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*Published in:*
Annals of Clinical Biochemistry

*DOI:*
10.1177/00045632211003605

*Publication date:*
2021

*Research Article*

The Association for Clinical Biochemistry & Laboratory Medicine
*Better Science, Better Testing, Better Care*

Annals of Clinical Biochemistry
2021, Vol. 58(5) 445–451

DOI: 10.1177/
00045632211003605
journals.sagepub.com/home/acb
SAGE

# Personalising laboratory medicine in the 'real world': Assessing clinical utility, by clinical indication, of serum total $B_{12}$ and Active-$B_{12}$® (holotranscobalamin) in the diagnosis of vitamin $B_{12}$ deficiency

**Michael J Murphy[1], Fiona Brandie[2], Mildred Ebare[3], Michelle Harrison[4], Ellie Dow[1], William A Bartlett[5] and David Craig[6]**

## Abstract

**Background:** Assessing the pre- and post-test probability of disease in the context of routine health care is challenging. We wished to study how test performance parameters relating to clinical utility vary by clinical indication in a 'real-world' setting.

**Methods:** The diagnostic accuracy of serum total $B_{12}$ and Active-$B_{12}$® (holotranscobalamin) was evaluated in a primary care population, using serum methylmalonic acid as the reference standard. We used electronic requesting to establish the clinical indication for each request. Routine requests from primary care for serum total $B_{12}$ were included if creatinine was also measured and estimated glomerular filtration rate was at least 60 mL/min/1.73 $m^2$.

**Results:** Clinical indications included peripheral neuropathy ($n = 168$), anaemia ($n = 168$), cognitive decline ($n = 125$), suspected dietary deficiency ($n = 76$), other ($n = 362$). For peripheral neuropathy, the area under the receiver operator curve ± 95% confidence interval (AUC ± CI) was 0.63 (0.54–0.71) ($P = 0.002$) for total $B_{12}$ and 0.68 (0.60–0.77) ($P < 0.0001$) for Active-$B_{12}$®. For anaemia, AUC ± CI was 0.56 (0.47–0.66) ($P = 0.10$) for total $B_{12}$ and 0.69 (0.59–0.78) ($P < 0.0001$) for Active-$B_{12}$®. For cognitive decline, AUC ± CI was 0.54 (0.43–0.65) ($P = 0.26$) for total $B_{12}$ and 0.69 (0.58–0.80) ($P = 0.0002$) for Active-$B_{12}$®. The pre–post-test change in probability of disease varied by clinical indication.

**Conclusion:** Combining diagnostic accuracy studies and electronic testing in a 'real-world' setting allows clinical utility to be assessed by clinical indication. Wider application of this would permit more personalised laboratory medicine. In this study, diagnostic performance of total $B_{12}$ and Active-$B_{12}$® varied across all indications. Active-$B_{12}$® provided better discrimination, but this may have reflected the cut-offs used.

## Keywords

Evidence-based medicine, clinical utility, Youden cut-offs, diagnostic uncertainty, electronic test requesting, 'real-world' setting, vitamin $B_{12}$

Accepted: 26th February 2021

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## Introduction

Diagnostic investigations are most useful at the point of maximum diagnostic uncertainty, i.e. when the potential gap between pre- and post-test probability of disease is greatest. In the context of routine health care, it is difficult to study the application of this principle to laboratory investigations. Practical challenges include: first, the existence of multiple requests and request sources, each with a potentially different pretest probability of disease; second, the need for information about the clinical indication for the request; third, the need for a validated reference standard. As a result of these difficulties, 'real-world' studies of the clinical utility of tests, and how this varies by clinical indication, are rare.

Separately, accurate assessment of vitamin $B_{12}$ status is problematic.[1] Defining deficiency in terms of tissue stores (e.g. red cell cobalamin)[2] is not always feasible, and no single analytical measurement is ideal, leading some to propose the use of combined indicators of vitamin $B_{12}$ status.[3] Several diagnostic reports[4–8] have used methylmalonic acid (MMA) as reference standard, despite its acknowledged limitations.[9] We adopted this approach in a study of the diagnostic accuracy of serum total $B_{12}$ and Active-$B_{12}$® (holotranscobalamin, or holoTC) in the assessment of vitamin $B_{12}$ status in an otherwise unselected primary care population. We used electronic requesting to establish the clinical indication for each request. Our study is the first to report how test performance parameters relating to clinical utility vary by clinical indication in a 'real-world' setting.

## Methods

General practitioners in 68 practices in Tayside, Scotland (population approximately 400,000) were advised of the intention to perform a study of the diagnostic accuracy of the existing measure of vitamin $B_{12}$ status (i.e. total serum $B_{12}$), and Active-$B_{12}$® (see below for details of methods), in the setting of routine health care. Their cooperation was sought in the provision of information about the clinical indication for individual requests. Clinical information was delivered through an electronic order communications system, the Integrated Clinical Environment (ICE) (Sunquest Information Systems, Uxbridge, UK). The study was performed between 12 June 2014 and 31 July 2014.

### Participants

The study population consisted of consecutive patients from whom samples were received for routine measurement of total serum vitamin $B_{12}$. In order to minimise the impact of reduced glomerular filtration rate (GFR) on serum MMA,[10] patients were excluded if creatinine was not concurrently requested or if estimated glomerular filtration rate (eGFR) was 60 mL/min/1.73 $m^2$ or less. Only the results of total serum vitamin $B_{12}$ were reported.

### Clinical indications

Health-care professionals ordering vitamin $B_{12}$ through the electronic order communications system chose from the following 'drop-down' list of clinical indications: unexplained anaemia; macrocytic anaemia; cognitive decline; suspected dietary deficiency; peripheral neuropathy; other.

### Analytical methods

Serum total $B_{12}$ was measured by chemiluminescent microparticle-based competitive immunoassay on an Advia Centaur (Siemens Healthineers, Tarrytown, NY, USA), Active-$B_{12}$® by chemiluminescent microparticle-based immunoassay on an Abbott Architect i2000 (Abbott Diagnostics, Abbott Park, IL, USA), both at Ninewells Hospital in Dundee. MMA was measured using a MultiPurpose Sampler (Gerstel GmbH, Mülheim an der Ruhr, Germany) coupled to liquid chromatography tandem mass spectrometry (MPS-LC-MS/MS) at the Nutristasis Unit, Viapath, St Thomas Hospital, London.

### Cut-offs used to define result categories

The lower limit of the reference interval (200 pg/mL) for serum total $B_{12}$ was used, in line with the manufacturer's recommendations. The cut-off for serum holoTC (Active-$B_{12}$®) was $<35$ pmol/L, as used by other groups.[6,11] Two separate cut-offs were used for MMA, reflecting the impact of age-related changes in glomerular function on serum MMA: $>0.28$ μmol/L for patients $\leqslant 65$ years and $>0.36$ μmol/L for patients $>65$ years. These were defined using age-specific 97.5th percentiles in vitamin $B_{12}$-replete patients in a previous study.[12] Total $B_{12}$, Active-$B_{12}$® and MMA were measured in isolation from each other; respective assessors of each did not have access to results of the other measurements, and only laboratory staff involved in the routine measurement of total $B_{12}$ had access to the clinical indication for each request.

### Statistical methods

Descriptive statistics (mean, 2.5th and 97.5th centiles) were calculated for population characteristics. Test performance parameters and receiver operator curve (ROC) analysis were performed by Analyze-it (Version 2.21; Analyze-it Software Ltd, West

Yorkshire, UK). Youden cut-offs were established for the three commonest indications and test performance recalculated for total $B_{12}$ and Active-$B_{12}$® by indication.

## Results

During the study period, 899 routine requests for serum total $B_{12}$ were received. The breakdown of clinical indications for requests was as follows: unexplained anaemia ($n = 134$), macrocytic anaemia ($n = 34$), cognitive decline ($n = 125$), suspected dietary deficiency ($n = 76$), peripheral neuropathy (168), other ($n = 362$). (The anaemia categories were merged for the purposes of data analysis.) Table 1 summarises group characteristics for the three commonest indications. For the other indications, the characteristics (mean and 2.5–97.5 centiles) were as follows: (1) suspected dietary $B_{12}$ deficiency: age (years) 57 (15–91), haemoglobin (g/L) 137 (109–165), haematocrit 0.42 (0.35–0.50), mean cell volume (fL) 95 (85–110), white cell count ($\times 10^9$/L) 6.4 (3.5–10.9), platelets ($\times 10^9$/L) 254 (108–406), total vitamin $B_{12}$ (pg/mL) 370 (164–1004), Active-$B_{12}$® (pmol/L) 65 (12–256), MMA (μmol/L) 342 (96–1869); (2) other: age (years) 61 (23–92), haemoglobin (g/L) 134 (99–165), haematocrit 0.41 (0.30–0.49), mean cell volume (fL) 94 (81–111), white cell count ($\times 10^9$/L) 6.8 (3.7–11.9), platelets ($\times 10^9$/L) 256 (130–441), total vitamin $B_{12}$ (pg/mL) 388 (170–974), Active-$B_{12}$® (pmol/L) 70 (17–256), MMA (μmol/L) 320 (95–1121).

ROC curves for the three commonest specific indications are shown in Figure 1(a) to (c). For other indications, the areas under the curve ± 95% confidence intervals (AUC ± CI) were as follows for total $B_{12}$: suspected dietary deficiency 0.50 (0.36–0.64); other 0.47 (0.41–0.54). For Active-$B_{12}$®, the AUC ± CI were: suspected dietary deficiency 0.61 (0.47–0.75); other 0.66 (0.60–0.72). For the total population, the AUC ± CI for total $B_{12}$ was 0.53 (0.49–0.57) ($P = 0.075$), and for Active-$B_{12}$®, AUC ± CI was 0.68 (0.64–0.72) ($P < 0.0001$). These AUC were significantly different from each other ($P < 0.0001$).

Table 2 summarises the pre- and post-test probabilities of vitamin $B_{12}$ deficiency (as defined by raised serum MMA), before and after measurement of serum total $B_{12}$, in each of the three commonest specific indications for requesting total $B_{12}$. Data are presented using the preassigned cut-offs and using Youden cut-offs (designed to optimise differentiating ability).[13] We wished to calculate the post-test probability of disease in the presence of positive and negative results, respectively; therefore, we replaced the negative predictive values (NPV) by [1–NPV]. Table 3 summarises the corresponding data for Active-$B_{12}$®.

## Discussion

Optimal targeting of diagnostic tests in the setting of normal care is challenging. Evaluations of diagnostic accuracy are usually performed in well-defined and preselected populations, whereas tests in routine use are usually applied to multiple populations that vary in terms of relevant parameters (e.g. prevalence). In the current study, we have addressed this issue by performing a diagnostic evaluation in the setting of normal care.

We identified several distinct populations, based on the clinical indication for the vitamin $B_{12}$ request. Patients with cognitive decline were, as anticipated, older than other patient groups, and had higher creatinine, reflecting reduced glomerular function. Interestingly, prevalence of vitamin $B_{12}$ deficiency, as defined by raised serum MMA, was broadly similar in the patient groups defined by the three commonest specific indications, ranging from 32.7% in patients with peripheral neuropathy to 36.6% in patients with anaemia. This is important, since any differences in PPV and [1–NPV] across clinical indications are therefore not attributable to large differences in prevalence.

For all categories of indication, measurement of Active-$B_{12}$® reliably differentiated vitamin $B_{12}$ deficiency from non-deficiency, and for all the AUC for Active-$B_{12}$® was greater than for total $B_{12}$, although in the case of peripheral neuropathy, the difference was not statistically significant. However, these findings must be interpreted with caution in the context of the wider literature. The effect on the clinical utility of holoTC of applying different MMA cut-offs,[8] and more widely, the need to base cut-offs (for all vitamin $B_{12}$ biomarkers) on adverse outcomes,[14] have previously been highlighted. The age-specific MMA cut-offs applied here, particularly in patients >65 years, may have affected sensitivity and specificity; other limitations include those of using MMA as a reference standard,[15] and the exclusion of patients where creatinine was not requested, or where eGFR was 60 mL/min/1.73 $m^2$ or less. On the wider issue, the ability of holoTC to predict, for example, neurological outcomes like cognitive decline in well-designed studies[16–19] has been variable, and its role as a biomarker of clinically meaningful vitamin $B_{12}$ deficiency remains inconclusive. Recent assessments[15,20] acknowledge the limitations of holoTC and other markers and endorse the adoption of algorithm-based approaches that combine more than one measure of vitamin $B_{12}$ status.[3] This seems reasonable, and our findings do not provide a basis for challenging this position.

With these caveats, low Active-$B_{12}$® results were more useful than normal ones; the increase in probability of vitamin $B_{12}$ deficiency seen with a low result

**Table 1.** Characteristics of study population.

| Group | Parameter | | 2.5–97.5 percentile | % abnormal results (cut-off value) |
|---|---|---|---|---|
| Total | | *n* | | |
| | Group total | 899 | | |
| | Female | 560 | | |
| | >65 years | 479 | | |
| | | Mean | | |
| | Age (years) | 64 | 23–92 | |
| | Haemoglobin (g/L) | 133 | 96–165 | 27% (<120 female, <130 male) |
| | Haematocrit | 0.41 | 0.30–0.50 | 19% (<0.37) |
| | Mean Cell Volume (fL) | 94 | 81–110 | 8% (>105) |
| | White Cell Count ($\times 10^9$/L) | 6.8 | 3.5–11.9 | 6% ($<4 \times 10^9$) |
| | Platelets ($\times 10^9$/L) | 257 | 126–447 | 7% ($<150 \times 10^9$) |
| | Total $B_{12}$ (pg/mL) | 408 | 171–1195 | 5% (<200) |
| | Active-$B_{12}$® (pmol/L) | 67 | 15–256 | 24% (<35) |
| | MMA (μmol/L) | 345 | 98–1259 | 32% (>0.28 ⩽ 65yo; >0.36 > 65yo) |
| Cognitive decline | | *n* | | |
| | Group total | 125 | | |
| | Female | 80 | | |
| | >65 years | 104 | | |
| | | Mean | | |
| | Age (y) | 77 | 44–93 | |
| | Haemoglobin (g/L) | 132 | 94–166 | 30% (<120 female, <130 male) |
| | Haematocrit | 0.41 | 0.29–0.51 | 21% (<0.37) |
| | Mean cell volume (fL) | 95 | 83–107 | 6% (>105) |
| | White cell count ($\times 10^9$/L) | 6.6 | 3.4–10.3 | 6% ($<4 \times 10^9$) |
| | Platelets ($\times 10^9$/L) | 263 | 135–479 | 7% ($<150 \times 10^9$) |
| | Total $B_{12}$ (pg/mL) | 413 | 166–1269 | 7% (<200)yo |
| | Active-$B_{12}$® (pmol/L) | 62 | 16–256 | 34% (<35)yo |
| | MMA (μmol/L) | 346 | 80–1044 | 34% (>0.28 ⩽ 65yo; >0.36 > 65yo) |
| Peripheral neuropathy | | *n* | | |
| | Group total | 168 | | |
| | Female | 109 | | |
| | >65 years | 58 | | |
| | | Mean | | |
| | Age (y) | 58 | 23–89 | |
| | Haemoglobin (g/L) | 142 | 110–168 | 7% (<120 female, <130 male) |
| | Haematocrit | 0.43 | 0.35–0.50 | 5% (<0.37) |
| | Mean cell volume (fL) | 94 | 84–106 | 2% (>105) |
| | White cell count ($\times 10^9$/L) | 7.2 | 3.8–13.0 | 4% ($<4 \times 10^9$) |
| | Platelets ($\times 10^9$/L) | 258 | 126–434 | 4% ($<150 \times 10^9$) |
| | Total $B_{12}$ (pg/mL) | 410 | 205–838 | 1% (<200) |
| | Active-$B_{12}$® (pmol/L) | 66 | 12–256 | 27% (<35) |
| | MMA (μmol/L) | 394 | 119–2011 | 33% (>0.28 ⩽ 65yo; >0.36 > 65yo) |
| Unexplained anaemia combined with macrocytic anaemia | | *n* | | |
| | Group total | 168 | | |
| | Female | 97 | | |
| | >65 years | 105 | | |

(continued)

**Table 1.** Continued.

| Group | Parameter | | 2.5–97.5 percentile | % abnormal results (cut-off value) |
|---|---|---|---|---|
| | | Mean | | |
| | Age (years) | 69 | 26–91 | |
| | Haemoglobin (g/L) | 121 | 87–159 | 62% (<120 female, <130 male) |
| | Haematocrit | 0.38 | 0.28–0.50 | 43% (<0.37) |
| | Mean cell volume (fL) | 95 | 79–116 | 12% (>105) |
| | White cell count ($\times 10^9$/L) | 6.8 | 3.0–11.9 | 10% ($<4 \times 10^9$) |
| | Platelets ($\times 10^9$/L) | 258 | 110–448 | 11% ($<150 \times 10^9$) |
| | Total $B_{12}$ (pg/mL) | 391 | 144–1541 | 8% (<200) |
| | Active-$B_{12}$® (pmol/L) | 67 | 15–256 | 21% (<35) |
| | MMA (μmol/L) | 347 | 84–1149 | 34% (>0.28 ⩽ 65yo; >0.36 > 65yo) |

Note: the upper measuring limit of the Active-$B_{12}$® assay is 256 pmol/L; all samples ⩾256 pmol/L were recorded for analysis as 256 pmol/L. This does not affect % abnormal results nor the clinical performance calculations. 'Unexplained anaemia' and 'macrocytic anaemia' categories were merged. In the category 'unexplained anaemia', 8 patients from 134 had an elevated MCV (>105 fL). In the category 'macrocytic anaemia', 12 patients from 34 had an elevated MCV.

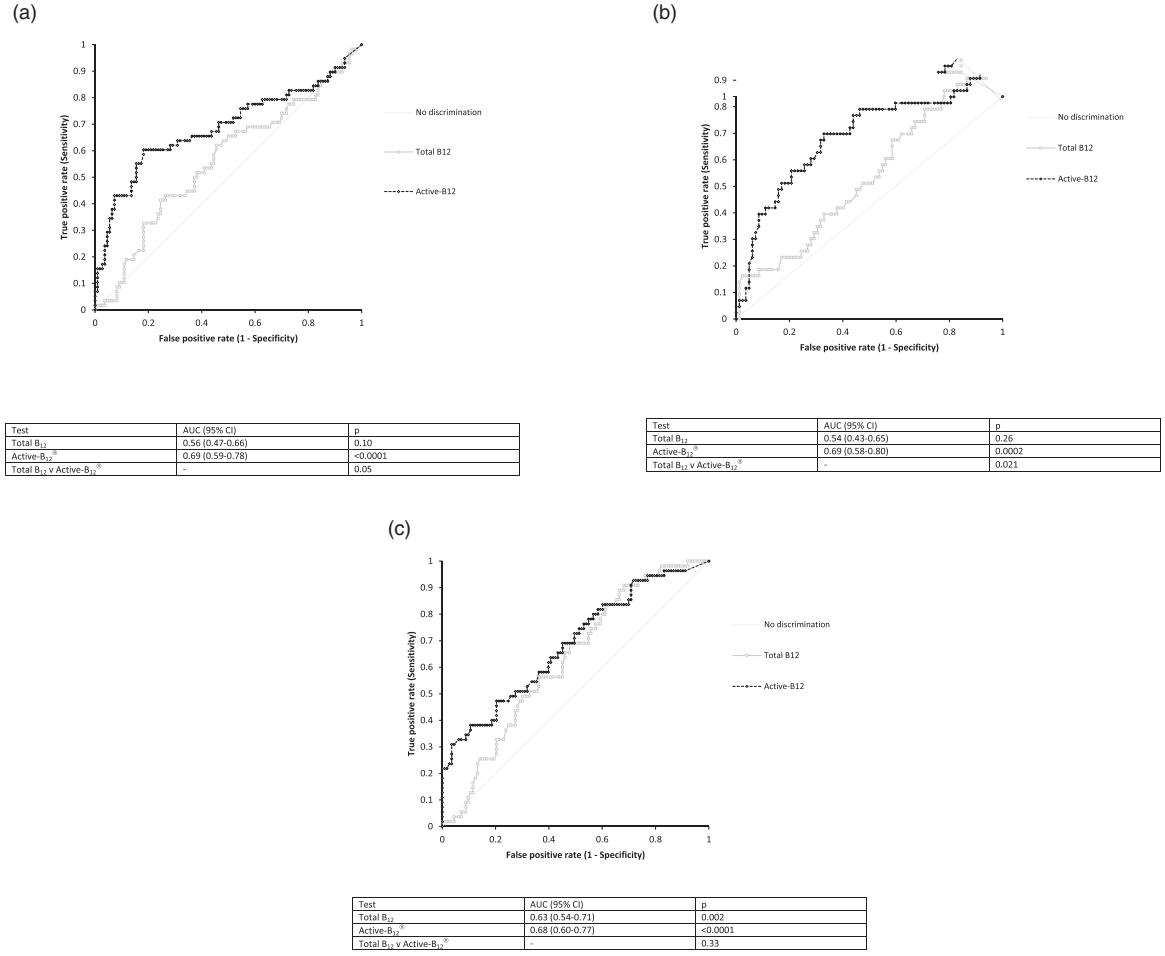


**Figure 1.** (a) ROC curves for total $B_{12}$ and Active-$B_{12}$® in the diagnosis of vitamin $B_{12}$ deficiency (as defined by raised serum MMA) in anaemia. (b) ROC curves for total $B_{12}$ and Active-$B_{12}$® in the diagnosis of vitamin $B_{12}$ deficiency (as defined by raised serum MMA) in cognitive decline. (c) ROC curves for total $B_{12}$ and Active-$B_{12}$® in the diagnosis of vitamin $B_{12}$ deficiency (as defined by raised serum MMA) in peripheral neuropathy.

**Table 2.** Clinical utility of Total $B_{12}$ by clinical indication.

| Using pre-assigned cut-off (200 pg/mL) | | | | | | Using Youden cut-offs[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Indication | Abnormal results (*n*) | PPV (%) | Δ in probability (%) with low result | 1 – NPV (%) | Δ in probability (%) with normal result | Youden cut-off (pg/mL) | Abnormal results (*n*) | PPV (%) | Δ in probability (%) with low result | 1–NPV (%) | Δ in probability (%) with normal result | Prevalence (%) |
| **Anaemia** *n* = 168 | 11 | 18.2 | –18.4 | 39.0 | +2.4 | 287 | 46 | 50.0 | +13.4 | 30.7 | –5.9 | 36.6 |
| **Cognitive decline** *n* = 125 | 9 | 77.8 | +43.4 | 31.0 | –3.4 | 200 | 9 | 77.8 | +43.4 | 31.0 | –3.4 | 34.4 |
| **Peripheral neuropathy** *n* = 168 | 2 | 50.0 | +17.3 | 32.5 | –0.2 | 481 | 127 | 39.4 | +6.7 | 12.2 | –20.5 | 32.7 |

Δ = change; PPV (positive predictive value) = post-test probability of vitamin $B_{12}$ deficiency in presence of a low total $B_{12}$ result; (1–NPV [negative predictive value]) = post-test probability of vitamin $B_{12}$ deficiency in presence of a normal total $B_{12}$ result.

Note: Probability of vitamin $B_{12}$ deficiency (as defined by raised serum MMA) before and after measurement of total $B_{12}$, for three clinical indications. Pretest probability (prevalence) is shown, along with the post-test probability associated with low and normal results. For ease of reference, the changes in probability are also shown. Data are presented using preassigned cut-offs, and separately using Youden cut-offs.

[a]The cut-offs that optimise differentiating ability when equal weight is given to sensitivity and specificity.

**Table 3.** Clinical utility of Active-$B_{12}$® by clinical indication.

| Using pre-assigned cut-off (35 pmol/L) | | | | | | Using Youden cut-offs[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Indication | Abnormal results (*n*) | PPV (%) | Δ in probability (%) with low result | 1 – NPV (%) | Δ in probability (%) with normal result | Youden cut-off (pg/mL) | Abnormal results (*n*) | PPV (%) | Δ in probability (%) with low result | 1 – NPV (%) | Δ in probability (%) with normal result | Prevalence (%) |
| **Anaemia** *n* = 168 | 29 | 75.9 | +39.3 | 26.7 | –9.9 | 43 | 44 | 68.2 | +31.6 | 22.2 | –14.4 | 36.6 |
| **Cognitive decline** *n* = 125 | 43 | 55.8 | +21.4 | 23.2 | –11.2 | 44 | 54 | 52.6 | +18.2 | 19.1 | –15.3 | 34.4 |
| **Peripheral neuropathy** *n* = 168 | 46 | 50.0 | +17.3 | 26.2 | –6.5 | 29 | 30 | 63.6 | +30.9 | 25.2 | –7.5 | 32.7 |

Δ = change; PPV (positive predictive value) = post-test probability of vitamin $B_{12}$ deficiency in presence of a low Active-$B_{12}$®result; (1–NPV [negative predictive value]) = post-test probability of vitamin $B_{12}$ deficiency in presence of a normal Active-$B_{12}$®result.

Note: Probability of vitamin $B_{12}$ deficiency (as defined by raised serum MMA) before and after measurement of Active-$B_{12}$®, for three clinical indications. Pretest probability (prevalence) is shown, along with the post-test probability associated with low and normal results. For ease of reference, the changes in probability are also shown. Data are presented using pre-assigned cut-offs, and separately using Youden cut-offs.

[a]The cut-offs that optimise differentiating ability when equal weight is given to sensitivity and specificity.

was higher, for all three of the commonest specific indications, than the decrease in probability associated with a normal result (see Table 3). This was true both when the preassigned cut-offs and when Youden cut-offs were used. As anticipated, the PPV ranking for these indications corresponded to the prevalence ranking (when the preassigned cut-off was used), with the highest PPV associated with anaemia and the lowest with peripheral neuropathy.

We have shown in the current study that it is possible to apply the principles of evidence-based medicine to routine requesting. Electronic requesting is now commonplace in health care, and, with the co-operation of clinical colleagues, can readily be harnessed to studies of diagnostic accuracy. In the presence of a validated reference standard, it is possible to establish prevalence by clinical indication and requesting source. The cost of diagnostic studies involving validated reference standards should be offset against the potential savings enabled by greater precision in laboratory requesting, for example through the application of local algorithms.

#### Acknowledgements

Reagents were provided by Axis-Shield Diagnostics.

#### Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: David Craig was an employee of Axis-Shield Diagnostics at the time of this research.

#### Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

#### Ethical approval

Not applicable.

#### Guarantor

MJM.

#### Contributorship

MJM had the idea for the study and wrote the first draft of the paper. DC performed data analysis. FB, ME and MH assisted with the laboratory processing of samples, and commented on drafts of the paper. ED and WAB commented on drafts of the paper.

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