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Homocysteine in the context of cobalamin metabolism and deficiency states

Review Article

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Summary. It is becoming increasingly clear that serum vitamin B12 (cobalamin) concentration is a dubious indicator of functional B12 status and, in contrast to long-standing convention, correlates poorly with haematological indices. This, in turn, has led to poorly defined reference intervals for serum B12. Patients presenting with neurological disturbance due to B12 deficiency are at risk of not being diagnosed if total reliance is placed on serum B12 levels and haematological parameters. Plasma homocysteine remethylation is uniquely placed at the metabolic end-point of B12 metabolism such that plasma total homocysteine is proving to be a sensitive marker of functional B12 status. Studies also show that plasma homocysteine correlates better with holotranscobalamin than serum B12. It is suggested that clinicians should cease to be guided by surrogate haematological markers when more specific tests of B12 deficiency, such as holotranscobalamin and total homocysteine, exist. These tests demand greater prevalence in routine diagnostic use.

Keywords: Vitamin B12 – Transcobalamin – Homocysteine – Cobalamin

Abbreviations and units: Cbl: cobalamin, vitamin B12; tHcy: total homocysteine; TCII: transcobalamin(II), MMA: methylmalonic acid; adomet: S-adenosyl methionine. For the sake of consistency all cobalamin values are cited as picomolar concentrations. Where necessary these have been obtained by conversion from the other commonly used mass concentration, nanogram/L, using the relationship: pmol/L $\times$ 1.35 = ng/L.

Introduction

The importance of plasma total homocysteine (tHcy) measurement in the diagnosis of vitamin B12 (cobalamin; Cbl) deficiency states is becoming increasingly evident. This is due to the poor sensitivity and specificity of both haematological parameters and the serum Cbl concentration as indicators of functional Cbl status (Lindenbaum et al., 1990). This may stem, in part, from the poor correlation between serum Cbl concentration and intracellular Cbl metabolism and also from the poorly defined reference intervals in use for serum Cbl. The historical practice of serum Cbl assays being performed by haematology laboratories may have contributed to this as a result of insufficient emphasis being placed on biochemical criteria when defining reference intervals. What therefore have come to be regarded as "normal" values may not necessarily be those which are desirable.

It is not intended here to give a detailed insight into Cbl metabolism (see Kapadia, 1995) but only to provide sufficient overview to place the measurement of homocysteine, as an indicator of functional Cbl status, into context.

Source of Cbl

Humans are unable to synthesise cobalamin. Ultimately, Cbl is derived from microbial metabolism and ingested in the form of animal products, particularly meat, although food products such as cereals may be fortified with vitamins, including B12. Efficient intestinal absorption of Cbl is crucial for human health. The natural decline of intestinal function with age, or chronic bowel disease, may lead to the insidious onset of Cbl deficiency which may go unrecognised.

Consequences of Cbl deficiency

The early detection and treatment of Cbl deficiency is essential in order to prevent irreversible neurological

damage, (Healton et al., 1991). Typical of overt deficiency is demyelinating disease of the nervous system. Commonly occurring neurological signs and symptoms include parasthesia, gait ataxia, faecal and urinary incontinence, urinary urgency, impaired dexterity and memory, and impotence. Non-neurological problems attributed to defective DNA synthesis consequent upon failure of the folate cycle include weakness, tiredness, headache, and bowel problems (Green and Kinsella, 1995). Due to the long half-life of tissue Cbl levels, some three years, manifestations of deficiency due to malabsorption may take several years to develop and be insidious in onset such that in the elderly symptoms are often conveniently ascribed to "old age".

Homocysteine remethylation: cause of neurological damage?

The homocysteine remethylation cycle (Fig. 1), is controlled primarily by three parameters. Two of these are essential cofactor and substrate for the enzyme methionine synthase (MS, 5-methyltetrahydrofolate-homocysteine methyltransferase, EC 2.1.1.13) namely methyl cobalamin and methyltetrahydrofolate (MTHF), the physiologically active forms of dietary Cbl and folate respectively. The third parameter is S-adenosyl cobalamin which is formed from methionine. S-adenosyl methionine (adomet) is an efficient donor of methyl groups for a variety of essential methylation reactions catalysed by methyltranferases present in all cells. These methylation reactions include creatine formation, methylation of myelin basic protein (Sponne et al., 2000) and the methylation of reduced cobalamin to methyl-Cbl. Methionine is supplied by dietary protein and is also formed by homocysteine remethylation (Fig. 1). Homocysteine formed from methionine is the substrate for cystathionine and cysteine synthesis via the transulphuration pathway, predominantly in the liver, and is also recycled, via the ubiquitous remethylation pathway, to reform methionine (Fig. 1). Adomet is a feedback regulator of 5,10-methylene tetrahydrofolate reductase (MTHFR; EC 1.1.1.68) and cystathionine β-synthase activity (CBS; EC 4.2.1.22), as is methionine with respect to methionine synthase (MS; EC 2.1.1.13). Sub-optimal concentrations of these compounds act to increase remethylation, and conserve methionine, by activation of MTHFR and reduce flux through the transulphuration pathway by inhibition of CBS (Fowler, 1997a). Mudd (1980) has demonstrated that the number of times a molecule of homocysteine will recycle is influenced by the level of dietary methionine supply. Adequate dietary levels of methionine, and adomet, will therefore down regulate the folate and remethylation cycles. However, even in the face of adequate dietary methionine intake the remethylation cycle has significant residual flux. This residual flux, seemingly independent of the methionine concentration, is probably that required for maintenance of the folate cycle (Patel and Briddon, 2000), and purine synthesis. Inhibition of the folate cycle results in reduction of purine synthesis and, consequently, DNA synthesis and cell division causing the megaloblastic anaemia associated with

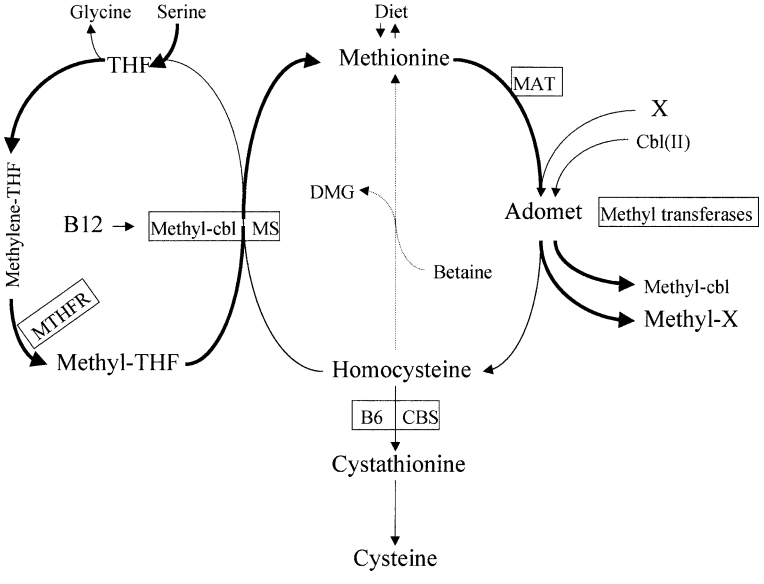

Fig. 1. Pathways of aminothiol metabolism showing (from left to right), the folate cycle, homocysteine remethylation and transmethylation reactions and (lower) the transulphuration pathway of homocysteine to cysteine. Key enzymes in boxes. Heavy lines indicate flow of methyl groups for transmethylation reactions, including the methylation of Cbl(II) to methyl-Cbl. *MS*, methionine synthase; *MTHFR*, methylene tetrahydrofolate reductase; *CBS*, cystathionine β-synthase; *MAT*, methionine adenosyl transferase; *DMG*, dimethyl glycine; *THF*, tetrahydrofolate

both Cbl and folate deficiency (Scott, 1999). Inhibition of the folate cycle also disrupts homocysteine remethylation resulting in increased concentrations of plasma homocysteine. As a result plasma tHcy has been cited as a valuable index of micronutrient status (Bates et al., 1997).

Neurological damage has been attributed to reduced transmethylation capacity consequent upon reduced flux through the remethylation cycle. This is an attractive theory since poor availability of methionine and adomet would be expected to affect essential transmethylation reactions, including the methylation of myelin phospholipids (Metz, 1992). Maintenance of methionine concentrations by an adequate dietary intake should protect against this and there is some evidence to support this (Patel and Briddon, 2000). However, severe Cbl deficiency can cause significantly low methionine levels to occur, especially if dietary intake is compromised (Hoey et al., 1982) and this might well be expected to adversely affect methylation reactions. An argument against this theory has been that disruption of the folate and remethylation cycles due to methyltetrahydrofolate deficiency may cause the typical haematological abnormalities but has not been generally associated with the neurological problems observed in Cbl deficiency (Green and Kinsella, 1995). Although there is now some indication that folate deficiency may cause spinal cord syndromes similar to that seen in Cbl deficiency (Green and Miller, 1999) the association is said to be rare (Ravakhah and West, 1995). Interestingly, Cbl deficiency in some other mammalian species is not associated with neurological pathology (Allen et al., 1993) or megaloblastic anaemia (Wickramasinghe, 1999). The cause of the abnormal neurology in Cbl deficiency thus remains unclear at this time and it has been suggested that there may be a Cbl dependent enzyme, or metabolic factor, which is as yet unidentified (Allen et al., 1998).

Serum Cbl: what is normal?

At a practical level the laboratory assay of Cbl is usually considered to be a haematology test since deficiency has been traditionally associated with haematological abnormalities such as megaloblastic anaemia and a raised mean cell volume (Ueland et al., 1993). Thus, there is often a perception among clinicians that if haematological parameters are normal then it can be assumed that functional Cbl status is adequate. However, there are now a number of documented reports of subjects with neurological abnormalities, demonstrably the result of poor Cbl status, but without abnormal haematology, (Carmel et al., 1987,1988; Lindenbaum et al., 1988). More worryingly, there have been patients with apparently normal serum Cbl concentrations who have been shown to have defects of Cbl metabolism (Nilsson-Ehle, 1998). Much of this confusion is probably caused by the poorly defined laboratory reference intervals for serum Cbl as evidenced by the large variation between centres. A poll of just six hospital laboratories in London and the south east of England revealed quoted lower reference limits for serum Cbl ranging from 111 to 165 pmol/L. Some laboratories cited deficiency as being less than 126 pmol/L with an indeterminate range between 126 and 165 pmol/L but without any clear idea as to the diagnostic relevance of this. Evidence of Cbl malabsorption has been reported in patients with serum Cbl within this indeterminate range (McCafferty et al., 1998). Reference intervals cited in the literature show a similar picture (Table 1). Clearly, assessment of Cbl status against haematological criteria alone is not appropriate. It has been said that "definitive plasma con-

Table 1. A representative selection of serum Cbl reference values cited in the literature

Method	pmol/L	ng/L	Reference
R	110–650	150–878	Brattstrom et al., 1988
I	185–815	250–1,100	Green and Kinsella, 1995
I	>260	>350	Stabler et al., 1996
I	163–839	223–1,132	McCafferty et al., 1998
?	141–667	190–900	Wallach, 1998
R	335 ± 124(SD)	452 ± 167(SD)	Abdelmouttaleb et al., 2000
I	156–674	211–910	Herrmann et al., 2001
I	200–600	270–810	Hvas et al., 2001

R, radioassay method; *I*, immunoassay method

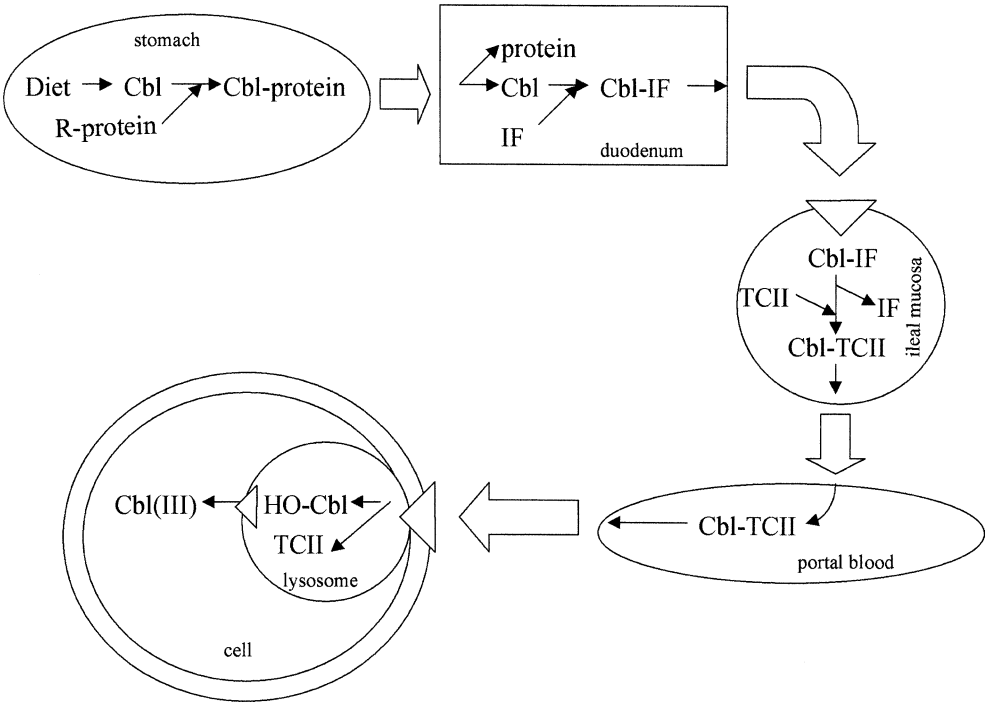

Fig. 2. Steps outlining the supply of cobalamin to the tissues via intestinal absorption and transport. *IF*, intrinsic factor; *TCII*, see text

centrations cannot be quoted for Cbl deficiency" (Loew et al., 1999). The questions then, appear to be, below what level of serum Cbl concentration are further diagnostic tests indicated or, indeed, whether serum Cbl is an appropriate first line test.

Rational of tHcy measurement for assessment of Cbl status

In addition to the poorly defined reference intervals for serum Cbl there are other factors which contribute to the poor sensitivity and specificity of this assay as an indicator of functional Cbl status.

Firstly, the delivery of Cbl to the cell is a complicated process involving various transport and binding proteins (Fig. 2). Defects at any of these steps, resulting in deficient Cbl absorption, might be expected to be reflected in the serum Cbl concentration. However, once in the circulation, approximately 20% of Cbl is transported bound to transcobalamin(II) (holotranscobalamin; holo-TCII) and it is this component of the total serum Cbl that represents the biologically active form necessary for transmembrane transport. Deficiencies in expression or function of TCII may cause functional Cbl deficiency not reflected by the total serum Cbl concentration. Metz et al. (1996) noted that Cbl deficiency becomes manifest in older patients at relatively higher concentrations of serum Cbl, possibly because of declining levels of TCII in the elderly.

Secondly, once within the cell, cobalamin released from TCII and the lysosome via CblF is subject to several further processing steps involving sequential reduction via Cbl E, Cbl C/D and Cbl E/G to attain the methyl cobalamin form in which it acts as an essential cofactor for methionine synthase and homocysteine remethylation (Fig. 3). Defects, generically known as methionine synthase deficiency, have been described affecting all of these steps (Fowler, 1997b). In addition, further mitochondrial cobalamin processing, via Cbl A and Cbl B, forms adenosyl cobalamin, the cofactor for methylmalonyl CoA mutase, deficiency of which leads to accumulation of plasma methylmalonic acid (MMA) and MMAuria. Whilst measurement of both plasma tHcy and MMA, or urinary MMA excretion, is therefore helpful in elucidating the site of any defect in intracellular cobalamin processing (Fig. 3), the isolated use of plasma MMA concentration has been reported to be of doubtful value in predicting functional Cbl deficiency (Metz et al., 1995; Hvas et al., 2001).

Thirdly, it has been reported that serum Cbl is unstable and losses may occur if samples are not protected from light and serum separated and frozen promtly (Komaromy-Hiller et al., 1997). Good laboratory practice should prevent losses where this problem

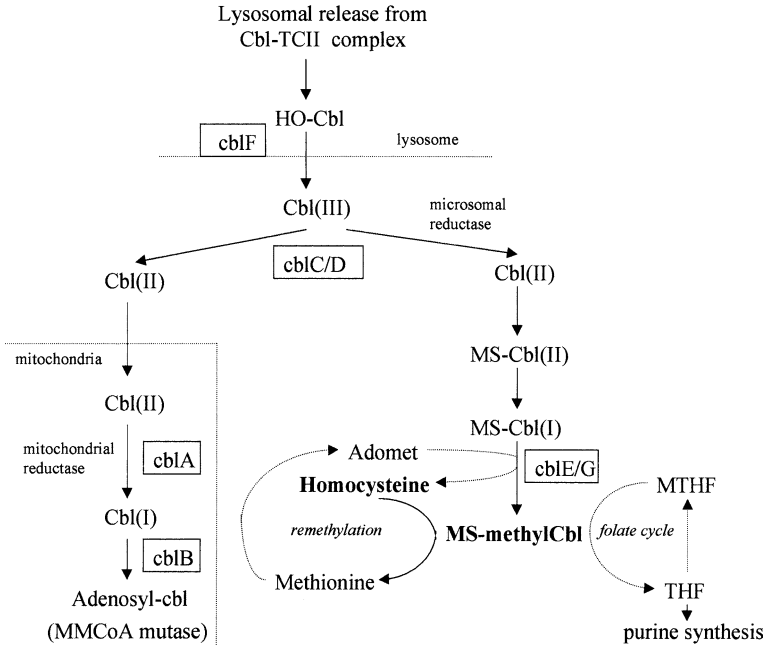

Fig. 3. Intracellular cobalamin processing

has been recognised but may otherwise result in false positive observations.

The methyl-Cbl activated remethylation of homocysteine to methionine is therefore uniquely placed at the metabolic end point of both Cbl absorption and intracellular processing. The rapid response of this pathway to changes in co-factor availability (Fowler, 1997a) make plasma tHcy concentration a useful and sensitive indicator of overall functional Cbl status.

tHcy in plasma

The reference interval for plasma tHcy is age and sex related (Fermo et al., 1993; McEvoy et al., 2001) but a commonly accepted overall working range is represented by 5–15 μmol/L (Ueland et al., 1993). Factors apparently unrelated to Cbl metabolism or inherited enzyme deficiencies may cause mildly increased plasma tHcy, in particular poor renal function (Refsum et al., 1997) and hypothyroidism (Nedrebo et al., 2001) must be considered as possible contributing factors, especially in the elderly population.

Effect of Cbl deficiency on sulphur amino acid metabolism

One of the biochemical consequences of insufficient methyl-Cbl is inhibition of homocysteine remethylation leading to increased plasma concentrations of homocysteine (Fig. 1). Provided the increase in homocysteine is relatively modest then the increase will be confined to the protein bound form and detectable only by measurement of plasma tHcy (Briddon, 1998; Moat et al., 1999). Although the inhibition of remethylation would be expected to decrease methionine concentration this will largely depend upon concurrent dietary methionine intake (Kapadia, 1995; Patel and Briddon, 2000) and methionine conservation by metabolic regulation. Depending on the degree of Cbl deficiency the homocysteine concentration is therefore the result of a balance between inadequate remethylation, formation of homocysteine from dietary and reformed methionine and disposal of homocysteine via transulphuration to cystathionine and cysteine. Methionine is more likely to be significantly reduced if dietary intake is poor and this may affect the relative activities of the pathways in an attempt to conserve methionine levels (Fowler, 1997a). Allen et al. (1993) reported most subjects with Cbl or folate deficiency to have a raised cystathionine. However, in that cohort of subjects methionine concentrations were generally normal, or even raised, and whilst this was attributed to metabolic regulation of the pathways conserving methionine this is to ignore the contribution of dietary methionine intake which, if adequate, should reduce the need for metabolic conservation. Increased transulphuration flux (Fig. 1) due to the raised homocysteine substrate catalysed by adomet

activated high Km CBS could explain the increased cystathionine concentrations. In contrast, if methionine levels are not maintained by dietary intake they may drop significantly, thereby triggering metabolic compensation with inhibition of transulphuration by down regulation of CBS, in an attempt to conserve methionine (see case 1).

Diagnostic use of thcy: illustrative cases

Case 1

A 28 year old man was first seen in India as a result of seizures. There was a history of learning disability and a report of a previous seizure at age 11. Neurological assessment showed him to be mildly dyscognitive, mood-labile, dysartthric, with extraocular movements, a brisk jaw-jerk and a broad-based gait. Cerebellar atrophy was present on MRI. Metabolic investigations were reported to be essentially normal except for a "borderline" serum Cbl. On this basis hydroxy-Cbl medication was prescribed. Four years later, after further seizures, investigations showed a normal serum Cbl but a raised MCV and a raised plasma tHcy, reported to be "greater than three times" normal but without homocystinuria or ocular defects. In the interim he had voluntarily adopted a vegetarian diet.

At age 32 he was referred to The National Hospital for Neurology and Neurosurgery (NHNN) in the UK for re-assessment. In summary, neurological examination revealed bilateral foot drop, ataxia, episodic worsening gait, brisk jaw-jerk and neuropathy. MRI of the head showed the cerebellum to be extremely atrophic with microencephaly. EMG showed definite, but relatively mild peripheral neuropathy, predominantly sensory, with no clear evidence of a primary demyelinating process. Cognitive functions were below average. Routine biochemistry and haematology were unremarkable except for a lowish plasma creatinine of 65 μmol/L (80–133). Serum Cbl was very high at 1,314 pmol/L (133–519) due to continued HO-Cbl therapy. Red cell folate was normal at all times. Further metabolic investigations, including white cell enzymes and very long chain fatty acids, were also normal except for plasma amino acids and urinary organic acids with results as follows:

Plasma amino acids: tHcy 191 umol/L (5–15); free homocystine 26 umol/L (not normally detectable); methionine 6 umol/L (13–40) and total cysteine 151 μmol/L (200–350). Urinary methylmalonic acid excretion was raised at 418 mmol/mol creatinine (0–30). Hydroxy cobalamin therapy was continued and oral betaine introduced to help reduce the homocysteine concentration and restore methionine levels for essential transmethylation reactions.

Comments on case 1

The grossly raised free and total homocysteine concentrations are in the range observed in untreated classical homocystinuria (cystathionine synthase deficiency; McKusick 23620). However, the very low methionine indicates that in this case the patient has a defect which is primarily affecting the remethylation pathway. Possible metabolic causes include Cbl malabsorption defects, intracellular Cbl processing defects and defects in the folate cycle such as methylene tetrahydrofolate reductase deficiency (McKusick 23625). The presence of a grossly raised tHcy and MMA excretion is consistent with a Cbl deficiency affecting the supply of adenosyl-Cbl (Fig. 3) and this, together with the fact that the biochemical abnormalities persist in the face of hydroxy-Cbl therapy (Table 2) narrows the diagnostic possibilities to an intracellular CblF or CblC/D defect (Fig. 3).

It should be noted that the original serum Cbl concentration was only reported as "borderline low" (data not available). No megaloblastic anaemia was found, the only haematological abnormality being a slightly raised MCV.

The low total plasma cysteine might be considered inappropriate for a remethylation defect but probably reflects down regulation of CBS activity and reduced flux through the trans-sulphuration pathway. Likewise, the low creatinine, despite the reasonable muscle mass of this patient, may also be a reflection of inadequate supply of methyl groups for transmethylation consequent upon the low methionine and adomet concentrations. It is probable that the vegetarian diet exacerbated the symptoms by contributing to the low methionine and thus further compromising transmethylation. Note that total cysteine increases as methionine is restored to normal following betaine therapy (Table 2). A low plasma total cysteine in the presence of both proven Cbl deficiency and hyperhomocysteinaemia may therefore be an additional indication of inadequate methionine and adomet supply for vital transmethylation reactions.

Clearly, some improvement in the biochemical parameters has occurred as judged by the moderate

Table 2. Metabolite response to cobalamin and betaine therapy in clinical cases

	B12 ng/L	tHcy μmol/L	fHcy μmol/L	tCys μmol/L	Met μmol/L	MMA mmol/mol creatinine	Hb g/dL	MCV fL
Case 1								
HO-Cbl injections								
Day 0	1,775	191	26	151	6	418	13.1	90.2
Day 5		154	17		13			
Day 11		146	2	185	11			
HO-Cbl plus betaine therapy								
Day 18	>2,000	122	4	209	63	305	13.5	87.5
Day 25		136	3	230	37	263		
Case 2.								
Day 0	133	75	n.d.	225	19	77	13.7	92.5
Day 13 HO-Cbl injections								
Day 17		6	n.d.	232	19			
Reference values	180–700	5–15	n.d.	200–350	13–40	0–30	13–17	80–99

tHcy/tCys, total aminothiol; *fHcy*, free (unbound) aminothiol; *Met*, methionine; *MMA*, urinary methyl malonic acid excretion; *n.d.*, not detected

decrease in tHcy and MMA excretion. However, this is most likely due to the mass effect of large doses of hydroxy-Cbl (Table 2).

It should also be noted that at the time of the original investigation, despite a raised total plasma homocystine, no homocystinuria was detected. This underlines the danger of relying on urine screening tests for the detection of hyperhomocysteinaemia when the plasma homocysteine may be entirely in the protein bound state (Briddon, 1998).

Fibroblast complementation studies confirmed late onset CblC disease in this patient.

Case 2

A 33 year old man, an ambulance paramedic by profession, was seen at his local hospital for a range of problems including progressive parathesia in both feet, gait ataxia, urinary urgency and frequency, and general weakness such that he was unable to work. Cognitive function was not impaired. Laboratory investigations were unremarkable. Serum Cbl was initially 126, then 177 and 156 pmol/L one month and six months later respectively, the local reference values being defined as greater than 126 pmol/L. No specific treatment was given other than physiotherapy. However, a neurological assessment was sought and on admission to NHNN his serum Cbl value was found to be unequivocally low at 98 pmol/L (133–519). Red cell folate and haematology indices, including MCV and haemoglobin, were normal. Gastric parietal cell and intrinsic factor antibodies were negative. Plasma tHcy was raised at 75 μmol/L, as was the urinary MMA excretion of 77 mmol/mol creatinine, strongly suggesting a functional Cbl deficiency state (Table 2). This was confirmed by the biochemical response to hydroxy-cobalamin.

Comments on case 2

At no time was any haematological abnormality noted in this patient.

The speed with which the tHcy normalised after starting Cbl injections can be compared to case 1 and suggests a generalised Cbl deficiency, possibly due to a non-specific absorption defect, and highlights the rapid response and sensitivity of remethylation enzymes to changes in micronutrient supply (Fowler, 1997a). The possibility in this patient, an ambulance paramedic, of mild, chronic exposure to nitrous oxide must be considered as a possible contributing factor since nitrous oxide inhalation will efficiently re-oxidise cobalamin, thus depleting methyl-Cbl and inhibiting MS activity in someone whose reserves may be marginal (Green and Kinsella, 1995).

Case 3

This case is presented as a contrast to the previous cases. This 35 year old man presented two years prior

to the final diagnosis with a history of tingling in his toes which gradually progressed to whole foot numbness. During routine blood donor screening he was found to be anaemic. In view of his symptoms this was attributed to Cbl deficiency and HO-Cbl treatment prescribed. Despite treatment over 2 years neurological signs and symptoms progressed with increased loss of sensation, some visual loss and neuropsychiatric symptoms.

On referral to NHNN the anaemia was still present, Hb 10.3 (13–17) g/dl but MCV normal at 83.1 (80–99) fL. On the basis of a normal plasma amino acid profile and low-normal tHcy of 5 (5–15) μmol/L it was not possible to confirm the presumed B12 deficiency and further investigations were undertaken. Abnormal results were obtained for iron and copper studies as follows: serum iron 3.6 (6–30) μmol/L; ferritin 151 (22–275) μmol/L and IBC saturation 4% (20–50). Serum copper and caerulplasmin concentrations were very low; copper 0.7 (11–21) μmol/L and caeruloplasmin 0.02 (1.1–2.7) μmol/L.

Comments on case 3

This case is a useful illustration of normal Cbl status despite clinical and laboratory indications to the contrary.

Although the plasma copper and caeruloplasmin values in this patient suggested a diagnosis of Wilsons disease it was felt that the clinical phenotype was not typical. Further investigations, including urinary copper excretion pre and post penicillamine, together with the abnormally low serum iron but normal ferritin values are consistent with a diagnosis of acaeruloplasminaemia. The resulting lack of ferroxidase activity is the likely cause of the low serum iron and anaemia.

General comments

In general, and as illustrated by the first two cases, intracellular Cbl processing defects result in higher plasma tHcy concentrations than absorption defects and has been used as a means of helping to distinguish the two groups (Fenton and Rosenberg, 1995). All three cases underline the danger of relying on serum Cbl and classical haematological parameters as indicators of true intracellular Cbl status. This may lead, as in case 2, to delay in treatment or, as in case 3, to a significant period of inappropriate treatment and continued clinical regression.

Serum Cbl and tHcy in pregnancy

Decreased serum vitamin Cbl concentration is a recognised feature of pregnancy and lactation, probably due to increased requirements at these times.

Metz et al. (1995), studied a cohort of pregnant patients and compared plasma MMA and tHcy concentrations between those with serum Cbl levels considered to be normal (154 to 430 pmol/L; n=25) and those with low serum Cbl (33–147 pmol/L; n=50). No significant difference between the two groups was detected. For tHcy the mean value for control and low groups respectively was 6.88 and 7.03 μmol/L. Overall, there was no correlation between Cbl and tHcy values. A significant increase in tHcy was found in only two patients, both of whom had subnormal Cbl values together with haematological evidence of Cbl deficiency. The authors concluded that the usual fall of serum Cbl during pregnancy did not reflect a Cbl deficiency at the biochemical level and that measurement of tHcy was of value in establishing a true deficiency state. This is important since a true deficiency state during pregnancy may have severe neurological consequences for the infant (Hoey et al., 1982; Fenton and Rosenberg, 1995). The authors also measured plasma MMA and their interpretation of the data agrees with that of Hvas et al. (2001) suggesting that isolated plasma MMA measurement is an unreliable indicator of functional Cbl status.

Plasma tHcy has been reported to be raised in cases of pre-eclampsia as compared to 3rd trimester uncomplicated pregnancies (Lopez-Quesada et al., 2001). In the pre-eclampsia patients (n=25) median (range) tHcy was 8.3 μmol/L (4.0–17.0) compared to the control group (n=64) of 6.3 (3.2–13.0) with an odds ratio of 6.42 (C.I.95%: 1.46–28.15). Hyperhomocysteinaemia of greater than 10.5 μmol/L (the 95th percentile of the control group), was found in 24% of patients and 5% of controls. Paradoxically, these results occurred despite higher folate levels in the patient group and serum Cbl concentrations which were not significantly different between groups. The biochemical basis of this finding is unclear, however higher levels of total cysteine (>95th percentile) have also been associated with pre-eclampsia (El-Khairy et al., 2001) which suggests an inhibition of remethylation rather than transulphuration. It is therefore worth speculating that the biochemical cause of the marginally higher tHcy is related to transcobalamin expression or Cbl-TCII binding, but whether any clini-

cal significance can be attached to these modest values is unclear.

Plasma tHcy and transcobalamin

There are three transcobalamin proteins which bind Cbl, TCI, II and III. Only TCII, which binds approximately 20% of total serum Cbl, is regarded as physiologically significant in transporting Cbl into tissues via specific cell surface receptors. This protein is expressed in all tissues (Seetharam, 1999). The remainder of the serum Cbl, bound to TCI and TCIII, constitutes the major fraction of the total serum Cbl but does not seem to contribute to the clinical phenotype of Cbl deficiency. This is supported by the finding that there is no correlation between TC1 and tHcy concentrations in plasma (Carmel et al., 2001). Uniquely, hepatocytes express a cell surface receptor which appears capable of binding and internalising Cbl bound to TC1 (Fenton and Rosenberg, 1995) although the relative contribution of this mechanism to the maintenance of normal tHcy concentrations in the face of other Cbl defects is debatable (see below). Defects in TCII expression (apo-TCII) or Cbl binding capacity (holo-TCII), therefore have the potential to seriously deplete the supply of Cbl available for intracellular cobalamin processing without this necessarily being evident from total serum Cbl values.

Metabolic deficiency of TCII generally presents during infancy with severe signs and symptoms including failure to thrive, vomiting, weakness and megaloblastic anaemia (Fenton and Rosenberg, 1995). Hyperhomocysteinaemia may not be a feature of this condition, this being ascribed to the unique ability of hepatocytes to internalise Cbl bound to TCI (Kapadia, 1995). This creates a paradox, since age related decline in TCII does seem to be associated with increased tHcy concentrations and further underlines the need for specific TCII assays. Milder and progressive TCII deficiency is a likely consequence of ageing (Metz et al., 1996) and together with age related gut atrophy makes Cbl deficiency increasingly prevalent with age. For this reason most studies of tHcy and Cbl parameters have been carried out on older age groups in relation to cognitive function.

Flynn et al. (1997) studied plasma tHcy, total serum Cbl and holo-TCII in a cohort of 171 subjects, mean age 66 (41–85). 83 (49%) of these patients had low holo-TCII concentrations (<60 ng/L) but only 13 (8%) also had concurrently low serum Cbl (<148 pmol/L). Of these 83 with low holo-TCII, 52 (63%) also had raised tHcy (>17.5 μmol/L) and 7 (8%) a low serum Cbl. The remaining 31 (37%) of the low TCII subset had normal tHcy with 6 (7%) showing a low serum Cbl. The reason for the normal tHcy in the latter group is not explained but it may be that they had not yet developed full functional Cbl deficiency and the contribution of hepatocyte processing of Cbl derived from TC1 must be considered.

The conclusions reached by these authors were that (i), measurement of holo-TCII, especially in the elderly, is important, (ii) that a low holo-TCII is the earliest sign of negative cobalamin balance, occurring prior to a fall in serum Cbl which is a late occurring event, and (iii) that plasma tHcy is a good marker for negative Cbl balance, giving better correlation with holo-TCII than with serum Cbl. Additionally, Lindgren et al. (1999) found that holo-TCII correlated better than serum Cbl in patients with symptoms compatible with Cbl malabsoprtion and that no patients with holo-TCII within the reference interval had symptoms of Cbl malabsorption.

tHcy and serum Cbl

Although RIA assays for holo-TCII exist they are not widely available in the routine laboratory and so, whilst TCII is almost certainly a better diagnostic marker than serum Cbl, the latter is well established as an HPLC assay and as an immunoassay on automated equipment and for convenience sake is likely to remain so for some time. Plasma tHcy therefore has a continuing and important role to play in the detection and investigation of Cbl malabsorption and deficiency.

Correlation between plasma tHcy and serum Cbl concentration shows a hyperbolic relationship with tHcy tending to increase above 15 μmol/L as serum Cbl drops below about 200 pmol/L (Fig. 4). This data confirms that previously reported (Ueland et al., 1993). It should be noted that this Cbl concentration is significantly above values generally regarded by laboratories as a lower limit of normal (Table 1). However, whilst the overall trend suggests that at serum Cbl concentrations below 200 pmol/L there is an adverse biochemical affect on Cbl dependant homocysteine remethylation due to sub-optimal Cbl (methyl-Cbl) availability, there remains an area where normal values for tHcy correspond to Cbl values well below 200 pmol/L (Fig. 4). Presumably these subjects are Cbl replete despite a lower serum Cbl value.

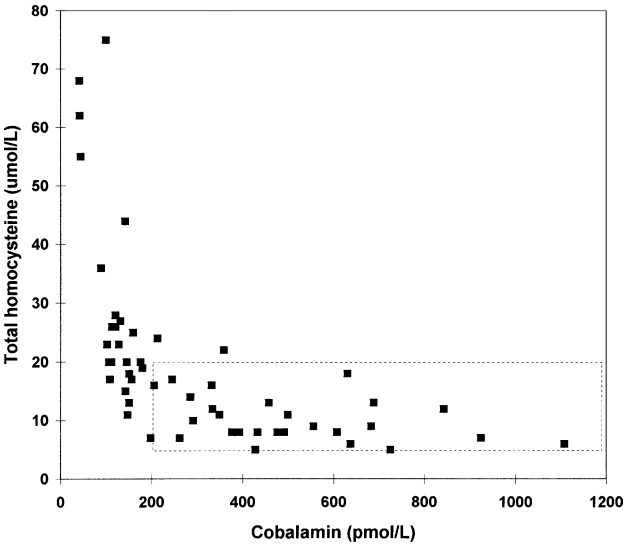

Fig. 4. Relationship between serum cobalamin concentration and plasma total homocysteine. Values within the boxed area satisfy recent recommendations (see text). 200 pmol/L = 270 ng/L Cbl

Although, in general, most studies concur that tHcy is a useful marker of Cbl deficiency not all support this. For example, Ray et al. (2000) in a Canadian study of 692 subjects did not find that a cut-off value of 15 μmol/L for tHcy discriminated those with a serum Cbl less than 120 pmol/L, nor did it reveal those with "indeterminate" levels between 120 and 150 pmol/L.

To answer the question 'below what level of serum Cbl are further tests indicated' the recent study reported by Clarke et al. (2001) at the 3rd International Conference on Homocysteine is based on a sufficiently large patient base to provide an answer with a high degree of confidence. From a population based study of 1559 persons over 65 years of age these workers statistically derived a set of cut-off values for serum Cbl and tHcy which exposed the greatest number of Cbl deficient subjects as judged by clinical signs and MMA concentrations. Of these only 12% had anaemia. They concluded that a serum Cbl of less than 200 pmol/L in the presence of a tHcy greater than 20 μmol/L should be taken as being consistent with Cbl deficiency.

Analytical notes

Like Cbl, homocysteine concentration in whole blood is also unstable. Continued homocysteine synthesis by red cell metabolism causes plasma concentrations to increase at a rate of about 1% per hour at room temperature (Ueland et al., 1993). Fortunately, total (but not free), homocysteine is stable in plasma which should be separated from the red cells as soon as possible, or at least within 30 minutes as a reasonable practical requirement unlikely to cause misinterpretation of results. In heparinised whole blood stored for 4 days at 4°C an increase from 10 to 16 umol/L was found (personal observation). Serum is likely to give slightly higher values than plasma (Flynn et al., 1997). There is good analytical agreement between the routinely used analytical methods, HPLC, RIA, immunoassay and conventional amino acid analysis, (Ueland et al., 1993; Donnelly and Pronovost, 2000). Differences between the immunoassay for serum Cbl and the radioligand assays have been reported but without any consistent correlation between them (Thomas, 1998). This may reflect assay imprecision which can be up to 12% (Chen et al., 1989).

Concluding remarks

It is clear that neurological dysfunction due to Cbl deficiency frequently occurs in the absence of abnormal haematological indices. There would, therefore, seem to be a good argument for ceasing to think of serum Cbl and related investigations as haematological tests and certainly not to regard haematology indices as adequate screening tests for Cbl deficiency. Holo-TCII is probably the best test for detection of Cbl malabsorption but is not widely available. Plasma tHcy is arguably the best test for assessment of overall functional Cbl deficiency in preference to serum Cbl assay but in isolation will not distinguish malabsorption defects from intracellular processing defects or specific TCII defects. Although a normal tHcy is likely to exclude a functional Cbl defect at the time of sampling, if other indices are abnormal, or a high degree of clinical suspicion is present, then continued monitoring would be sensible. Even within their normal ranges Cbl and folate are strong determinants of tHcy concentration (Brattstrom, 1996) suggesting that better diagnostic discrimination can be obtained by the use of more realistic reference intervals for serum Cbl, focusing on "desirable" rather than "normal" levels. Recent work strongly advocates the use of a higher cut-off for serum Cbl of 200 pmol/L which should be interpreted as consistent with Cbl deficiency if plasma tHcy is also elevated. Although tHcy assay is available as a single test immunoassay there is considerable value in also obtaining an amino acid profile to exclude other meta-

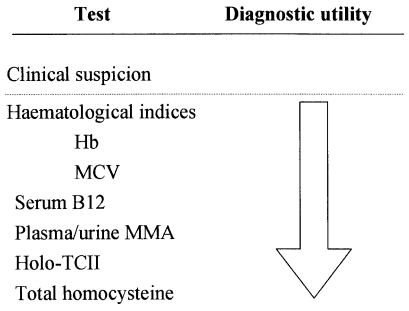

Fig. 5. Concluding recommendation for relative diagnostic utility of laboratory tests available for investigation of Cbl deficiency

bolic causes of hyperhomocysteinaemia. Knowledge of methionine and total cysteine concentrations are especially helpful diagnostically and may help to direct treatment aimed at maintaining methionine levels for transmethylation. In conclusion it is suggested that the relative utility of current diagnostic assays is as shown in Fig. 5. Due to the greater prevalence of malabsorption, especially in older age groups, there is little doubt that lack of a routinely available TCII assay is a serious shortfall in the routine laboratory repertoire. Therefore, total plasma homocysteine, with wider availability in the general laboratory, is arguably the most essential component in the initial investigation of cobalamin deficiency at the present time.

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