disease aggravating the situation (Savage and Lindenbaum 1986). Poor diet (Eichner and Hillman 1971) and increased urinary excretion also contribute to this (Russell, *et al* 1983).

3) Pregnancy: Pregnancy and lactation are associated with increased folate requirements, and preferential delivery of folate to the fetus may result in severe maternal deficiency in the presence of normal folate status in the baby (O'Connor, *et al* 1997). Multiparity and hyperemesis gravidarum increase the risk of developing deficiency in the mother.

Since women are advised to take folate supplements in the preconception period and in early pregnancy, there is no requirement to measure serum folate in pregnancy apart from poor diet, suspected malabsorption, hyperemesis, or development of macrocytic anaemia.

4) Increased requirements: Folate requirement increase in peripheral red cell destruction or abnormal haemopoiesis, manifested by increased red cell requirements or thrombocytopenia, e.g. sickle cell disease (Kennedy, *et al* 2001, Liu 1975) and haemolytic anaemias.

Exfoliative skin diseases also increase folate requirements (Gisoni, *et al* 2007).

5) Haemodialysis: Since serum folate levels are reduced in the immediate post-dialysis period, red cell folate may be the preferable assay soon after dialysis (Heinz, *et al* 2008). However, for pre-dialysis assessment, the serum folate assay may be preferable (De Vecchi, *et al* 2000).

(c) Other folate associated disorders

Low blood folate status is seen following chronic use of some medications (Lindenbaum 1983, Linnebank, *et al* 2011, Mountifield 1985, Neubauer 1970, Reynolds 1967, Wilson, *et al* 2011), particularly anticonvulsants (Eren, *et al* 2010, Linnebank, *et al* 2011) and in individuals homozygous for a well-described functional polymorphism in the *MTHFR* gene (HGNC:7436) which encodes the folate metabolising enzyme 5,10-methylenetetrahydrofolate reductase. The 677 C→T variant leads to a more labile enzyme form that is associated with lower folate status and has been linked to higher risk of several chronic disease conditions and of neural tube defects (Brouns, *et al* 2008). However, there are no specific recommendations for treatment of low folate levels in individuals who are homozygous for this polymorphism i.e. all patients with low folate levels are treated in the same way regardless of their genotype.

Assessment of folate status with respect to the risk of neural tube defects, cardiovascular and cerebrovascular risk is beyond the scope of these guidelines.

Recommendation

- **Folate status is generally checked in clinical situations similar to those of cobalamin deficiency (Grade 1A).**

- **Consultation of the British National Formulary and Summary of Product Characteristics is recommended for clarifying any suspicion of low serum folate levels associated with prescribed medications.**

C. Treatment of Folate deficiency

The dose of folic acid necessary for treatment depends on the cause of the deficiency. A meta-analysis showed that daily doses of 0.8 mg or more of folic acid are typically required to achieve the maximal reduction in plasma homocysteine concentrations produced by folic acid supplementation. Doses of 0.2 and 0.4 mg are associated with 60% and 90%, respectively, of this maximal effect (Homocysteine Lowering Trialists' Collaboration 2005). However, more recently, a dose of 0.2 mg/day over at least six months was shown to have optimal effects (Tighe 2011).

The BNF has outlined the treatment of folate deficiency as follows

(<http://www.medicinescomplete.com/mc/bnf/current/PHP5867-drugs-used-in-megaloblastic-anaemias.htm>):

Folate deficient megaloblastic anaemia (due to dietary insufficiency, pregnancy, or antiepileptics): 5 mg of folic acid daily is taken for 4 months, except in pregnancy where it is continued until term, and up to 15 mg daily for 4 months is suggested in malabsorptive states.

Chronic haemolytic states and renal dialysis: the prophylactic dose suggested is 5 mg daily to weekly, depending on the diet and rate of haemolysis.

Pregnancy: the prophylactic dose suggested is 200-500 µg daily.

Recommendation

- **Treatment of folate disorders should follow the schedules in the British National Formulary (Grade 1A).**

Recommendations for future research

Well designed reference range studies for cobalamin, folate, homocysteine, methylmalonic acid, and holotranscobalamin in target populations addressing variables such as diet, gender, pregnancy, vitamin intake etc.

Prospective long term studies on the natural history of subclinical cobalamin deficiency or LCUS with or without low dose cobalamin therapy.

Research on discovery of more sensitive and specific tests is needed.

Research on ways of assessing adequate replacement would be of benefit in titrating the treatment with clinical efficacy.

Recommendations for audit

Laboratory performance of serum cobalamin assay in relation to UK NEQAS.

Clinical reason for serum cobalamin request as specified on request forms.

Haemoglobin concentration and mean cell volume, if available, taken at same time as serum cobalamin in the deficiency level and 25% below the local laboratory reference range cut off level.

Clinical audit investigating deficiency states in relation to local reference ranges.

Establishment of clinical cut-off points for deficiency with holotranscobalamin assay.

Acknowledgements

All the authors contributed to the writing of these guidelines.

Conflict of interest

AM has received funding from Axis Shield Diagnostics to study the clinical utility of holotranscobalamin.

Table I: Outline of Causes of Cobalamin and Folate deficiency

Population Sector	Cause	Cobalamin deficiency	Folate deficiency
All Ages	Infections	<i>H.pylori, Giardia lamblia</i> , fish tapeworm	-
	Malabsorption	Pernicious anaemia	Poor bioavailability
	Medical conditions	Gastric resection for obesity or cancer, inflammation of small intestine due to coeliac disease, tropical sprue, Crohn's disease	Ileal or gastric resection for obesity or cancer, inflammation of small intestine due to coeliac disease, tropical sprue, Crohn's disease
	Inadequate dietary intake	Low intake of cobalamin-rich foods	Low intake of folate-rich or folic acid-fortified foods
Infants and children	Genetic disorders	Transcobalamin deficiency, Imerslund Grasbeck syndrome, other cbl mutations	Mutations in the proton coupled folate transporter gene (PCFT deficiency)
	Inadequate dietary intake	Maternal strict vegetarian (vegan) diet in pregnancy.	-

		Adherence to a vegan diet post-weaning	
Women of child-bearing age	Pregnancy and lactation	-	Maternal deficiency secondary to high foetal and infant requirements
Older persons	Malabsorption	Achlorhydria due to atrophic gastritis and proton pump inhibitors: results in malabsorption of food-bound cobalamin. Symptoms may develop slowly because secretion of intrinsic factor continues therefore the enterohepatic recycling of cobalamin is not affected	Poor bioavailability

Table II. Useful questions to guide clinicians in suspected cobalamin or folate deficiency

Clinical feature	Possible cause of cobalamin or folate deficiency
Macrocytic anaemia	Consider alcohol excess or myelodysplasia in elderly
Evaluation of diet Is patient vegan or vegetarian? Is patient anorexic or have food fads/ poor diet?	Dietary deficiency
Personal and Family history of autoimmune disease Does patient, parent or sibling have vitiligo, hypothyroidism or pernicious anaemia ?	Positive family history or personal autoimmune conditions increase pretest probability of pernicious anaemia
History of glossitis or mouth ulceration	Glossitis is common presentation of low cobalamin, and mouth ulcers may reflect folate deficiency
History of paraesthesiae, unsteadiness, peripheral neuropathy (particularly proprioception).	Wide differential including cobalamin deficiency, diabetes, carpal tunnel, paraproteinaemia, other causes. Note that neurological presentation of cobalamin deficiency may occur despite normal haematology indices

<p>Features of malabsorption Ask about pale stool, bowel movements at night, abdominal pain, mouth or perianal ulceration. Consider history of pancreatitis, due to alcohol excess.</p>	<p>Steatorrhoea may be due to pancreatic disease, or small bowel disease such as coeliac disease. Terminal ileal disease with mouth and perianal ulceration may be due to Crohn's .</p>
<p>Ask about previous stomach surgery including partial gastrectomy, bariatric surgery, or small bowel resection, which may have occurred as part of a hemicolectomy.</p>	<p>Gastrectomy will result in gradual depletion of cobalamin stores, deficiency occurring 1-2 years post surgery.</p>
<p>Ask about drug history Prolonged proton pump use</p> <p>Metformin</p> <p>Oestrogen contraceptive pill</p>	<p>Omeprazole or equivalent result in a gastric pH of 3.0, this may affect cobalamin release from food, and causes low cobalamin levels Metformin may be associated low cobalamin levels.</p> <p>May be associated with mild reduction in cobalamin levels</p>
<p>Pregnancy</p>	<p>Prepregnancy folate supplements important to reduce risk of neural tube defects. Low cobalamin levels found in 3rd trimester may be physiological.</p>
<p>Neurocognitive impairment in elderly</p>	<p>Low cobalamin levels of uncertain significance in the elderly may be associated with neurocognitive impairment. Low cobalamin or</p>

	folate levels may reflect poor diet as part of poor general condition.
--	--

Appendix 1: Strength of recommendation and quality of evidence

STRENGTH OF RECOMMENDATIONS:

Strong (grade 1): Strong recommendations (grade 1) are made when there is confidence that the benefits do or do not outweigh harm and burden. Grade 1 recommendations can be applied uniformly to most patients. Regard as 'recommend'.

Weak (grade 2): Where the magnitude of benefit or not is less certain a weaker grade 2 recommendation is made. Grade 2 recommendations require judicious application to individual patients. Regard as 'suggest'.

QUALITY OF EVIDENCE

The quality of evidence is graded as high (A), moderate (B) or low (C). To put this in context it is useful to consider the uncertainty of knowledge and whether further research could change what we know or our certainty.

(A) High Further research is very unlikely to change confidence in the estimate of effect. Current evidence derived from randomised clinical trials without important limitations.

(B) Moderate Further research may well have an important impact on confidence in the estimate of effect and may change the estimate. Current evidence derived from randomised clinical trials with important limitations (e.g. inconsistent results, imprecision - wide confidence intervals or methodological flaws - e.g. lack of blinding, large losses to follow up, failure to adhere to intention to treat analysis), or very strong evidence from observational studies or case series (e.g. large or very large and consistent estimates of the magnitude of a treatment effect or demonstration of a dose-response gradient).

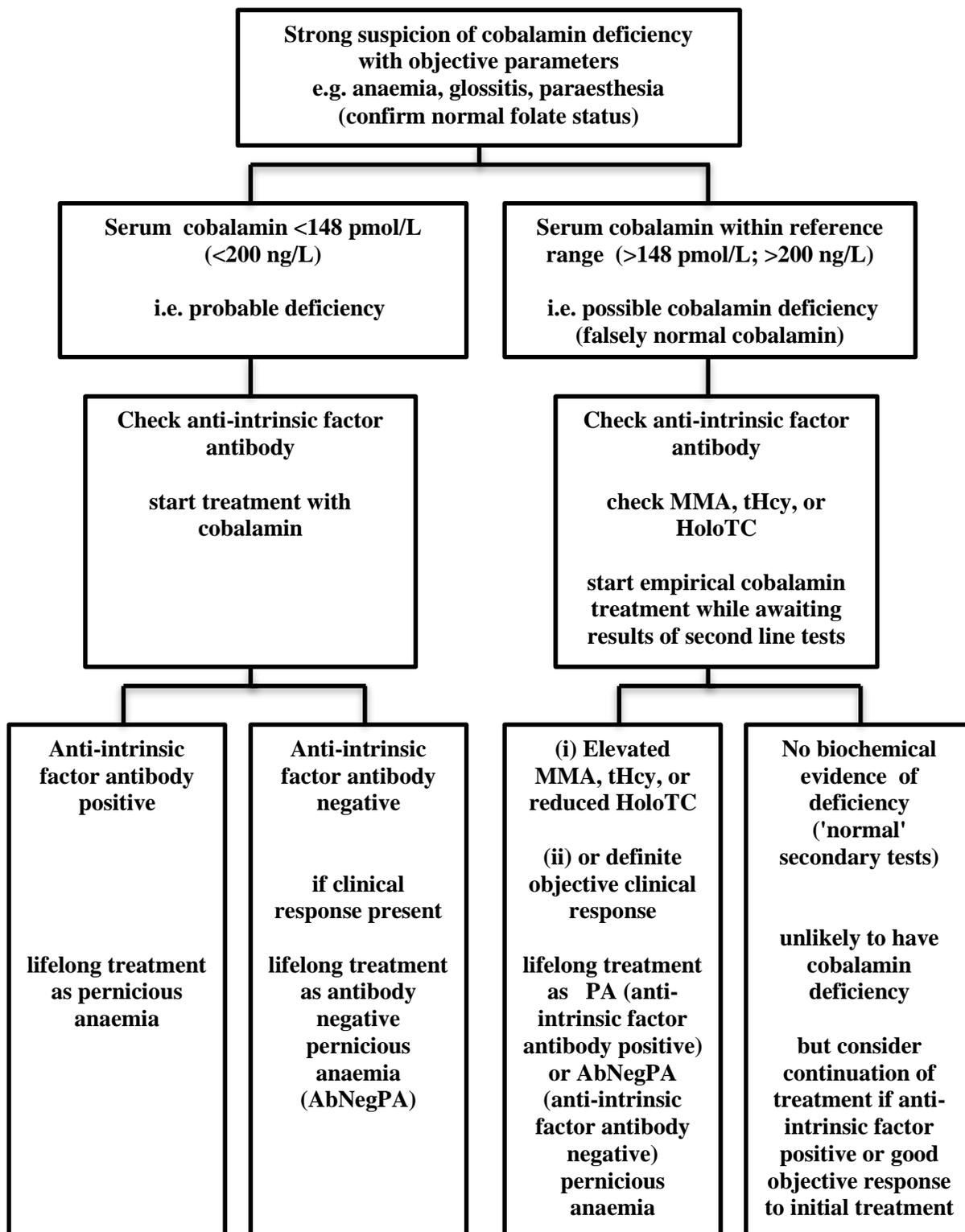
(C) Low Further research is likely to have an important impact on confidence in the estimate of effect and is likely to change the estimate. Current evidence from observational studies, case series or just opinion.

Appendix 2. Problems associated with serum cobalamin assays

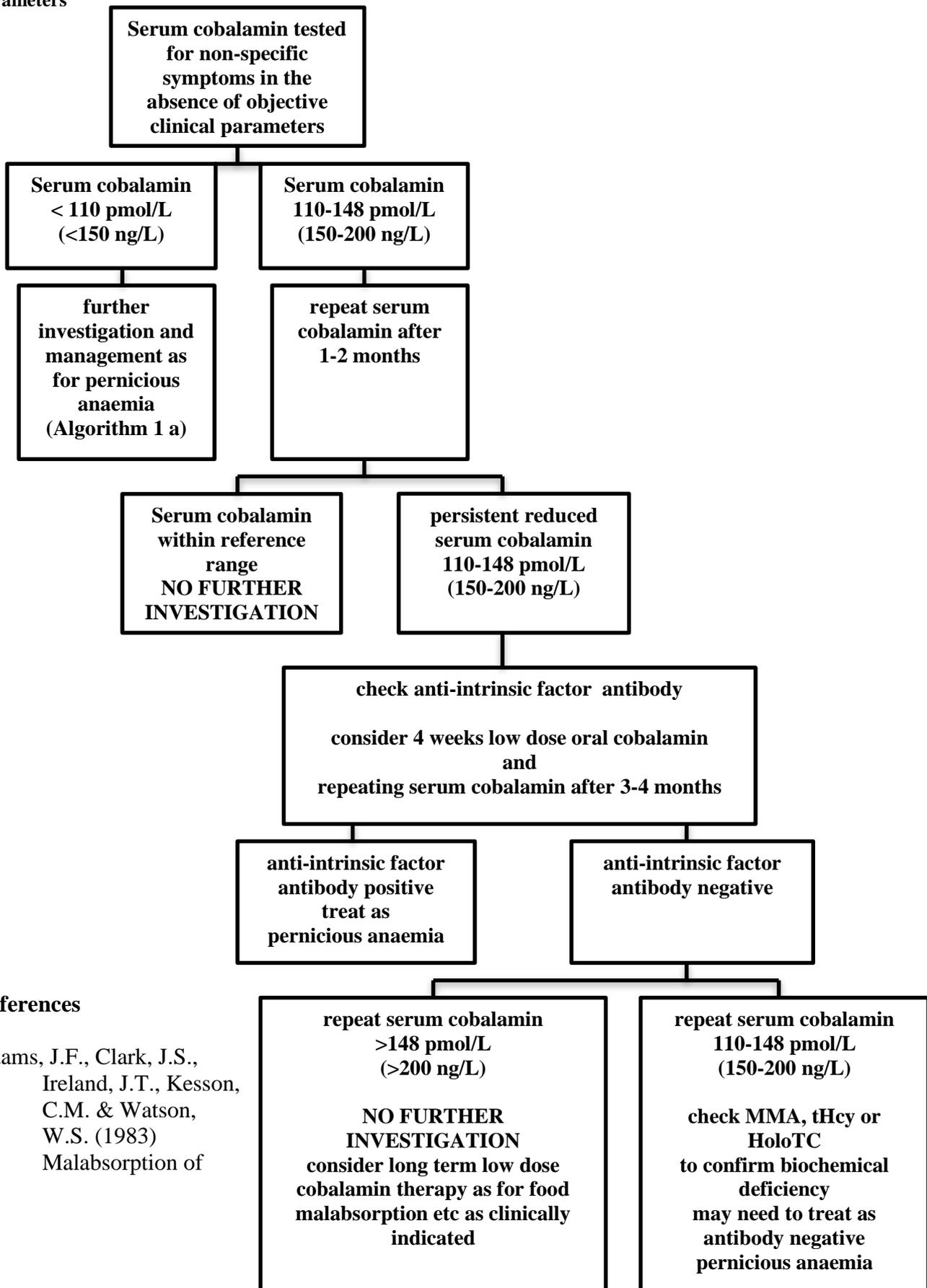
In the case of cobalamin testing there are 3 pitfalls:

1. The definition of a disease state is not clear since individuals identified with low levels or “deficient” includes three categories:
 - a] Individuals with clinical disease state e.g. megaloblastic anaemia or neuropathy.
 - b] Individuals with no symptoms but with evidence of metabolic disturbance i.e. subclinical deficiency identified by metabolic changes but no clinical features.
 - c] Individuals with levels below two standard deviations from the mean of the population studied but no clinical or identifiable metabolic abnormality
2. The diagnostic cut off point may be chosen to identify only 1(a) or to include 1(a) and 1(b). The cut off point of 148 pmol/L (200 ng/l) results in 95% of category 1(a) being identified, ie 95% sensitivity, however 50% of individuals with results below this cut off point will have no identifiable clinical sequelae of cobalamin deficiency i.e. specificity of 50%. Choosing a lower cut off point e.g. 110 pmol/L (150ng/l) will reduce the sensitivity of the test to approximately 75% but will enhance the specificity reducing the false positives and enhancing the true positives. The choice of cut off point can be explored by the use of ROC curves, in studies where a gold standard test can be used to identify deficient patients – however, no entirely suitable gold standard test exists.
3. The definition of a reference range is the mean of a suitably large number of individuals in a study population, plus or minus two standard deviations and may be easily determined by taking the entire database of cobalamin results and taking the 2.5th and 97.5th centile as lower and upper cut-off points. The distribution is not normally distributed, unless log transformed. Some cobalamin distributions are bimodal suggesting interference with the assay at high levels or vitamin supplementation. The range of cobalamin results in the study population itself is affected by age, ethnicity and dietary differences e.g. low levels in vegetarians and vegans, pregnancy, the pill, and drug therapy e.g. metformin, or in long term gastric acid suppression by proton pump inhibitors.

Algorithm 1. Investigation and management of patients presenting with a strong clinical suspicion of cobalamin deficiency and objective parameters to support this



Algorithm 2. Investigation of low serum cobalamin in patients without objective clinical parameters



References

Adams, J.F., Clark, J.S., Ireland, J.T., Kesson, C.M. & Watson, W.S. (1983) Malabsorption of

- vitamin B12 and intrinsic factor secretion during biguanide therapy. *Diabetologia*, **24**, 16-18.
- Allen, L.H. (1994) Vitamin B12 metabolism and status during pregnancy, lactation and infancy. *Adv Exp Med Biol*, **352**, 173-186.
- Allen, L.H. (2008) Causes of vitamin B12 and folate deficiency. *Food Nutr Bull*, **29**, S20-34; discussion S35-27.
- Andres, E., Dali-Youcef, N., Vogel, T., Serraj, K. & Zimmer, J. (2009) Oral cobalamin (vitamin B(12)) treatment. An update. *Int J Lab Hematol*, **31**, 1-8.
- Annibale, B., Lahner, E. & Fave, G.D. (2011) Diagnosis and management of pernicious anemia. *Curr Gastroenterol Rep*, **13**, 518-524.
- Baker, H., DeAngelis, B., Holland, B., Gittens-Williams, L. & Barrett, T., Jr. (2002) Vitamin profile of 563 gravidas during trimesters of pregnancy. *J Am Coll Nutr*, **21**, 33-37.
- Banka, S., Roberts, R., Plews, D. & Newman, W.G. (2010) Early diagnosis and treatment of cobalamin deficiency of infancy owing to occult maternal pernicious anemia. *J Pediatr Hematol Oncol*, **32**, 319-322.
- Banka, S., Ryan, K., Thomson, W. & Newman, W.G. (2011) Pernicious anemia - genetic insights. *Autoimmun Rev*, **10**, 455-459.
- Bauman, W.A., Shaw, S., Jayatilleke, E., Spungen, A.M. & Herbert, V. (2000) Increased intake of calcium reverses vitamin B12 malabsorption induced by metformin. *Diabetes Care*, **23**, 1227-1231.
- Beck, W.S. (1991) Diagnosis of megaloblastic anemia. *Annu Rev Med*, **42**, 311-322.
- Bell, D.S. (2010) Metformin-induced vitamin B12 deficiency presenting as a peripheral neuropathy. *South Med J*, **103**, 265-267.
- Bizzaro, N. & Antico, A. (2014) Diagnosis and classification of pernicious anemia. *Autoimmun Rev*.
- Bjorke Mosen, A.L., Ueland, P.M., Vollset, S.E., Guttormsen, A.B., Markestad, T., Solheim, E. & Refsum, H. (2001) Determinants of cobalamin status in newborns. *Pediatrics*, **108**, 624-630.
- Bjorke-Mosen, A.L. & Ueland, P.M. (2011) Cobalamin status in children. *J Inherit Metab Dis*, **34**, 111-119.
- Blackmore, S., Pfeiffer, C.M., Lee, A., Fazili, Z. & Hamilton, M.S. (2011) Isotope dilution-LC-MS/MS reference method assessment of serum folate assay accuracy and proficiency testing consensus mean. *Clin Chem*, **57**, 986-994.
- Bradford, G.S. & Taylor, C.T. (1999) Omeprazole and vitamin B12 deficiency. *Ann Pharmacother*, **33**, 641-643.
- Brady, J., Wilson, L., McGregor, L., Valente, E. & Orning, L. (2008) Active B12: a rapid, automated assay for holotranscobalamin on the Abbott AxSYM analyzer. *Clin Chem*, **54**, 567-573.
- British Committee for Standards in Haematology (1994) Guidelines on the investigation and diagnosis of cobalamin and folate deficiencies. *Clin Lab Haematol*, **16**, 101-115.
- Brouns, R., Ursem, N., Lindemans, J., Hop, W., Pluijm, S., Steegers, E. & Steegers-Theunissen, R. (2008) Polymorphisms in genes related to folate and cobalamin metabolism and the associations with complex birth defects. *Prenat Diagn*, **28**, 485-493.

- Carmel, R. (1988) Treatment of severe pernicious anemia: no association with sudden death. *Am J Clin Nutr*, **48**, 1443-1444.
- Carmel, R. (1992) Reassessment of the relative prevalences of antibodies to gastric parietal cell and to intrinsic factor in patients with pernicious anaemia: influence of patient age and race. *Clin Exp Immunol*, **89**, 74-77.
- Carmel, R. (1995) Malabsorption of food cobalamin. *Baillieres Clin Haematol*, **8**, 639-655.
- Carmel, R. (2011) Biomarkers of cobalamin (vitamin B-12) status in the epidemiologic setting: a critical overview of context, applications, and performance characteristics of cobalamin, methylmalonic acid, and holotranscobalamin II. *Am J Clin Nutr*, **94**, 348S-358S.
- Carmel, R. (2012a) Holotranscobalamin: not ready for prime time. *Clin Chem*, **58**, 643-645; author reply 645-646.
- Carmel, R. (2012b) Subclinical cobalamin deficiency. *Curr Opin Gastroenterol*, **28**, 151-158.
- Carmel, R. & Agrawal, Y.P. (2012) Failures of cobalamin assays in pernicious anemia. *N Engl J Med*, **367**, 385-386.
- Carmel, R., Green, R., Jacobsen, D.W. & Qian, G.D. (1996a) Neutrophil nuclear segmentation in mild cobalamin deficiency: relation to metabolic tests of cobalamin status and observations on ethnic differences in neutrophil segmentation. *Am J Clin Pathol*, **106**, 57-63.
- Carmel, R., Howard, J.M., Green, R., Jacobsen, D.W. & Azen, C. (1996b) Hormone replacement therapy and cobalamin status in elderly women. *Am J Clin Nutr*, **64**, 856-859.
- Carmel, R. & Sarrai, M. (2006) Diagnosis and management of clinical and subclinical cobalamin deficiency: advances and controversies. *Curr Hematol Rep*, **5**, 23-33.
- Chanarin, I. (1972) Pernicious anaemia as an autoimmune disease. *Br J Haematol*, **23**, Suppl:101-107.
- Chery, C., Barbe, F., Lequere, C., Abdelmoutaleb, I., Gerard, P., Barbarino, P., Boutroy, J.L. & Gueant, J.L. (2002) Hyperhomocysteinemia is related to a decreased blood level of vitamin B12 in the second and third trimester of normal pregnancy. *Clin Chem Lab Med*, **40**, 1105-1108.
- Davidson, R.J., Atrah, H.I., Sewell, H.F. (1989) Longitudinal study of circulating gastric antibodies in pernicious anaemia. *J Clin Pathol*, **42**, 1092-1095.
- De Block, C.E., De Leeuw, I.H. & Van Gaal, L.F. (2008) Autoimmune gastritis in type 1 diabetes: a clinically oriented review. *J Clin Endocrinol Metab*, **93**, 363-371.
- de Jager, C.A., Oulhaj, A., Jacoby, R., Refsum, H. & Smith, A.D. (2012) Cognitive and clinical outcomes of homocysteine-lowering B-vitamin treatment in mild cognitive impairment: a randomized controlled trial. *Int J Geriatr Psychiatry*, **27**, 592-600.
- de Jager, J., Kooy, A., Lehert, P., Wulffele, M.G., van der Kolk, J., Bets, D., Verburg, J., Donker, A.J. & Stehouwer, C.D. (2010) Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial. *BMJ*, **340**, c2181.

- De Vecchi, A.F., Bamonti-Catena, F., Finazzi, S., Campolo, J., Taioli, E., Novembrino, C., Colucci, P., Accinni, R., De Franceschi, M., Fasano, M.A. & Maiolo, A.T. (2000) Homocysteine, vitamin B12, and serum and erythrocyte folate in peritoneal dialysis and hemodialysis patients. *Perit Dial Int*, **20**, 169-173.
- Devalia, V. (2006) Diagnosing vitamin B-12 deficiency on the basis of serum B-12 assay. *BMJ*, **333**, 385-386.
- Dittmar, M. & Kahaly, G.J. (2003) Polyglandular autoimmune syndromes: immunogenetics and long-term follow-up. *J Clin Endocrinol Metab*, **88**, 2983-2992.
- Donadelli, S.P., Junqueira-Franco, M.V., de Mattos Donadelli, C.A., Salgado, W., Jr., Ceneviva, R., Marchini, J.S., Dos Santos, J.E. & Nonino, C.B. (2012) Daily vitamin supplementation and hypovitaminosis after obesity surgery. *Nutrition*, **28**, 391-396.
- Dupre, A., Albarel, N., Bonafe, J.L., Christol, B. & Lassere, J. (1979) Vitamin B-12 induced acnes. *Cutis*, **24**, 210-211.
- Edholm, D., Svensson, F., Naslund, I., Karlsson, F.A., Rask, E. & Sundbom, M. (2012) Long-term results 11 years after primary gastric bypass in 384 patients. *Surg Obes Relat Dis*.
- Eichner, E.R. & Hillman, R.S. (1971) The evolution of anemia in alcoholic patients. *Am J Med*, **50**, 218-232.
- Eren, E., Yegin, A., Yilmaz, N. & Herken, H. (2010) Serum total homocystein, folate and vitamin B12 levels and their correlation with antipsychotic drug doses in adult male patients with chronic schizophrenia. *Clin Lab*, **56**, 513-518.
- Eussen, S.J., de Groot, L.C., Clarke, R., Schneede, J., Ueland, P.M., Hoefnagels, W.H. & van Staveren, W.A. (2005) Oral cyanocobalamin supplementation in older people with vitamin B12 deficiency: a dose-finding trial. *Arch Intern Med*, **165**, 1167-1172.
- Farrell, C.J., Kirsch, S.H. & Herrmann, M. (2013) Red cell or serum folate: what to do in clinical practice? *Clin Chem Lab Med*, **51**, 555-569.
- Fedosov, S.N. (2012) Physiological and molecular aspects of cobalamin transport. *Subcell Biochem*, **56**, 347-367.
- Galloway, M. & Hamilton, M. (2007) Macrocytosis: pitfalls in testing and summary of guidance. *BMJ*, **335**, 884-886.
- Galloway, M. & Rushworth, L. (2003) Red cell or serum folate? Results from the National Pathology Alliance benchmarking review. *J Clin Pathol*, **56**, 924-926.
- Gisondi, P., Fantuzzi, F., Malerba, M. & Girolomoni, G. (2007) Folic acid in general medicine and dermatology. *J Dermatolog Treat*, **18**, 138-146.
- Gloria, L., Cravo, M., Camilo, M.E., Resende, M., Cardoso, J.N., Oliveira, A.G., Leitao, C.N. & Mira, F.C. (1997) Nutritional deficiencies in chronic alcoholics: relation to dietary intake and alcohol consumption. *Am J Gastroenterol*, **92**, 485-489.
- Graham, S.M., Arvela, O.M. & Wise, G.A. (1992) Long-term neurologic consequences of nutritional vitamin B12 deficiency in infants. *J Pediatr*, **121**, 710-714.

- Grant, J.A., Bilodeau, P.A., Guernsey, B.G. & Gardner, F.H. (1982) Unsuspected benzyl alcohol hypersensitivity. *N Engl J Med*, **306**, 108.
- Gregory, J.F. (1995) The bioavailability of folate. In: *Folate in health and disease* (ed. by Bailey, L.B.), pp. 195-223. Marcel Dekker, New York.
- Gregory, M.E. (1975) Reviews of the progress of Dairy Science. Water-soluble vitamins in milk and milk products. *J Dairy Res*, **42**, 197-216.
- Greibe, E., Andreasen, B.H., Lildballe, D.L., Morkbak, A.L., Hvas, A.M. & Nexø, E. (2011) Uptake of cobalamin and markers of cobalamin status: a longitudinal study of healthy pregnant women. *Clin Chem Lab Med*, **49**, 1877-1882.
- Greibe, E., Lildballe, D.L., Strey, S., Vestergaard, P., Rejnmark, L., Mosekilde, L. & Nexø, E. (2013) Cobalamin and haptocorrin in human milk and cobalamin-related variables in mother and child: a 9-mo longitudinal study. *Am J Clin Nutr*, **98**, 389-395.
- Gueant, J.L., Champigneulle, B., Gaucher, P. & Nicolas, J.P. (1990) Malabsorption of vitamin B12 in pancreatic insufficiency of the adult and of the child. *Pancreas*, **5**, 559-567.
- Guez, S., Chiarelli, G., Menni, F., Salera, S., Principi, N. & Esposito, S. (2012) Severe vitamin B12 deficiency in an exclusively breastfed 5-month-old Italian infant born to a mother receiving multivitamin supplementation during pregnancy. *BMC Pediatr*, **12**, 85.
- Hamilton, M.S., Blackmore, S. & Lee, A. (2006) Possible cause of false normal B-12 assays. *BMJ*, **333**, 654-655.
- Hamilton, M.S., Lee, A., Blakemore, S., Bonser, J. & Valente, E. (2010) Ability of holotranscobalamin assay (Active B12) to detect severe cobalamin deficiency evidenced by high methylmalonic acid in the presence of high titre intrinsic factor antibody and false normal B12 results. *Br J Haematol*, **149**, 54.
- Hay, G., Johnston, C., Whitelaw, A., Trygg, K. & Refsum, H. (2008) Folate and cobalamin status in relation to breastfeeding and weaning in healthy infants. *Am J Clin Nutr*, **88**, 105-114.
- Healton, E.B., Savage, D.G., Brust, J.C., Garrett, T.J. & Lindenbaum, J. (1991) Neurologic aspects of cobalamin deficiency. *Medicine (Baltimore)*, **70**, 229-245.
- Heil, S.G., de Jonge, R., de Rotte, M.C., van Wijnen, M., Heiner-Fokkema, R.M., Kobold, A.C., Pekelharing, J.M., Adriaansen, H.J., Sanders, E., Trienekens, P.H., Rammeloo, T. & Lindemans, J. (2012) Screening for metabolic vitamin B12 deficiency by holotranscobalamin in patients suspected of vitamin B12 deficiency: a multicentre study. *Ann Clin Biochem*, **49**, 184-189.
- Heinz, J., Domrose, U., Westphal, S., Luley, C., Neumann, K.H. & Dierkes, J. (2008) Washout of water-soluble vitamins and of homocysteine during haemodialysis: effect of high-flux and low-flux dialyser membranes. *Nephrology (Carlton)*, **13**, 384-389.
- Herrmann, W. & Obeid, R. (2012) Cobalamin deficiency. *Subcell Biochem*, **56**, 301-322.
- Herrmann, W., Schorr, H., Obeid, R. & Geisel, J. (2003) Vitamin B-12 status, particularly holotranscobalamin II and methylmalonic acid concentrations, and hyperhomocysteinemia in vegetarians. *Am J Clin Nutr*, **78**, 131-136.

- Heyworth-Smith, D. & Hogan, P.G. (2002) Allergy to hydroxycobalamin, with tolerance of cyanocobalamin. *Med J Aust*, **177**, 162-163.
- Homocysteine Lowering Trialists' Collaboration (2005) Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials. *Am J Clin Nutr*, **82**, 806-812.
- Hovding, G. (1968) Anaphylactic reaction after injection of vitamin B12. *Br Med J*, **3**, 102.
- Hvas, A.M., Morkbak, A.L., Hardlei, T.F. & Nexo, E. (2011) The vitamin B12 absorption test, CobaSorb, identifies patients not requiring vitamin B12 injection therapy. *Scand J Clin Lab Invest*, **71**, 432-438.
- Hvas, A.M. & Nexo, E. (2006) Diagnosis and treatment of vitamin B12 deficiency--an update. *Haematologica*, **91**, 1506-1512.
- Institute of Medicine (2000) *Dietary reference intakes: Thiamine, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline*. National Academy Press, Washington D.C.
- James, J. & Warin, R.P. (1971) Sensitivity to cyanocobalamin and hydroxocobalamin. *Br Med J*, **2**, 262.
- Joelson, D.W., Fiebig, E.W. & Wu, A.H. (2007) Diminished need for folate measurements among indigent populations in the post folic acid supplementation era. *Arch Pathol Lab Med*, **131**, 477-480.
- Karnad, A.B. & Krozser-Hamati, A. (1992) Pernicious anemia. Early identification to prevent permanent sequelae. *Postgrad Med*, **91**, 231-234, 237.
- Kennedy, T.S., Fung, E.B., Kawchak, D.A., Zemel, B.S., Ohene-Frempong, K. & Stallings, V.A. (2001) Red blood cell folate and serum vitamin B12 status in children with sickle cell disease. *J Pediatr Hematol Oncol*, **23**, 165-169.
- Khan, S., Del-Duca, C., Fenton, E., Holding, S., Hirst, J., Dore, P.C. & Sewell, W.A. (2009) Limited value of testing for intrinsic factor antibodies with negative gastric parietal cell antibodies in pernicious anaemia. *J Clin Pathol*, **62**, 439-441.
- Klee, G.G. (2000) Cobalamin and folate evaluation: measurement of methylmalonic acid and homocysteine vs vitamin B(12) and folate. *Clin Chem*, **46**, 1277-1283.
- Koebnick, C., Heins, U.A., Dagnelie, P.C., Wickramasinghe, S.N., Ratnayaka, I.D., Hothorn, T., Pfahlberg, A.B., Hoffmann, I., Lindemans, J. & Leitzmann, C. (2002) Longitudinal concentrations of vitamin B(12) and vitamin B(12)-binding proteins during uncomplicated pregnancy. *Clin Chem*, **48**, 928-933.
- Lacut, K., Oger, E., Abalain, J.H., Moineau, M.P. & Mottier, D. (2004) Effects of oral and transdermal 17 beta-estradiol combined with progesterone on homocysteine metabolism in postmenopausal women: a randomised placebo-controlled trial. *Atherosclerosis*, **174**, 173-180.
- Lam, J.R., Schneider, J.L., Zhao, W. & Corley, D.A. (2013) Proton pump inhibitor and histamine 2 receptor antagonist use and vitamin B12 deficiency. *JAMA*, **310**, 2435-2442.
- Ledoux, S., Msika, S., Moussa, F., Larger, E., Boudou, P., Salomon, L., Roy, C. & Clerici, C. (2006) Comparison of nutritional consequences of conventional

- therapy of obesity, adjustable gastric banding, and gastric bypass. *Obes Surg*, **16**, 1041-1049.
- Leung, S., Mattman, A., Snyder, F., Kassam, R., Meneilly, G. & Nexo, E. (2010) Metformin induces reductions in plasma cobalamin and haptocorrin bound cobalamin levels in elderly diabetic patients. *Clin Biochem*, **43**, 759-760.
- Lindenbaum, J. (1983) Drugs and vitamin B12 and folate metabolism. *Curr Concepts Nutr*, **12**, 73-87.
- Lindenbaum, J., Heaton, E.B., Savage, D.G., Brust, J.C., Garrett, T.J., Podell, E.R., Marcell, P.D., Stabler, S.P. & Allen, R.H. (1988) Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. *N Engl J Med*, **318**, 1720-1728.
- Linnebank, M., Moskau, S., Semmler, A., Widman, G., Stoffel-Wagner, B., Weller, M. & Elger, C.E. (2011) Antiepileptic drugs interact with folate and vitamin B12 serum levels. *Ann Neurol*, **69**, 352-359.
- Linnell, J.C. & Bhatt, H.R. (1995) Inherited errors of cobalamin metabolism and their management. *Baillieres Clin Haematol*, **8**, 567-601.
- Liu, Y.K. (1975) Folic acid deficiency in sickle cell anaemia. *Scand J Haematol*, **14**, 71-79.
- Lussana, F., Zighetti, M.L., Bucciarelli, P., Cugno, M. & Cattaneo, M. (2003) Blood levels of homocysteine, folate, vitamin B6 and B12 in women using oral contraceptives compared to non-users. *Thromb Res*, **112**, 37-41.
- Mahood, J.M. (1977) Pernicious anemia with neuromyelopathy and normal serum-vitamin-B12. *Lancet*, **2**, 977.
- McKillop, D.J., Pentieva, K., Daly, D., McPartlin, J.M., Hughes, J., Strain, J.J., Scott, J.M. & McNulty, H. (2002) The effect of different cooking methods on folate retention in various foods that are amongst the major contributors to folate intake in the UK diet. *Br J Nutr*, **88**, 681-688.
- Miller, J.W., Garrod, M.G., Rockwood, A.L., Kushnir, M.M., Allen, L.H., Haan, M.N. & Green, R. (2006) Measurement of total vitamin B12 and holotranscobalamin, singly and in combination, in screening for metabolic vitamin B12 deficiency. *Clin Chem*, **52**, 278-285.
- Morkbak, A.L., Hvas, A.M., Milman, N. & Nexo, E. (2007) Holotranscobalamin remains unchanged during pregnancy. Longitudinal changes of cobalamins and their binding proteins during pregnancy and postpartum. *Haematologica*, **92**, 1711-1712.
- Mountifield, J.A. (1985) Effects of oral contraceptive usage on b(12) and folate levels. *Can Fam Physician*, **31**, 1523-1526.
- Murphy, M.M., Molloy, A.M., Ueland, P.M., Fernandez-Ballart, J.D., Schneede, J., Arija, V. & Scott, J.M. (2007) Longitudinal study of the effect of pregnancy on maternal and fetal cobalamin status in healthy women and their offspring. *J Nutr*, **137**, 1863-1867.
- Neubauer, C. (1970) Mental deterioration in epilepsy due to folate deficiency. *Br Med J*, **2**, 759-761.
- Nexo, E. & Hoffmann-Lucke, E. (2011) Holotranscobalamin, a marker of vitamin B-12 status: analytical aspects and clinical utility. *Am J Clin Nutr*, **94**, 359S-365S.

- Nilsson, K., Gustafson, L. & Hultberg, B. (2001) Improvement of cognitive functions after cobalamin/folate supplementation in elderly patients with dementia and elevated plasma homocysteine. *Int J Geriatr Psychiatry*, **16**, 609-614.
- O'Connor, D.L., Green, T. & Picciano, M.F. (1997) Maternal folate status and lactation. *J Mammary Gland Biol Neoplasia*, **2**, 279-289.
- Obeid, R., Jung, J., Falk, J., Herrmann, W., Geisel, J., Friesenhahn-Ochs, B., Lammert, F., Fassbender, K. & Kostopoulos, P. (2013) Serum vitamin B12 not reflecting vitamin B12 status in patients with type 2 diabetes. *Biochimie*, **95**, 1056-1061.
- Ottesen, M., Feldt-Rasmussen, U., Andersen, J., Hippe, E. & Schouboe, A. (1995) Thyroid function and autoimmunity in pernicious anemia before and during cyanocobalamin treatment. *J Endocrinol Invest*, **18**, 91-97.
- Perros, P., Singh, R.K., Ludlam, C.A. & Frier, B.M. (2000) Prevalence of pernicious anaemia in patients with Type 1 diabetes mellitus and autoimmune thyroid disease. *Diabet Med*, **17**, 749-751.
- Quadros, E.V. (2010) Advances in the understanding of cobalamin assimilation and metabolism. *Br J Haematol*, **148**, 195-204.
- Quadros, E.V., Lai, S.C., Nakayama, Y., Sequeira, J.M., Hannibal, L., Wang, S., Jacobsen, D.W., Fedosov, S., Wright, E., Gallagher, R.C., Anastasio, N., Watkins, D. & Rosenblatt, D.S. (2010) Positive newborn screen for methylmalonic aciduria identifies the first mutation in TCbIR/CD320, the gene for cellular uptake of transcobalamin-bound vitamin B(12). *Hum Mutat*, **31**, 924-929.
- Rajan, S., Wallace, J.I., Brodtkin, K.I., Beresford, S.A., Allen, R.H. & Stabler, S.P. (2002) Response of elevated methylmalonic acid to three dose levels of oral cobalamin in older adults. *J Am Geriatr Soc*, **50**, 1789-1795.
- Rasmussen, S.A., Fernhoff, P.M. & Scanlon, K.S. (2001) Vitamin B12 deficiency in children and adolescents. *J Pediatr*, **138**, 10-17.
- Refsum, H., Johnston, C., Guttormsen, A.B. & Nexø, E. (2006) Holotranscobalamin and total transcobalamin in human plasma: determination, determinants, and reference values in healthy adults. *Clin Chem*, **52**, 129-137.
- Refsum, H., Yajnik, C.S., Gadkari, M., Schneede, J., Vollset, S.E., Orning, L., Guttormsen, A.B., Joglekar, A., Sayyad, M.G., Ulvik, A. & Ueland, P.M. (2001) Hyperhomocysteinemia and elevated methylmalonic acid indicate a high prevalence of cobalamin deficiency in Asian Indians. *Am J Clin Nutr*, **74**, 233-241.
- Reynolds, E.H. (1967) Schizophrenia-like psychoses of epilepsy and disturbances of folate and vitamin B12 metabolism induced by anticonvulsant drugs. *Br J Psychiatry*, **113**, 911-919.
- Riedel, B., Bjorke Monsen, A.L., Ueland, P.M. & Schneede, J. (2005) Effects of oral contraceptives and hormone replacement therapy on markers of cobalamin status. *Clin Chem*, **51**, 778-781.
- Russell, R.M., Rosenberg, I.H., Wilson, P.D., Iber, F.L., Oaks, E.B., Giovetti, A.C., Otradovec, C.L., Karwoski, P.A. & Press, A.W. (1983) Increased urinary excretion and prolonged turnover time of folic acid during ethanol ingestion. *Am J Clin Nutr*, **38**, 64-70.

- Sarafoglou, K., Rodgers, J., Hietala, A., Matern, D. & Bentler, K. (2011) Expanded newborn screening for detection of vitamin B12 deficiency. *JAMA*, **305**, 1198-1200.
- Savage, D. & Lindenbaum, J. (1986) Anemia in alcoholics. *Medicine (Baltimore)*, **65**, 322-338.
- Schrempf, W., Eulitz, M., Neumeister, V., Siegert, G., Koch, R., Reichmann, H. & Storch, A. (2011) Utility of measuring vitamin B12 and its active fraction, holotranscobalamin, in neurological vitamin B12 deficiency syndromes. *J Neurol*, **258**, 393-401.
- Seal, E.C., Metz, J., Flicker, L. & Melny, J. (2002) A randomized, double-blind, placebo-controlled study of oral vitamin B12 supplementation in older patients with subnormal or borderline serum vitamin B12 concentrations. *J Am Geriatr Soc*, **50**, 146-151.
- Smith, A.D. & Refsum, H. (2009) Vitamin B-12 and cognition in the elderly. *Am J Clin Nutr*, **89**, 707S-711S.
- Smith, A.D., Smith, S.M., de Jager, C.A., Whitbread, P., Johnston, C., Agacinski, G., Oulhaj, A., Bradley, K.M., Jacoby, R. & Refsum, H. (2010) Homocysteine-lowering by B vitamins slows the rate of accelerated brain atrophy in mild cognitive impairment: a randomized controlled trial. *PLoS One*, **5**, e12244.
- Snow, C.F. (1999) Laboratory diagnosis of vitamin B12 and folate deficiency: a guide for the primary care physician. *Arch Intern Med*, **159**, 1289-1298.
- Sobczynska-Malefora, A., Gorska, R., Pelisser, M., Ruwona, P., Witchlow, B. & Harrington, D.J. (2014) An audit of holotranscobalamin ("Active" B12) and methylmalonic acid assays for the assessment of vitamin B12 status: application in a mixed patient population. *Clin Biochem*, **47**, 82-86.
- Solomon, L.R. (2005) Cobalamin-responsive disorders in the ambulatory care setting: unreliability of cobalamin, methylmalonic acid, and homocysteine testing. *Blood*, **105**, 978-985.
- Solomon, L.R. (2011) Diabetes as a cause of clinically significant functional cobalamin deficiency. *Diabetes Care*, **34**, 1077-1080.
- Specker, B.L., Black, A., Allen, L. & Morrow, F. (1990) Vitamin B-12: low milk concentrations are related to low serum concentrations in vegetarian women and to methylmalonic aciduria in their infants. *Am J Clin Nutr*, **52**, 1073-1076.
- Stabler, S.P. (2013) Clinical practice. Vitamin B12 deficiency. *N Engl J Med*, **368**, 149-160.
- Stabler, S.P. & Allen, R.H. (2004) Vitamin B12 deficiency as a worldwide problem. *Annu Rev Nutr*, **24**, 299-326.
- Sumner, A.E., Chin, M.M., Abrahm, J.L., Berry, G.T., Gracely, E.J., Allen, R.H. & Stabler, S.P. (1996) Elevated methylmalonic acid and total homocysteine levels show high prevalence of vitamin B12 deficiency after gastric surgery. *Ann Intern Med*, **124**, 469-476.
- Sutterlin, M.W., Bussen, S.S., Rieger, L., Dietl, J. & Steck, T. (2003) Serum folate and Vitamin B12 levels in women using modern oral contraceptives (OC) containing 20 microg ethinyl estradiol. *Eur J Obstet Gynecol Reprod Biol*, **107**, 57-61.

- Thorpe, S.J., Heath, A., Blackmore, S., Lee, A., Hamilton, M., O'Broin, S., Nelson, B.C. & Pfeiffer, C. (2007) International Standard for serum vitamin B(12) and serum folate: international collaborative study to evaluate a batch of lyophilised serum for B(12) and folate content. *Clin Chem Lab Med*, **45**, 380-386.
- Tighe, P., Ward, M., McNulty, H., Finnegan, O., Dunne, A., Strain, J., Molloy, A.M., Duffy, M., Pentieva, K., and Scott, J.M. (2011) A dose-finding trial of the effect of long-term folic acid intervention: implications for food fortification policy. *Am J Clin Nutr*, **93**, 11-18.
- Ting, R.Z., Szeto, C.C., Chan, M.H., Ma, K.K. & Chow, K.M. (2006) Risk factors of vitamin B(12) deficiency in patients receiving metformin. *Arch Intern Med*, **166**, 1975-1979.
- Toh, B.H., van Driel, I.R. & Gleeson, P.A. (1997) Pernicious anemia. *N Engl J Med*, **337**, 1441-1448.
- Tomkin, G.H., Hadden, D.R., Weaver, J.A. & Montgomery, D.A. (1971) Vitamin-B12 status of patients on long-term metformin therapy. *Br Med J*, **2**, 685-687.
- Torsvik, I., Ueland, P.M., Markestad, T. & Bjorke-Monsen, A.L. (2013) Cobalamin supplementation improves motor development and regurgitations in infants: results from a randomized intervention study. *Am J Clin Nutr*, **98**, 1233-1240.
- Ungar, B., Whittingham, S., Francis, C.M. (1967) Pernicious anaemia: incidence and significance of circulating antibodies to intrinsic factor and to parietal cells. *Australas Ann Med*, **16**, 226-229.
- Valente, E., Scott, J.M., Ueland, P.M., Cunningham, C., Casey, M. & Molloy, A.M. (2011) Diagnostic accuracy of holotranscobalamin, methylmalonic acid, serum cobalamin, and other indicators of tissue vitamin B(1)(2) status in the elderly. *Clin Chem*, **57**, 856-863.
- van Asselt, D.Z., Pasman, J.W., van Lier, H.J., Vingerhoets, D.M., Poels, P.J., Kuin, Y., Blom, H.J. & Hoefnagels, W.H. (2001) Cobalamin supplementation improves cognitive and cerebral function in older, cobalamin-deficient persons. *J Gerontol A Biol Sci Med Sci*, **56**, M775-779.
- Vargas-Ruiz, A.G., Hernandez-Rivera, G. & Herrera, M.F. (2008) Prevalence of iron, folate, and vitamin B12 deficiency anemia after laparoscopic Roux-en-Y gastric bypass. *Obes Surg*, **18**, 288-293.
- Vidal-Alaball, J., Butler, C.C., Cannings-John, R., Goringe, A., Hood, K., McCaddon, A., McDowell, I. & Papaioannou, A. (2005) Oral vitamin B12 versus intramuscular vitamin B12 for vitamin B12 deficiency. *Cochrane Database Syst Rev*, CD004655.
- Vinker, S., Krantman, E., Shani, M. & Nakar, S. (2013) Low clinical utility of folate determinations in primary care setting. *Am J Manag Care*, **19**, e100-105.
- Vlasveld, L.T. (2003) Low cobalamin (vitamin B12) levels in multiple myeloma: a retrospective study. *Neth J Med*, **61**, 249-252.
- Wertalik, L.F., Metz, E.N., LoBuglio, A.F. & Balcerzak, S.P. (1972) Decreased serum B 12 levels with oral contraceptive use. *JAMA*, **221**, 1371-1374.
- Westerman, D.A., Evans, D. & Metz, J. (1999) Neutrophil hypersegmentation in iron deficiency anaemia: a case-control study. *Br J Haematol*, **107**, 512-515.

- Whitehead, V.M. (2006) Acquired and inherited disorders of cobalamin and folate in children. *Br J Haematol*, **134**, 125-136.
- Wile, D.J. & Toth, C. (2010) Association of metformin, elevated homocysteine, and methylmalonic acid levels and clinically worsened diabetic peripheral neuropathy. *Diabetes Care*, **33**, 156-161.
- Wilson, S.M., Bivins, B.N., Russell, K.A. & Bailey, L.B. (2011) Oral contraceptive use: impact on folate, vitamin B(6), and vitamin B(1)(2) status. *Nutr Rev*, **69**, 572-583.
- Wulffele, M.G., Kooy, A., Lehert, P., Bets, D., Ogterop, J.C., Borger van der Burg, B., Donker, A.J. & Stehouwer, C.D. (2003) Effects of short-term treatment with metformin on serum concentrations of homocysteine, folate and vitamin B12 in type 2 diabetes mellitus: a randomized, placebo-controlled trial. *J Intern Med*, **254**, 455-463.
- Ziegler, O., Sirveaux, M.A., Brunaud, L., Reibel, N. & Quilliot, D. (2009) Medical follow up after bariatric surgery: nutritional and drug issues. General recommendations for the prevention and treatment of nutritional deficiencies. *Diabetes Metab*, **35**, 544-557.