The limited value of methylmalonic acid, homocysteine and holotranscobalamin in the diagnosis of early B12 deficiency

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Treatment of B12 deficiency is important to prevent progressive neurological and/or hematologic disease but requires a secure diagnosis. The aim of this study was to evaluate second line tests of B12 status as prognostic indicators of a hematologic response to vitamin B12 therapy. Forty-nine patients referred with low, serum vitamin B12 concentrations were treated with intramuscular B12 and re-assessed after 3 months. Methylmalonic acid, homocysteine, holotranscobalamin and neutrophil hypersegmentation index were measured before and after treatment. Before treatment 27/49 patients were anemic or macrocytic of whom 15 had a clear hematologic response. All the tests had a similar prognostic accuracy. Symptomatic improvement did not correlate with hematologic response. Supplementary tests of vitamin B12 status were not significantly better than total serum B12 concentration as predictors of a hematologic response to vitamin B12 therapy.

Key words: vitamin B12 deficiency, B12 therapy, methylmalonic acid, homocysteine, transcobalamin, neutrophil hypersegmentation index.

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Low serum B12 concentrations are common, especially amongst the elderly[1,2] although up to 90% may not manifest clinical signs of vitamin B12 deficiency.[3] Nevertheless, these people may be at risk from progressive neurological and/or hematologic disease.[3] Neurological symptoms have been reported to occur in the absence of anemia in up to 28% of cases.[4] The need for a secure diagnosis is therefore important but the role of supplementary tests of B12 status remains unclear. Methylmalonic acid (MMA) or total homocysteine (tHCY)[5] and holotranscobalamin (holoTC)[6,7] have been advocated as additional tests in this setting. The aim of this study was to evaluate the added value of *second line* tests of B12 status (MMA, tHCY, holoTC) as prognostic indicators of hematologic response to B12 therapy. We also evaluated the neutrophil hypersegmentation index.[8]

Design and Methods

Patients were recruited from those referred to the Hematology Laboratory of the Cardiff and Vale NHS Trust for B12 estimation. From June 2002 to July 2003, 5984 samples were received from general practitioners for vitamin B12 assay including 479 with levels < 170 ng/L (the laboratory lower reference limit). After excluding those with abnormal liver function tests, hypothyroidism (free T4, TSH), alcohol abuse, folate deficiency (serum folate <2.1 μg/L) or renal failure (raised serum creatinine) there were 151 patients under 75 years old with serum B12 <170 ng/L. These were invited to take part in the study through their general practitioners. Fifty-two (34 %) agreed to take part and 49 completed the study. Following clinical assessment in the Hematology Clinic, further blood samples were taken and treatment was administered with intramuscular B12 (1 mg per week for 4 weeks).[9] The patients were re-assessed at 3 months. The treating physician (AG) was provided only with the routine laboratory information (full blood count, total B12 and ferritin) during the course of the study.

Blood was drawn with the patient seated and was centrifuged within 1 hour. Laboratory investigations included the full blood count (ABX Pentra 120 analyzer), a peripheral blood smear, B12/folate and ferritin assays (Roche E170 immunoassay analyser), holoTC (Axis Shield), homocysteine (fluorescence polarization immunoassay, Abbot IMX) and methylmalonic acid (in house GCMS) and neutrophil hyper-segmentation index.[8] The laboratory reference limits for vitamin B12 deficiency were: hemoglobin (Hb) <13.0 g/dL (men) or <12.0 g/dL (women), mean corpuscular volume (MCV) >99 fL, serum B12 <170 ng/L, holoTC <38 pmol/L, MMA >0.47 μmol/L, tHCY >15 μmolL and neutrophil hypersegmentation index >16.9. The reference range for MMA (mean±1.96 SD) was derived from analysis of plasma levels in 82 healthy adults with no clinical or laboratory evidence of

Table 1. Hematologic and biochemical measurements for patients (n=49) at first clinic visit and after treatment.

Analyte	*Women (n=35)**			*Men (n=14)**		
	1st clinic visit	*After treatment*	*p*	*1st clinic visit*	*After treatment*	*p*
Hb (g/dL)°	13.4 (1.3)	13.4 (1.4)	NS	14.5 (1.8)	14.6 (1.1)	NS
MCV (fL)	94.7 (10.0)	91.4 (10.0)	NS	98.5 (9.2)	94.2 (6.0)	NS
Serum B12 (ng/L)	147 (27.6)	621 (47.5)	<0.001	147 (59.8)	721 (59.5)	0.003
Serum ferritin (μg/L)+	59 (59)	58 (61)	NS	123 (78)	116 (757)	NS
Serum holoTC (pmol/L)	19.9 (8.7)	94 (78)	<0.001	18.0 (12.3)	105 (87)	0.001
Serum MMA (μmol/L)	0.665 (0.86)	0.274 (0.114)	0.011	1.54 (1.98)	0.255 (0.099)	0.031
Serum homocysteine (μmol/L)	28.1 (26.8)	17.6 (15.8)	0.055	34.8 (28.0)	13.0 (8.5)	0.014
Neutrophil hyperseg. index	17 (11)	14 (9)	NS	13 (15)	14 (10)	0.097

*Values are mean (SD); *Mean age of women: 48±14.7 years; mean age of men: 60±12 years (p=0.006); °Hb concentration was significantly higher in men than in women at the first visit (p=0.046); +Serum ferritin concentration was significantly higher in men than in women at the first visit (p=0.012).*

cobalamin deficiency.[10] Statistical analyses were carried out with Minitab (v13); differences between mean values were evaluated using the t test and differences between median values were evaluated with the Kruskal-Wallis test. Receiver operator characteristics (ROC) curves were plotted using SPSS (v12). This study was approved by the local ethics committee and all patients gave informed consent.

Results and Discussion

At the end of the study patients were divided into two groups based on initial Hb level and red cell (*hematologic*) response. Group 1 subjects had a low Hb concentration at their first hospital visit and/or macrocytosis (pre-treatment MCV of 97 fL or more); these patients were divided into those who responded to treatment (group 1a) and those who did not (group 1 b). Response to treatment is defined as an increase in Hb concentration of 1.0 g/dL or more and/or a decrease in MCV of 3 fl or more. This value of 3 fL represents twice the expected total, intraindividual variation over a period of several months.[11] The threshold for macrocytosis of 97 fL or more also allows for this variability). Group 2 patients had a normal, initial Hb concentration and MCV < 97 g/dL.

Baseline results

The laboratory indices at the first clinic visit are shown in Table 1. The mean ages, Hb, MCV, serum B12 and folate levels for the 63 women and 36 men who did not take part were not significantly different from those of the 35 women and 14 men who did take part. At the first clinic visit correlations were observed between vitamin B12 concentrations and holoTC (r^2=0.397, p<0.001), and inversely with MMA (r^2=0.583, p<0.001), tHCY levels (r^2=0.182, p=0.001) and the degree of neutrophil hypersegmentation (r^2=0.303, p<0.001).

Response to B12 therapy

After treatment mean serum B12 and holoTC concentrations increased about 5-fold. Overall, there were no significant changes in mean Hb, MCV and serum ferritin concentration (Table 1) whereas there were significant decreases in MMA (p=0.013), tHCY (p=0.001) and the neutrophil hypersegmentation index (p=0.013). HoloTC concentrations increased more than 2-fold in all cases but three: there were three patients who showed a hematologic response but whose holoTc remained below the lower limit of normal. In all but one case MMA values were less than 0.47 μmol/L. In 14 patients (29%) abnormal tHCY levels did not return to the reference range although in all but two cases the level dropped by more than 25% (a significant change).[12] In 12 patients the neutrophil hypersegmentation index remained high. At the initial visit to the clinic 27 of the 49 patients were either anemic or macrocytic. Of these, 15 showed a hematologic response (group 1a) and 12 did not (group 1b). Of the 49 patients who completed the study six were anemic before treatment. Three, with normal serum ferritin concentrations, responded and the ferritin concentration fell by more than 20%. The other three patients, with ferritin concentrations < 15 μg/L, did not respond. MMA values were normal although one had a raised level of tHCY. Another four female patients were iron deficient. One, with a raised tHCY concentration (19.9 μmol/L), showed a hematologic response. Mean B12 and holoTC levels at the first clinic visit (Table 2) were significantly lower in group 1a than in group 2. The mean level of tHCY was significantly higher in group 1a than in group 2. The mean reticulocyte count for the 49 patients, which was 67×10^9/L, did not change significantly with treatment and did not vary between the three response groups. Pre-treatment analyte concentrations for groups 1a, 1b and 2 are shown in Table 3. For patients with anemia and/or macrocytosis the likelihood of a response to

Table 2. Hematologic and biochemical indices in the response groups before treatment. Results are mean (SD).

Variable	*Group 1a (n=15)*	*Group 1b (n=12)*	*Group 2 (n=22)*
Serum B12 (ng/L)	121 (38)°	158 (45)	160 (27)
Serum ferritin (μg/L)	78 (62)	98 (94)	65 (61)
Serum MMA (μmol/L)	1.74 (2.1)	0.55 (0.45)	0.55 (0.51)
tHCY (μmol/L)	51.8 (34.1)°	29.3 (22.7)	14.3 (5.7)
holoTC (pmol/L)	13.4 (9.0)*	22.0 (10.6)	21.9 (8.2)
Neutrophil hypersegmentation index	25.3 (13.7)	15.3 (11.9)	15.1 (9.5)

**Significantly different to group 2 ($p < 0.05$) °$p < 0.01$.*

Table 3. Number of patients with abnormal analyte concentrations in the three treatment response groups.

Group (no of patients)	*MMA↑*	*tHCY↑*	*holoTC↓*	*NSI↑*	*B12↓*
1a.Responders (15)					
Low Hb (and raised MCV) (3)	3	3	3	3	3
High MCV only (12)	8	12	12	7	10
All (15)	11	15	15	10	13
1b. Non responders (12)					
Low Hb only+ (3)	0	1	3	2	2
High MCV only (9)	4	6	9	1*	6
All (12)	4	7	12	3	8
2. Normal Hb and MCV (22)	8	5*	18	6	15

*NSI: neutrophil hyper segmentation index. *No result obtained for one patient; +these three patients had serum ferritin concentrations ≤10 μg/L.*

B12 therapy was 0.73, 0.68, 0.77 and 0.62 if MMA, tHCY, hypersegmention index or B12, respectively, were outside the laboratory reference range. If the values were normal the likelihood of a response was 0.33, 0.0, 0.38 and 0.33. HoloTC values were abnormal in all but one patient.

ROC curves for predicting a response were plotted for the 27 patients who were anemic and/or macrocytic. The areas under the ROC curves (95% CI) were 0.73 (0.54-0.93) for MMA, 0.74 (0.55-0.93) for tHCY, 0.75 (0.55-0.94) for holoTC and 0.67 (0.46-0.93) for the neutrophil hypersegmentation index. None of these differed significantly from the area under the ROC curve for vitamin B12 for predicting a hematologic response - 0.72 (CI 0.52 – 0.92) nor from that for the initial serum vitamin B12 (0.84, CI 0.68 – 1.00). The optimum diagnostic thresholds differed from laboratory thresholds (MMA >0.62 μmol/L, tHCY >43 μmol/L, holoTC < 16 pmol/L, hypersegmentation index >11.8 and vitamin B12 <130 ng/L).

Most patients complained of general non-specific tiredness with or without symptoms of peripheral neuropathy or muscle cramps. There was no correlation between hematologic responses and patients' assessment of their overall well-being after treatment: 53% of patients not having a hematologic response claimed to feel better compared to 73 % of those with a hematologic response (χ^2=1.05, p=0.31). However serum B12 and holoTC levels prior to treatment were lower in those who felt better than those who did not (Kruskal-Wallis test, p=0.04). One patient presented with classical symptoms of early peripheral neuropathy with a normal full blood count and a normal repeat B12 level (172 ng/L). Her MMA was markedly increased (2.6 μmol/L), holoTC low (4.9 pmol/L) and tHCY mildly raised (17.3 μmol/L). Her symptoms resolved entirely after treatment suggesting that MMA was reflecting functional B12 status in neurological terms. Six patients described a major, short-lived improvement in well-being. All had low B12 levels (112-164 ng/L) prior to treatment. Two were hematologic responders. Two had mildly raised MMA levels, three had raised tHCY levels. Final B12 levels were low compared with those of the other patients (Kruskal-Wallis test, p=0.028).

The seven women whose serum ferritin and B12 concentrations were both low may have been at an increased risk of autoimmune gastritis. This would be a likely explanation if the Hb and serum ferritin levels failing to respond to oral iron therapy.[13] An elevated MMA has been proposed as a sensitive indicator of B12 deficiency and in this study MMA levels increased rapidly as the serum B12 levels fell below 100 ng/L. The upper limit of the reference range is contentious, varying from 0.26 μmol/L to 0.40 μmol/L.[14] These limits have been derived from B12 replete populations and may be an ideal rather than normal range.[15] The intra-individual variation of MMA in serum is moderate[16] and age or gender-related differences are small. Hvas *et al*. found that vitamin B12 treatment normalized levels of MMA and tHCY in most patients[9] but had only a limited clinical effect and little impact on health-related quality of life.[17]

Homocysteine was a sensitive though non-specific marker of B12 responsiveness. All 15 hematologic responders had an initial tHCY >15 μmol/L but so did 7 of the 12 non-responders. Furthermore samples should be collected under carefully controlled conditions. All patients had low holoTC levels. If the threshold was set at < 16 pmol/L (a clinically significant range rather than a normal one), holoTC levels were of similar efficiency to B12, MMA and tHCY in predicting hematologic response. Wickramasinghe and Tatnayaka[18] concluded that holoTC was of limited value in the differential diagnosis of macrocytosis.

Our findings show that measurements of MMA, tHCY and holoTC were not significantly better than a measurement of vitamin B12 concentration for predicting a hematologic response to vitamin B12. Any patient with anemia and/or macrocytosis with a low vitamin B12 concentration should be treated. Further tests will

not significantly improve the ability to predict the response. However, the role of additional *metabolic markers* in predicting neurological dysfunction remains unanswered by this study. For any patient displaying neurological effects of vitamin B12 deficiency, but with normal hematology, MMA determination may be helpful. The symptomatic effects of high dose B12 therapy warrant further investigation in larger studies. Our findings are in keeping with the recent report by Solomon[19] that the *metabolite markers* MMA and tHCY cannot be regarded as the gold standards for assessing B12 deficiency.

AG, RE, IMD, JV-A, CB, MW all contributed to the design of the study. A Goringe assessed all the patients. RE was responsible for the collection of samples and for hematologic analyses including holo-TC. IMD was responsible for analysis of MMA and total tHCY. CJ measured the hypersegmentation index. MW wrote the first draft of the manuscript with contributions from RE and AG. All authors took part in the revision of the manuscript. We thank Cardiff and Vale NHS Trust for financial support (Small Research Grant). Axis Shield kindly supplied holoTC kits at a reduced price. We acknowledge and thank Heather Wheatley, Rachel Still, Stuart Moat and other laboratory staff for their assistance with MMA and homocysteine assays.

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