Vitamin B12 testing

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Topics

‣ B12 physiology
  ‣ Total B12 (TB12) and Holo-transcobalamin (HoloTC)
  ‣ Different binding proteins in blood for B12

‣ Laboratory assessment for B12 status
  ‣ TB12 vs HoloTC assays:
    ‣ Questions: Are all the TB12 assays the same?
  ‣ Functional markers:
    ‣ homocysteine (HCY); methyl-malonic acid (MMA)
    ‣ FBE: Hb, MCV, film

‣ Questions to cover:
  ‣ 1. Is the HoloTC assay better test than the TB12 assays?
  ‣ 2. What is an equivocal range for TB12 assays?
  ‣ 3. How do we interpret the HoloTC results?
Vitamin B12

- **Rich sources:**
  - Meat, egg, milk and dairy

- **Recommended Daily Allowance (RDA):**
  - Adult: 2 ug/day
  - Pregnancy & lactation: 2.6 ug/day
  - Toddler-Children: 0.7 - 2 ug/day

- **Western diet:**
  - Average 3-30 ug/day, 1-5 ug/day absorbed

- **Total body store:**
  - 2 - 5 mg (50% in liver)
  - Takes many years to deplete without intake
**B12 absorption**

**In food:**
B12-binds to animal protein

**In Stomach and proximal SI:**
- B12 is cleaved off from the food binding protein
- B12–Intrinsic factor complex formed
- B12-IF transported to ileum

**In distal ileum (80 cm):**
- B12-IF complex bound to IF receptor
- B12 absorbed into blood

Andrés E et al. CMAJ 2004; 171: 251-259
B12 Binding Protein in Blood

Transcobalamin (TC II) (HoloTC)
- Active form → binds to receptor → cells

Haptocorrin (HC)
- Binds ~75% of B12 (HoloHC)
- glycoprotein,Inactive form
- function unknown

Total B12 influenced by changes in binding proteins → poor indicator of bioavailable cobalamin
Metabolic Functions of B12: a cofactor of

- Methyl Malonic Acid Mutase
- Methionine Synthase

In B12 deficiency:

- $\uparrow$ Homocysteine
- $\uparrow$ MMA

HoloTC
B12 physiology
- Total B12 (TB12) and Holo-transcobalamin (HoloTC)
- Different binding proteins in blood for B12

Laboratory assessment for B12 status
- TB12 vs HoloTC assays:
  - Questions: Are all the TB12 assays the same?
- Functional markers:
  - homocysteine (HCY); methyl-malonic acid (MMA)
  - FBE: Hb, MCV, film

Questions to cover:
- 1. Is the HolotTC assay better test than the TB12 assays?
- 2. What is an equivocal range for TB12 assays?
- 3. How do we interpret the HoloTC results?
Markers of B12 status

- What’s the gold standard? – none

**Clinical:**
- Signs and symptoms

**Laboratory:**
- Total B12 – routine, readily available
- HoloTC – limited labs

**Functional markers:**
- HCY (Non-specific)
- MMA (Non-specific)
- FBE: Hb, MCV, film
Total B12 Assay:

Method principle

Limitations
Serum total B12 assay: Problem 1

Total B12 assay measure both fractions:

Haptocorrin (HC)
- >80% of B12 (Holo-HC)
- glycoprotein, Inactive form, function unknown

Transcobalamin (TC II)
- ~20% of B12 (Holo-TC)
- Active form, TC II-Cbl complex binds to receptor → enter cells by endocytosis

Total B12 influenced by changes in binding proteins → poor indicator of bioavailable cobalamin
Are all the Total B12 assays the same?

Inter-ab comparison study

November, 2014
Methods Studied

Total B12:
- Roche e602
  - Melbourne Pathology (Routine)
- Abbott Architect
  - Melbourne Pathology (Laurence Hardy, Abbott)
  - Douglass Hanly Moir (Andy Liu)
- Siemens Centaur
  - QML (Dr Julia Chang, Donna Liong)
- Beckman Dxi800
  - Monash Medical Centre (George Streitberg & Julie Newman)

HoloTC: Melbourne Pathology (Laurence Hardy, Abbott)

MMA: by GCMS at Mater Pathology (Dr Janet Warner)
Total B12 Inter-lab comparison, Nov 2014

N=189
Total B12 Methods vs HoloTC
Problem 2: Method not standardised

Different laboratories have:

- different methods
- report in different units
- different reference ranges

Can not just look at the values !!!

Same specimen can measure low in one lab, normal in another lab.

Who is right???
Other Limitations of Total B12

- No diagnostic gold standard
  - True lower limits of normal is poorly defined
- Low sensitivity
- Raw +ve predictive values: 22-72%
  - In different study population
- True B12 deficiency but normal B12
  - Chronic myeloproliferative disease
  - Liver disease
Holotranscobalamin (HoloTC) assay (Active B12)
B12 Binding Protein in Blood

- **Total B12 assay**
  - Haptocorrin (HC)
    - Binds ~75% of B12 (HoloHC)
    - glycoprotein, Inactive form
    - function unknown

- **Active B12 assay**
  - Transcobalamin (TC II) (HoloTC)
    - Active form → binds to receptor → cells
Specimen type: Serum or Li Hep

Sample stability:
- 28 days at 2-8 °C
- Stable for up to 3 freeze-thaw cycles

Minimal Sample volume:
- 200 uL assay vol + 100 ul dead space
The Automatic HoloTC assay – “Active B12”

- Currently produced by one company – Abbott

- 2006-2012: Abbott AxSYM

- 2012 Jul – now: Abbott Architect

Melbourne Pathology have been using HoloTC since 2007
AxSYM Active B12 Assay

Two-step sandwich assay 2008-2012
[Microparticle Enzyme Immunoassay (MEIA)]

Active-B12 specific Mab (mouse, monocl.) immobilised on latex microparticle.
Sample B12 bound to transcobalamin (red) and haptocorrin (magenta).
Only B12-TC (Active-B12) will bind to solid phase.
Suspension moved to glass fiber matrix and washed to remove unbound sample.

Anti-TC ALP conjugate (mouse, monocl.) is added.
Conjugate binds to TC bound to solid phase.
Unbound conjugate is removed.
Rate of fluorescent MU formation is directly proportional to [Active-B12] in sample.
Architect Active B12

From July 2012
[Chemiluminescent microparticle Immunoassay (CMIA)]

Active-B12 specific Mab (mouse, monoclonal) immobilised on latex microparticle.

Sample B12 bound to transcobalamin (red) and haptocorrin (magenta).

Only B12 TC (Active-B12) will bind to solid phase.

Chemiluminescent reaction measured as
Relative light units (RLUs)

Acrdinium-labelled conjugate

Rate of fluorescent MU formation is directly proportional to [Active-B12] in sample.

Unbound conjugate is removed.

WASH
%Macrocytosis  %HCY >11 umol/L

Figure 2. Cut-off values for HoloTC. 2A: Deficiency ; 2B: insufficiency

AxSYM® Active-B12

central 95% of the population defined the expected range of 19.1 to 119.3 pmol/L.
Active B12: Architect vs AxSYM-2015Feb

\[ y = 1.5 \times  + 3.1 \]
### tHCY & MMA for detecting ↓ B12

<table>
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<th>Sensitivity</th>
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<tr>
<td>tHCY</td>
<td>95.9%</td>
</tr>
<tr>
<td>MMA</td>
<td>98.4%</td>
</tr>
<tr>
<td>tHCY + MMA</td>
<td>99.8%</td>
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Both have high negative predictive values. However, if elevated, need to exclude other causes.

If ↑ in tHCY/MMA and normalised after B12 replacement → also diagnostic for B12 deficiency
Plasma tHcy - Limitations

- 95% sensitivity for B12 deficiency
  - High NPV: normal exclude B12 deficiency
- Poor specificity
  - Non-fasting
  - Incorrect sample collection (Clin Chem 2004; 50: 3-32)
  - Aging / renal impairment
  - Smoking, coffee, wine
  - ↓ folate / Vit B6
  - Hyperproliferative disorder
  - Genetic defect
  - MTHFR gene mutation (677C→T)
MMA - Limitations

High MMA can be seen in:

- Elderly
- Renal impairment
- Hypovolaemic state
- Urine MMA is more influenced by food intake than serum MMA
Anti-IF antibodies

50-70% sensitivity

Near 100% specific for PA → Diagnostic

False +ve if assayed following recent B12 injection
Serum gastrin

- Produced by G-cells in gastric antrum
- Binds to parietal cells stimulates acid
- Interpretation:
  - Normal/Low: excludes PA
  - Usually very high in PA
- Pitfalls
  - ↑ with age
  - ↑ in proton pump / H2 blockers (need to be off PPI for 3 weeks)
Is macrocytosis a sensitive marker for B12 deficiency?
Active B12 and RBC morphology

- **Macrocytic**: MCV > 98
- **Normocytic**: MCV 83-98
- **Microcytic**: MCV < 83

N > 20,000
TB12, aB12, MCV & Ferritin available on the same episode
Microcytosis vs Macrocytosis

- **Microcytosis**
- **Macrocytosis**

Active B12 (pmol/L) vs Low Ferritin (%)

- Low Ferritin: 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%
**Case: 51 y/o woman, vegetarian**

- **Hb**: 96 ↓ g/L (115-160)
- **MCV**: 81 ↓ fL (80-100)
- **Ferritin**: 12 ↓ ug/L (20-350)
- **Total B12**: 175 pmol/L (140-650)
- **Active B12**: 12 ↓ pmol/L (23-100)

Iron & B12 Deficiencies often coexist in vegetarians. B12 deficiency can be overlooked by TB12.
Topics

- **B12 physiology**
  - Total B12 (TB12) and Holo-transcobalamin (HoloTC)
  - Different binding proteins in blood for B12

- **Laboratory assessment for B12 status**
  - TB12 vs HoloTC assays:
    - Questions: Are all the TB12 assays the same?
  - Functional markers:
    - homocysteine (HCY); methyl-malonic acid (MMA)
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- **Questions to cover:**
  - 1. Is the HolotC assay better test than the TB12 assays?
  - 2. What is an equivocal range for TB12 assays?
  - 3. How do we interpret the HoloTC results?
Question 1:

Is the HoloTC assay better than the total B12 assays for assessing B12 status?
Total B12: Low Diagnostic Accuracy

Fact Sheet

Vitamin B12 tests

The diagnostic accuracy of serum B12 tests is low due to:

- Lack of a gold standard.\(^6\)
- Inconsistent cutoff values used to define deficiency
  - differences in methods
  - differences in approaches to define deficiency.\(^4\)
- The fraction of vitamin B12 bound to haptocorrin, an inactive carrier protein.\(^7\)
Haptocorrin Levels can Vary

Decreased in:
- In pregnancy
- Genetic haptocorrin deficiency

Increased in:
- Some haematological conditions
Case 1. 33y, pregnant women

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<tr>
<td>S Total B12: 119 L pmol/L</td>
<td>(140-650)</td>
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<tr>
<td>S Active B12: 47 pmol/L</td>
<td>(23-100)</td>
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Active B12: AxSYM
Longitudinal studies in uncomplicated pregnancy

Data obtained from Koebnick et al. Clin Chem 2002; 48: 928

Data obtained from Morkbak et al. Haematologica 2007; 92: 1711

HoloTC not performed in this study
Changes in Total B12

Introduction
Low serum total vitamin B12 (TB12) is often not bound to haptocorrin (HoloHC) or transcobalamin (including placenta). In the postpartum period.

Methods
Samples from 65 women with blood available and postpartum were analysed for TB12 and HoloT (Architect). HoloHC was compared using one-way ANOVA followed by Tukey.

Results
Mean TB12 levels were significantly lower during pregnancy than those of the 2nd and 3rd trimesters. Approximately half of the women had TB12 levels above the analytical point. Data from 44 women were used in the analysis.

Discussion and Conclusion
There is a significant decline in both TB12 and HoloT from the 1st to the 3rd trimester. The absolute decline in pmol/L is larger than the decline in HoloTG (+14.2 pmol/L). Most of the TB12 decline is due to a reduction in HoloT fraction.

The results of this study indicate that HoloT is a total B12 for assessing B12 status during pregnancy.
Total B12 & HoloTC concordance: 68%

Low TB12, normal HoloTC:
- 9.6%
  - Pregnancy
- 17.7%
  - Genetic Haptocorrin deficiency

50.5%

Total pairs: n=79,363

Architect TB12
AxSYM HoloTC
## Genetic Haptocorrin Deficiency?

### Case 2. 66y M, well, routine check up

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<tr>
<th>Name: G.H</th>
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### B12/FOLATE

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<td>pmol/L</td>
<td>(200-700)</td>
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| S Active B12 | 54 | 50 | pmol/L | (23-100) |
| P Homocyst.   | 10 | umol/L | (6-15) |

FBE normal
Haptocorrin Deficiency:

Heterozygote: 15% - more common than previous considered

Homozygote: 0.6%

Low Haptocorrin but normal HCY & MMA → B12 was functionally adequate
(Trancobalamin bound fraction (aB12) not affected)
Both sons have normal homocysteine

Modified from Carmel R. Clin Chem 2003; 49: 1367
Total B12 & HoloTC

Haptocorrin Deficiency?
Haptocorrin can be Falsely High – certain Haem conditions

Non-Hodgkins lymphoma

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**B12/FOLATE**

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<td>Lab Id.</td>
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<td>92420329</td>
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**Units Range**

- **RBC FOLATE**: 1329 pmol/L (600-3000)
- **S Active B12**: 10 L 12 L pmol/L (23-100)
- **S Total B12**: 601 373 pmol/L (140-650)
HoloTC vs Homocysteine (tHCY) / MMA

Receiver Operating Characteristic (ROC) curve: for assay performance

B12 def defined as:
MMA>400 nmol/L
(normal renal function)

Active B12 assay: RIA method

Black et al 2007, Alfred Hospital
- 187 patients
- Data: clinical history, blood films and biochem tests (Hcy & MMA)
- reviewed by 2 haematologists

Active B12 assay: AxSYM
Inter-lab comparison study

HoloTC vs TB12 for MMA $\geq 0.50 \text{ umol/L}$

- **AUC**
  - HoloTC: 0.87
  - TB12_e602: 0.79 *
  - TB12_Architect: 0.81 *
  - TB12_Dxl: 0.79 *
  - TB12_Centaur: 0.75 **

HoloTC: Architect
Is the HoloTC assay better than the total B12 assays for assessing B12 status?

Answer: Yes.

- ROC curve:
  - HoloTC better than any of the 4 current TB12 assays
- HoloTC is better is some specific groups:
  - In pregnancy
  - Haematological conditions
  - Haptocorrin deficiency
HoloTC assay has been added to MBS

Medicare Benefits Schedule Book
Category 6
Operating from 01 November 2014

New item 66839
Quantification of vitamin B12 markers such as holoTranscobalamin or mehtylmalonic acid, where initial serum vitamin B12 result is low or equivocal
Question 2.

What is a low or equivocal total B12 range?

Markers:

- FBE: MCV, Hb
- MMA
- tHCY
- HoloTC
Roche Total B12 assay: ROC

MCV >98
MelbPath data

HCY >14 umol/L
MelbPath data, n=5,743
Adult, eGFR>60

MMA
Inter-lab comparison study
n=195
TB12 (Roche) for MMA ≥0.5 umol/L

Inter-lab comparison study, Nov 2014

TB12_Roche

AUC 0.79
P<0.001

110
(95% specificity)

170
(95% sensitivity)

270

N=195
95% sensitivity: 95% of people with B12 deficiency are below this cut-off.

95% specificity: 95% of the people without B12 deficiency are above this cut-off.
Roche assay

Normal

B12 deficiency

95% Specificity 110 pmol/L

95% sensitivity 270 pmol/L

Deficient

Not deficient

Total B12 – Roche (pmol/L)
HoloTC for predicting MMA $\geq 0.50$ umol/L

Central 95%:
25.1-165.0 pmol/L,
n=181 apparently healthy

$>$50 pmol/L: not deficient
$<$25 pmol/L: deficient

95% specificity
95% sensitivity

Active B12
AUC 0.87
$p<0.0001$
Answer to Question 3.

- **< 25 pmol/L:** B12 deficiency most probable
- **> 50 pmol/L:** B12 deficiency unlikely
- **Indeterminate**
**WHAT IS AN EQUIVOCAL SERUM TOTAL VITAMIN B12 CONCENTRATION?**

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**Introduction:** Medicare Australia has recently approved rebates for holotranscobalamin (HoloTC) or methylmalonic acid (MMA) as confirmatory tests when serum total B12 (TB12) is low or equivocal. We examined (1) How well do different TB12 assays agree? (2) Is HoloTC better than TB12 for diagnosis of B12 deficiency? (3) What are the equivocal TB12 ranges for the commonly used assays?

**Methods:** Serum samples (n=195) selected from routine TB12 requests were sent frozen to different laboratories for the analysis of HoloTC (Abbott Architect), MMA (GCMS) and TB12 using Roche (e602), Abbott (Architect), Siemens (Centaur) and Beckman (DXI800). B12 deficiency was defined as MMA>0.5 umol/L. For each of the aims: (1) All methods were compared with the Roche assay using Passing-Bablok and Bland-Altman plots; (2) Receiver Operating Characteristic (ROC) curve analysis was used to predict MMA>0.50 umol/L; (3) 95% sensitivity and 95% specificity values, respectively, obtained from ROC curves were used to indicate B12 sufficiency or deficiency and the limits of the equivocal range.

**Results:** (1) Abbott TB12 results were similar to Roche (Abbott=1.02Roche+2.6 pmol/L). Siemens TB12 were similar at values > 200 pmol/L (Siemens=0.85Roche+50 pmol/L) but appeared to be proportionally higher at values ≤200 pmol/L. Beckman-TB12 results were approximately 35% lower (Beckman=0.76Roche-20 pmol/L).

(2) The area under the ROC curve was significantly higher for HoloTC (0.87) than for all of the TB12 assays (Roche: 0.79; Architect: 0.81; Centaur: 0.75; Beckman: 0.79).

(3) The equivocal ranges from ROC curve analysis for TB12 (pmol/L) were: Roche 115-272, Abbott 121-268, Siemens 137-263 and Beckman 64-176. The equivocal range for HoloTC was 22-53 pmol/L, rounding to 25-50 pmol/L.

**Conclusion:** HoloTC has better diagnostic accuracy for vitamin B12 deficiency than current TB12 assays when increased plasma MMA is used as the reference point. Significant differences exist between different TB12 assays meaning that equivocal ranges are assay-dependent.
In Summary

- HoloTC has better sensitivity and specificity for assessing B12 status (not affected by haptocorrin levels)
  - It’s not used as a first line test yet
    - Limited availability and high cost

- TB12 assay differences exist. The low or equivocal range for the Roche TB12 is likely to be <270 pmol/L (by ROC curve using MMA≥0.5 umol/L)

- Current strategy of using TB12 + HoloTC in the low or equivocal range improving diagnostic accuracy.
  - HoloTC <25 pmol/L: most probably deficient
  - HoloTC >50 pmol/L: most probably sufficient
  - HoloTC 25-49 pmol/L: uncertain (~13% of all the B12 requests)
Acknowledgement

Dr Janet Warner, Mater Pathology and Dr Julia Chang, QML for coordinating the vitamin B12 comparison study

Dr Ken Sikaris and Dr Alan McNeil, Melbourne Pathology for helping with this presentation

Companies provided the reagents:

- Abbott diagnostics: Active B12 kits
- Siemens: Centaur total B12 kit

People and labs that participated in the inter-lab comparison study

- Dr George Streitberg, Dr Julie Newman & Dr Jim Doery, Monash Medical Centre
- Ms Donna Liong, QML
- Mr Laurence Hardy, Abbott Diagnostics
- Mr Andy Liu, Douglass Hanly Moir Pathology