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

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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$_{12}$

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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\textsubscript{12} status is problematic.\textsuperscript{1} Defining deficiency in terms of tissue stores (e.g. red cell cobalamin)\textsuperscript{2} is not always feasible, and no single analytical measurement is ideal, leading some to propose the use of combined indicators of vitamin B\textsubscript{12} status.\textsuperscript{3} Several diagnostic reports\textsuperscript{4–8} have used methylmalonic acid (MMA) as reference standard, despite its acknowledged limitations.\textsuperscript{9} We adopted this approach in a study of the diagnostic accuracy of serum total B\textsubscript{12} and Active-B\textsubscript{12} (holotranscobalamin, or holoTC) in the assessment of vitamin B\textsubscript{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\textsubscript{12} status (i.e. total serum B\textsubscript{12}, and Active-B\textsubscript{12} (holotranscobalamin, or holoTC) in the assessment of vitamin B\textsubscript{12} status in an otherwise unselected primary care population. We used electronic requesting to establish the clinical indication for each request. Our 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\textsubscript{12}. In order to minimise the impact of reduced glomerular filtration rate (GFR) on serum MMA,\textsuperscript{10} patients were excluded if creatinine was not concurrently requested or if estimated glomerular filtration rate (eGFR) was 60 mL/min/1.73 m\textsuperscript{2} or less. Only the results of total serum vitamin B\textsubscript{12} were reported.

Clinical indications

Health-care professionals ordering vitamin B\textsubscript{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\textsubscript{12} was measured by chemiluminescent microparticle-based competitive immunoassay on an Advia Centaur (Siemens Healthineers, Tarrytown, NY, USA), Active-B\textsubscript{12} (holotranscobalamin, or holoTC) in the assessment of vitamin B\textsubscript{12} status in an otherwise unselected primary care population. We used electronic requesting to establish the clinical indication for each request. Our study was performed between 12 June 2014 and 31 July 2014.

Cut-offs used to define result categories

The lower limit of the reference interval (200 pg/mL) for serum total B\textsubscript{12} was used, in line with the manufacturer’s recommendations. The cut-off for serum holoTC (Active-B\textsubscript{12}) was <35 pmol/L, as used by other groups.\textsuperscript{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 ≤65 years and >0.36 μmol/L for patients >65 years. These were defined using age-specific 97.5th percentiles in vitamin B\textsubscript{12}-replete patients in a previous study.\textsuperscript{12} Total B\textsubscript{12}, Active-B\textsubscript{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\textsubscript{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 B12 and Active-B12® by indication.

Results
During the study period, 899 routine requests for serum total B12 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 B12 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 (×10⁹/L) 6.4 (3.5–10.9), platelets (×10⁹/L) 254 (108–406), total vitamin B12 (pg/mL) 370 (164–1004), Active-B12® (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 (×10⁹/L) 6.8 (3.7–11.9), platelets (×10⁹/L) 256 (130–441), total vitamin B12 (pg/mL) 388 (170–974), Active-B12® (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 B12: suspected dietary 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 (×10⁹/L) 6.4 (3.5–10.9), platelets (×10⁹/L) 254 (108–406), total vitamin B12 (pg/mL) 370 (164–1004), Active-B12® (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 (×10⁹/L) 6.8 (3.7–11.9), platelets (×10⁹/L) 256 (130–441), total vitamin B12 (pg/mL) 388 (170–974), Active-B12® (pmol/L) 70 (17–256), MMA (µmol/L) 320 (95–1121).

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 B12 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 B12 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-B12® reliably differentiated vitamin B12 deficiency from non-deficiency, and for all the AUC for Active-B12® was greater than for total B12, 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 B12 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 m² or less. On the wider issue, the ability of holoTC to predict, for example, neurological outcomes like cognitive decline in well-designed studies16–19 has been variable, and its role as a biomarker of clinically meaningful vitamin B12 deficiency remains inconclusive. Recent assessments15,20 acknowledge the limitations of holoTC and other markers and endorse the adoption of algorithm-based approaches that combine more than one measure of vitamin B12 status.3 This seems reasonable, and our findings do not provide a basis for challenging this position.

With these caveats, low Active-B12® results were more useful than normal ones; the increase in probability of vitamin B12 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**                    |                                       |                     |                                   |
| Group total                  | n                                     | 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 (×10⁹/L)    | 6.8                                   | 3.5–11.9            | 6% (<4 × 10⁹)                    |
| Platelets (×10⁹/L)           | 257                                   | 126–447             | 7% (<150 × 10⁹)                  |
| Total B₁₂ (pg/mL)            | 408                                   | 171–1195            | 5% (<200)                        |
| Active-B₁₂⁺ (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 (×10⁹/L)    | 6.6                                   | 3.4–10.3            | 6% (<4 × 10⁹)                    |
| Platelets (×10⁹/L)           | 263                                   | 135–479             | 7% (<150 × 10⁹)                  |
| Total B₁₂ (pg/mL)            | 413                                   | 166–1269            | 7% (<200)yo                      |
| Active-B₁₂⁺ (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 (×10⁹/L)    | 7.2                                   | 3.8–13.0            | 4% (<4 × 10⁹)                    |
| Platelets (×10⁹/L)           | 258                                   | 126–434             | 4% (<150 × 10⁹)                  |
| Total B₁₂ (pg/mL)            | 410                                   | 205–838             | 1% (<200)                        |
| Active-B₁₂⁺ (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                           | 26–91              |                                   |
| Age (years)   | 69                             | 87–159             | 62% (<120 female, <130 male)      |
| 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 (×10^9/L) | 6.8                          | 3.0–11.9           | 10% (<4 × 10^9)                   |
| Platelets (×10^9/L) | 258                         | 110–448            | 11% (<150 × 10^9)                 |
| Total B12 (pg/mL) | 391                        | 144–1541           | 8% (<200)                         |
| Active-B12 (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-B12 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 B12 and Active-B12 in the diagnosis of vitamin B12 deficiency (as defined by raised serum MMA) in anaemia. (b) ROC curves for total B12 and Active-B12 in the diagnosis of vitamin B12 deficiency (as defined by raised serum MMA) in cognitive decline. (c) ROC curves for total B12 and Active-B12 in the diagnosis of vitamin B12 deficiency (as defined by raised serum MMA) in peripheral neuropathy.
### Table 2. Clinical utility of Total B₁₂ by clinical indication.

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

Δ = change; PPV (positive predictive value) = post-test probability of vitamin B₁₂ deficiency in presence of a low total B₁₂ result; (1–NPV [negative predictive value]) = post-test probability of vitamin B₁₂ deficiency in presence of a normal total B₁₂ result.

Note: Probability of vitamin B₁₂ deficiency (as defined by raised serum MMA) before and after measurement of total B₁₂, 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.

aThe cut-offs that optimise differentiating ability when equal weight is given to sensitivity and specificity.

### Table 3. Clinical utility of Active-B₁₂ by clinical indication.

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

Δ = change; PPV (positive predictive value) = post-test probability of vitamin B₁₂ deficiency in presence of a low Active-B₁₂ result; (1–NPV [negative predictive value]) = post-test probability of vitamin B₁₂ deficiency in presence of a normal Active-B₁₂ result.

Note: Probability of vitamin B₁₂ deficiency (as defined by raised serum MMA) before and after measurement of Active-B₁₂, 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.

aThe 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 cooperation 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.

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Guarantor
MJM.

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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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