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An audit of holotranscobalamin ("Active" B_{12}) and methylmalonic acid assays for the assessment of vitamin B_{12} status: Application in a mixed patient population

Agata Sobczyńska-Malefora [a,*], Renata Gorska [a], Michel Pelisser [a], Patricia Ruwona [a], Bernie Witchlow [b], Dominic J. Harrington [a]

[a] The Nutristasis Unit, GSTS Pathology (part of King's Healthcare Partners), St. Thomas' Hospital, London, UK
[b] Laboratory Informatics, GSTS Pathology (part of King's Healthcare Partners), St. Thomas' Hospital, London, UK

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ABSTRACT

Background: Vitamin B_{12} insufficiency/deficiency is common in mixed patient populations. However there is no single marker which can reliably diagnose B_{12} insufficiency/deficiency. Elevated concentrations of methylmalonic acid (MMA) are considered the most representative marker of metabolic vitamin B_{12} insufficiency, but poor assay availability limits clinical utility. Low concentrations of serum vitamin B_{12} are often used to assess vitamin B_{12} status but this approach generates a high rate of false negative results. Emerging evidence indicates that holotranscobalamin (holoTC) may be a more reliable indicator of vitamin B_{12} status.

Aims and methods: We substituted serum vitamin B_{12} measurement with holoTC, supported by MMA in patients referred for assessment of vitamin B_{12} status. A service evaluation was undertaken of the pattern of MMA values obtained for patients with holoTC 25–50 pmol/L (an indeterminate result). MMA cut-offs of 280 and 360 nmol/L were applied for patients ≤65 or >65 years respectively.

Results: A total of 4,175 consecutive patients were investigated and MMA was analysed for 19% of patients. The incidence of elevated MMA was 41% (holoTC, 25–29 pmol/L), 32% (30–34 pmol/L), 33% (35–39 pmol/L), 30% (40–44 pmol/L), and 26% (45–50 pmol/L).

Conclusions: Our results indicate that in the clinical setting a holoTC between 25 and 50 pmol/L is a poor predictor for the concentration of MMA provided the goal is to identify patients with MMA values above the limits used in the present study. Further studies are needed to evaluate to what extent holoTC <25 and >50 pmol/L reflect circulatory MMA concentrations.

1. Introduction

Vitamin B_{12} (cobalamin) has the largest and most complicated structure of all the 13 universally recognised vitamins. Vitamin B_{12} is synthesised by micro-organisms and enters the food chain in food of animal origin [1]. In humans the activity of two enzymes, methionine synthase and (R)-methylmalonyl-CoA mutase, are both dependent on vitamin B_{12} [2]. Impaired vitamin B_{12} status, which may lead to clinical deficiency, is common in mixed patient populations, especially in those older than 60 years. Vitamin B_{12} deficiency can occur in patients with autoimmune disease (pernicious anaemia), severe primary hypothyroidism, those with ileal disease, patients on chronic therapy with antacids, proton pump inhibitor or H2 antagonists, or colchicine, and in chronic malnutrition states including alcoholism. Deficiency is also common in those who adhere to vegetarian and vegan diets [3].

The timely detection, and correction, of vitamin B_{12} deficiency prevents macrocytic anaemia, elevated homocysteine (possible thrombotic risk factor), potentially irreversible peripheral neuropathy, memory loss and other cognitive deficits. The clinical manifestation and combination of these symptoms are variable in their presence and severity [4,5].

However, the prompt diagnosis of impaired vitamin B_{12} status has long been recognised as problematic. Several laboratory tests are currently used to determine status. Of these, red cell mean volume (MCV) is a poor indicator and only reflects an advanced deficient state with regards to haematological dysfunction. Metabolic markers of status include circulatory concentrations of homocysteine and methylmalonic acid (MMA). Unfortunately, the utility of an elevated plasma homocysteine concentration to indicate impaired methionine synthase function as a consequence of poor vitamin B_{12} availability (specifically the methylcobalamin form) has a co-dependency on the optimal supply of 5-methyltetrahydrofolate. MMA does not share this limitation and its measurement is considered as the gold standard and the most representative marker of metabolic vitamin B_{12} insufficiency. In short,

* Corresponding author at: The Nutristasis Unit, GSTS Pathology, St. Thomas' Hospital, London, SE1 7EH, UK. Fax: +44 207 188 2726.
E-mail address: agata.malefora@gsts.com (A. Sobczyńska-Malefora).

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vitamin B_{12} (specifically the adenosyl-cobalamin form) is a cofactor for the methylmalonyl-CoA mutase catalysed conversion of methylmalonyl-CoA to succinyl-CoA. MMA is a by-product of this pathway with no known biological function. In vitamin B_{12} insufficiency, excess methylmalonyl-CoA is hydrolysed to MMA causing the circulatory concentration of MMA to increase. Assays for the measurement of MMA remain analytically challenging and relatively expensive to perform. It is also recognised that interpretation is particularly complex in elderly populations and those with impaired renal function [6,7].

The current convention is to estimate the abundance of vitamin B_{12} using total serum vitamin B_{12} as the first-line screen for deficiency. However this test has a low sensitivity [5,8]. Up to 45% of vitamin B_{12}-deficient subjects may be overlooked if only serum B_{12} is used as a screening test [9]. Holotranscobalamin (holoTC) is the metabolically active fraction of B_{12}. Emerging evidence indicates that a low concentration of holoTC is a more reliable marker of impaired vitamin B_{12} status than a low concentration of serum vitamin B_{12} [10]. Moreover unlike serum B_{12}, holoTC is believed to be stable in pregnancy [11]. Several studies support holoTC measurement as a first-line diagnostic test, and suggest that holoTC may be the earliest marker for B_{12} depletion [3,10,12–14]. However, reports of the use of this assay in the clinical setting are limited and discrepancies exist with regards to the application of cut-off values. Our main objective was to perform a preliminary service evaluation of the pattern of MMA values obtained for patients with holoTC 25–50 pmol/L (defined as indeterminate values).

2. Materials and methods

The evaluation included all samples received for the assessment of vitamin B_{12} status between January 17th and April 16th 2012 from in- and out-hospital patients from both Guy's and St. Thomas' Hospitals, London, UK. All data were extracted from our Laboratory Information System (Pathnet, version Classic 306) using the Cerner Corporation Limited (US) variant of the industry standard query tool (SQL).

Serum holoTC was measured by the AxSYM analyser (Abbott Diagnostics) [15]. The AxSYM "Active" B_{12} assay is based on microparticle enzyme immunoassay (MEIA) technology. The intra-assay CVs for duplicate samples from our data ranged from 0.6 to 6.2%. The inter-assay CVs for two quality control samples with a low and normal holoTC concentrations were 12.8 and 13.0% respectively (92 determinations each, analysed between September 2010 and April 2012). Our laboratory participates in the UK NEQAS scheme for holoTC ("Active" B_{12}) (Heart of England Foundation Trust, UK). HoloTC values exceeding 128 pmol/L (above the dynamic assay range) were reported as >128 pmol/L.

The assay manufacturer's suggested cut-off for holoTC is 35 pmol/L. This cut-off is also used by most of the UK NEQAS participants and by experts in the field [16–18]. When the cut-off value for holoTC was set at 35 pmol/L in the study by Herrmann et al., the sensitivity was 0.87 and the specificity was 0.75 [16]. To further evaluate the vitamin B_{12} status of our patients an indeterminate holoTC range between 25 pmol/L and 50 pmol/L was implemented [13]. Samples with holoTC values within this range were referred for MMA analysis, subject to an estimated glomerular filtration rate (eGFR) of ≥60 mL/min/1.73 m^2 within 2 weeks from the blood collection. Serum MMA analysis was performed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) with electrospray ionisation as previously described [19]. The chromatographic system — HPLC (Dionex Ultimate 3000) was coupled with the tandem mass spectrometer (Bruker, amaZon X). For separation, a Symmetry C18, 2.1 × 100 mm 3.5 μm column (Waters) was used and the mobile phase contained 10% of methanol and 0.4% of formic acid. The transitions m/z 117 to 73 and 120 to 76 were used in the selected reaction monitoring mode for MMA and MMA-d3 respectively. The intra-assay CVs for duplicate analysis ranged from 0.0 to 10.9%, while the inter-assay CVs (concentration range: 177–1114 nmol/L) were between 6 and 12%. Our laboratory participates in the DEKS (Aarhus University Hospital) Quality Assurance scheme for MMA. An MMA concentration >280 nmol/L was considered to be elevated (>360 nmol/L was applied to patients >65 years) [6,13,20].

Leucocytes (WBC), erythrocytes (RBC), haemoglobin (Hb), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) were determined by Beckman Coulter analysers (LH750, LH500, DxH and Ac75) by the Haematology Department (Guy's and St. Thomas' Hospitals). Serum creatinine was analysed on Modulars, Integras and c501 analysers by Roche. eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) formula [21]. Ethnicity was not included in the calculations of eGFR because the ethnic origins of patients were not known to us and are not a compulsory requirement when renal function is requested at our host hospitals. The conversion factor for Africans of 1.21 is given separately with each eGFR result. Serum folate and ferritin were analysed using the Architect System (Abbott Diagnostics). Specimens with serum folate concentration exceeding 45.3 nmol/L were reported as >45.3 nmol/L.

Statistical analyses were carried out using SPSS for Windows (SPSS Inc., US). All the continuous variables were not normally distributed, and statistical analyses using either nonparametric tests or $\log_{10}$ transformed values were performed. The Mann–Whitney test was used to compare values between patients with known and unknown eGFR. Spearman rank correlation coefficients were used to assess simple correlation between holoTC and MMA and the selected characteristics of the population. Linear regression analyses were used to look into relationships between holoTC with renal function and MMA with its main determinants. HoloTC results reported as ≥128 pmol/L and folate ≥45.3 nmol/L (above the dynamic range of the assay) were excluded from the regression and correlation analysis. The χ^2 test was used to compare a frequency of an elevated MMA within the holoTC indeterminate range between patients with known and unknown renal function. Results were considered statistically significant if the observed two sided p value was <0.05.

3. Results

3.1. Characteristics of the study population and the distribution of holoTC and MMA results

A total of 4,175 samples were received for holoTC measurements as part of the routine investigations of vitamin B_{12} status. The median age of all patients was 56 (range 0–101) years, with 55% being female. Female patients were significantly younger than male patients ($P < 0.001$). There was no difference in holoTC concentrations between sexes ($p = 0.545$). Male patients had higher MMA concentrations than female patients ($p = 0.021$).

The prevalence of holoTC values ≥128 pmol/L and folates ≥45.3 nmol/L was 20% and 7% respectively. Interestingly 72% of samples with holoTC ≥128 pmol/L had an eGFR <60 mL/min/1.73 m^2.

Using holoTC alone, impaired vitamin B_{12} status was suggested and excluded in 5% and 71% of patient's samples respectively (Fig. 1). After the exclusion of those with eGFR <60 mL/min/1.73 m^2, MMA analyses were carried out for all samples with a holoTC concentration between 25 and 50 pmol/L ($n = 802$, 19% of total patients), including samples for patients with unknown renal status. For analytical reasons e.g. insufficient sample, MMA analysis was performed for 778 samples only. MMA above our cut-offs was found in 6% of these samples (Fig. 1). Based on our holoTC and MMA cut-offs, the total prevalence of patients with low holoTC and/or elevated MMA in our mixed patient population was 11%. This excludes 5% of patients with a holoTC concentration within the indeterminate range and eGFR <60 mL/min/1.73 m^2.

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