Should holoTC be the first line diagnostic procedure for assessment of vitamin B12 status?

A comparison of the performance of blood indicators of tissue B12 status

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TRINITY COLLEGE DUBLIN

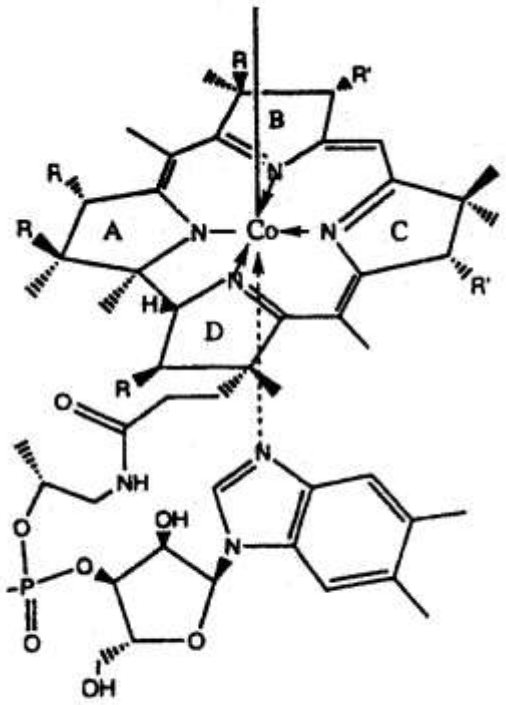
Introduction

- **Low blood vitamin B12 concentrations are common in the elderly. However, it is not clear what proportion of those with low B12 will eventually develop clinically relevant consequences.**

- **Poor sensitivity and specificity of assays to determine vitamin B12 status contribute to this confusion.**

- **Measurement of holoTC has been proposed as a better marker of B12 status than the total serum B12 level and is becoming more widely used**
  - **There are no standardized cut-offs for the different methods**
  - **None of the methods have been evaluated systematically**

X



β–axial ligand

tetra-corrin ring

α–axial ligand

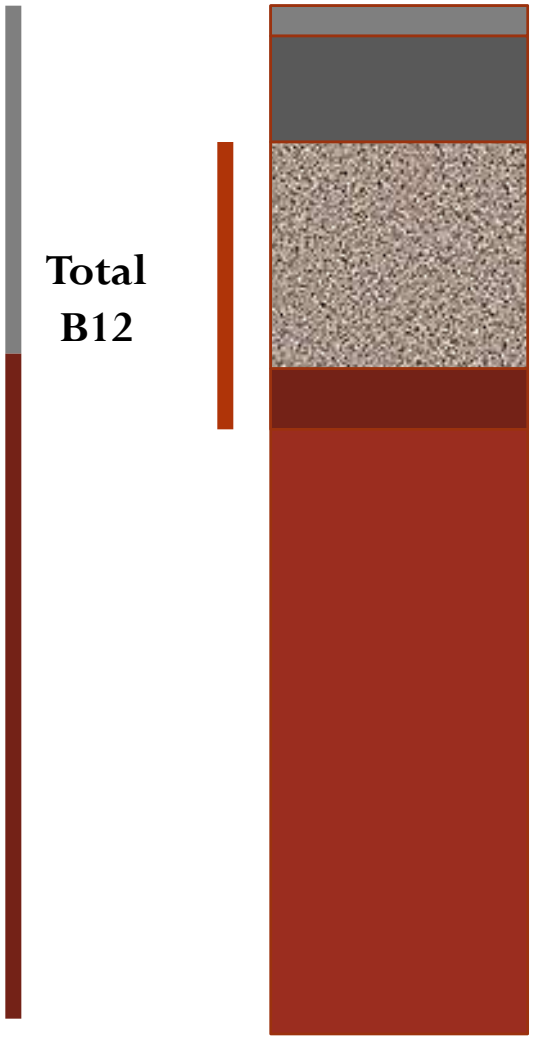
$R = CH_2CONH_2$

$R' = CH_2CH_2CONH_2$

X =

- OH: Hydroxycobalamin
- CH3: Methylcobalamin
- Ado: 5'Deoxyadenosylcobalamin
- CN: Cyanocobalamin
- GS: Glutathionylcobalamin

B12 binding proteins in blood



ApoHC (free haptocorrin– no ligand)

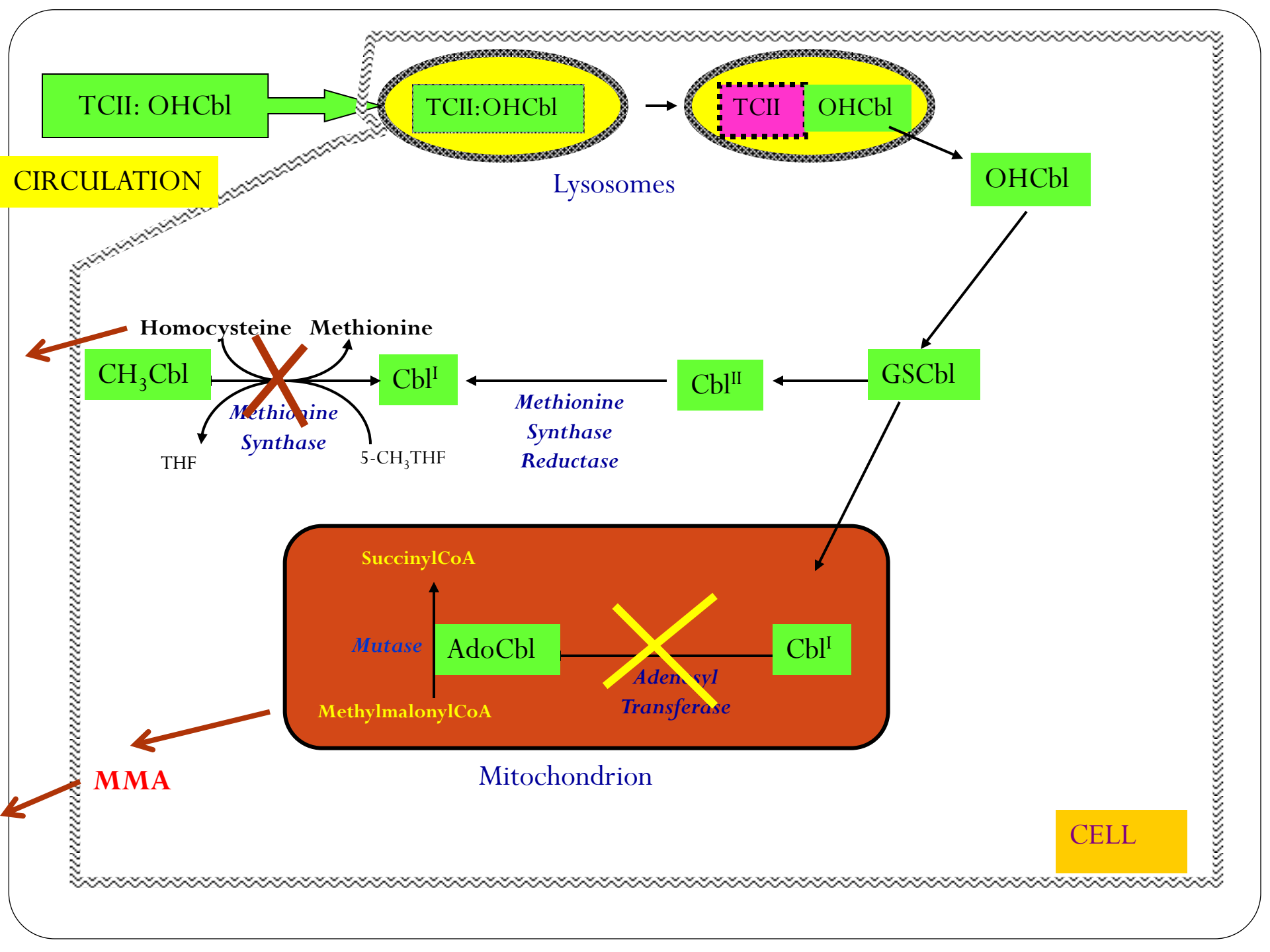
AnaHC (haptocorrin with inactive B12 analogues)

B12HC (haptocorrin with B12)

HoloTC (transcobalamin with B12)

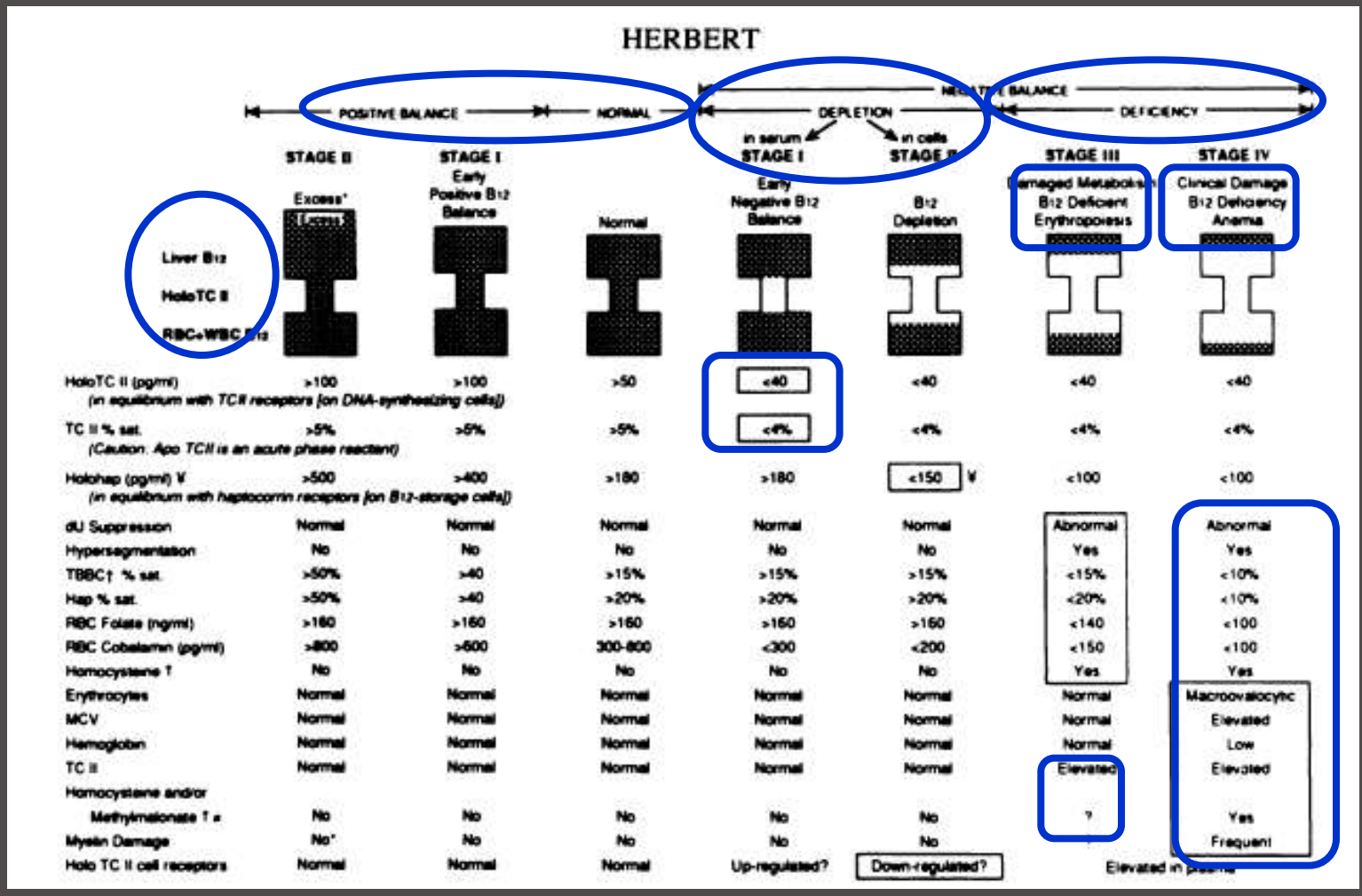
ApoTC (free transcobalamin-no ligand)

Adapted from Nexo and Hoffmann-Luke (2011)


TCII: OHCbl
TCII:OHCbl
TCII
OHCbl
CIRCULATION
Lysosomes
OHCbl
Homocysteine
Methionine
CH3Cbl
CblI
Methionine Synthase
THF
5-CH3THF
CblII
Methionine Synthase Reductase
GSCbl
SuccinylCoA
Mutase
AdoCbl
CblI
Adenosyl Transferase
MethylmalonylCoA
MMA
Mitochondrion
CELL

Staging vitamin B12 deficiency

Victor Herbert AJCN 1994



Laboratory tests to determine inadequate vitamin B12 status

- **Serum total B12**
  - agreement between methods poor
  - measures B12 bound to two circulating proteins – only one carries B12 for uptake
  - not reliable in mild to marginal B12 deficiency

- **Methylmalonic acid** (plasma MMA) – specific product of B12 metabolism
  - low levels considered to indicate normal status
  - high levels may be due to renal disease
  - assays not widely available

- **Homocysteine** (total plasma tHcy)
  - low levels probably compatible with normal status
  - high levels may be due to multiple causes - renal disease, folate deficiency, genotype interactions with riboflavin status, etc

- **HoloTC** carries about 20% of total circulating B12 and represents B12 destined for uptake by tissues

  – a reliable early marker of deficiency?

Current dilemma with early stage B12 deficiency

- **No unique clinical marker of disease**
  - Often absence of classical symptoms –anemia, macrocytosis,etc
  - Often diffuse non-specific neurological symptoms
  - Elderly patients often have confounding neurological symptoms due to other causes
- **Forced to choose a surrogate marker**
  - MMA – functional marker
    - Very high specificity in ruling out overt disease
    - Very high sensitivity if elevation is large and renal dysfunction is ruled out.
  - Red cell B12 – tissue status marker

The Performance of a Diagnostic Test:

The ability of the test to diagnose the true patient status

Disease Status (surrogate)	Test Result	
	Abnormal	**Normal**
Disease	**True Positive**	**False Negative**
No Disease	**False Positive**	**True Negative**

Disease Status	Test Result		
	Abnormal	Normal	
Disease	True Positive (a)	False Negative (c)	All people with disease (a+c)
No disease	False Positive (b)	True Negative (d)	All healthy people (b+d)
	All people with abnormal tests (a+b)	All people who tested normal (c+d)	

Disease based information:

Sensitivity: proportion of disease patients correctly identified by the test (a/a+c)

Specificity: proportion of healthy people correctly identified by the test (d/b+d)

Test based information:

Positive Predictive Value (PPV): proportion of people with positive test that are correctly diagnosed (a/a+b)

Negative Predictive Value (NPV): proportion of people negative test that are correctly diagnosed (d/c+d)

Aims of EU Project

- To compare the performance of holoTC against total B12 in detecting:
  - 'definite' B12 deficiency (MMA≥0.75 µmol/L)
  - 'probable' B12 deficiency (MMA≥0.45 µmol/L)

Based on

- Three available monoclonal holoTC immuno-assays
  - Commercial RIA (Axis Shield Diagnostics)
  - In-house ELISA assay (Aarhus University Hospital)
  - Automated ELISA assay on Abbott AxSym

- Three methods to measure total B12;
  - Access (Beckman) immuno-assay
  - Advia Centaur (Bayer) immuno-assay
  - L. Leichmannii microbiological assay.

Study subsets used in the systematic evaluation of holoTC versus total B12

	Banbury N=1002 Median (IQR)	OHAP N=1649 Median (IQR)	Aarhus N=934 Median (IQR)	Belfast N=1160 Median (IQR)
Final comparison set	613 (61%)	1052 (64%)	708 (76%)	915 (79%)

The final study set consisted of:

Those with B12 < 1000 pmol/L AND

Complete data for all assays carried out in the study AND

Unimpaired renal function

(creatinine <120 µmol/L for males and <97 µmol/L for females).

Vitamin B12 measurements (pmol/L)

	Banbury N=1002 Median (IQR)	OHAP N=1649 Median (IQR)	Aarhus N=934 Median (IQR)	Belfast N=1160 Median (IQR)
Access (Beckman)	216 (127)	-	-	207 (133)
Advia Centaur (Bayer)	238 (126)	251 (122)	279 (140)	243 (125)
Microbiological Assay *L. Leichmannii** *Colistin sulphate resistant	-	-	-	257 (163)

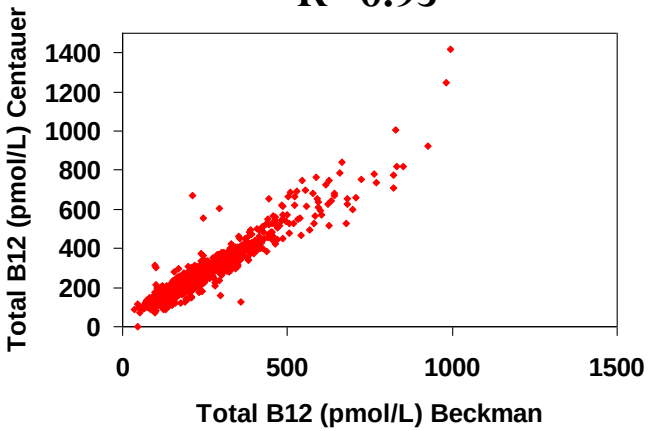
All populations have a B12 measurement by Advia Centaur
One population has a B12 measurement by all three methods

HoloTC measurements (pmol/L)

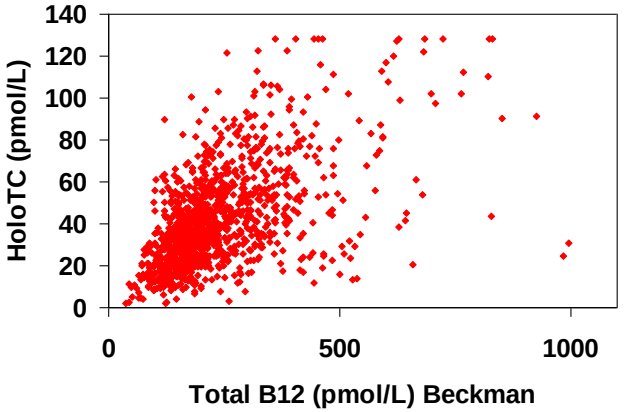
	Banbury N=1002 Median (IQR)	OHAP N=1649 Median (IQR)	Aarhus N=934 Median (IQR)	Belfast N=1160 Median (IQR)
Commercial RIA	65 (47)		56 (44)	
ELISA-I In-house	-	65 (45)	62 (44)	-
ELISA-II (AxSym)	-	-	-	38 (28)

Correlations between assays

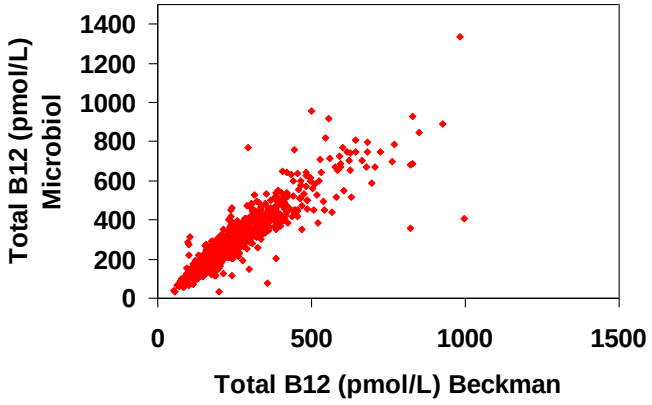
R=0.95



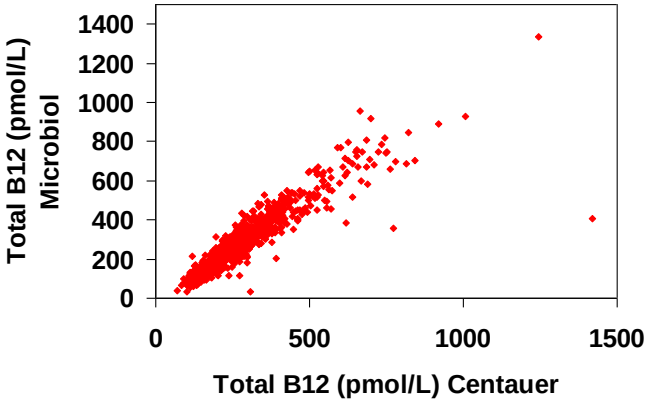
R=0.57



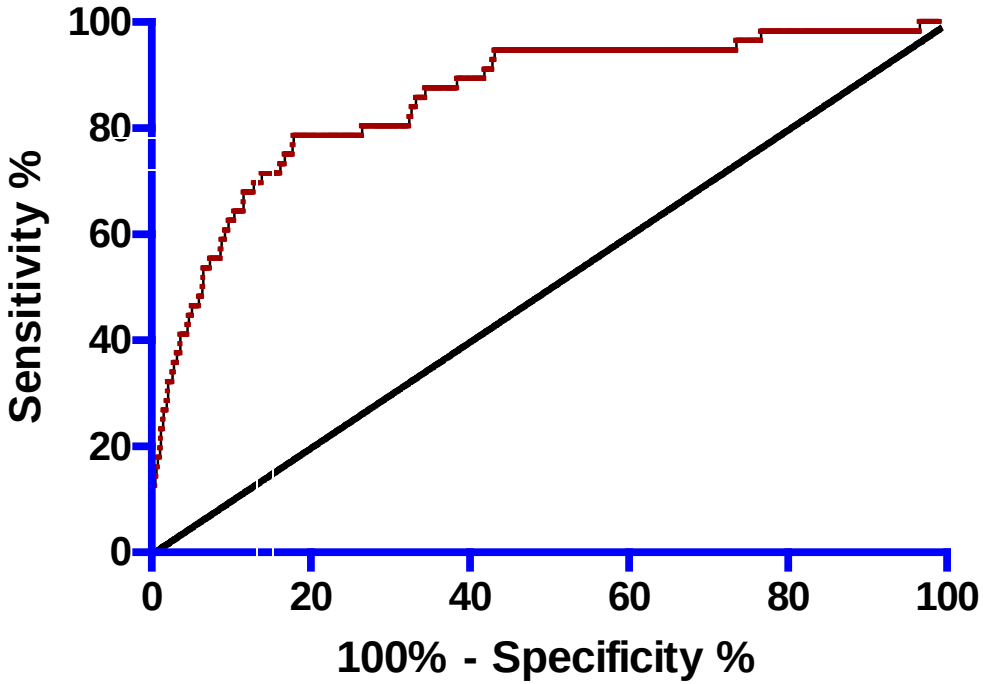
R=0.90



R=0.91

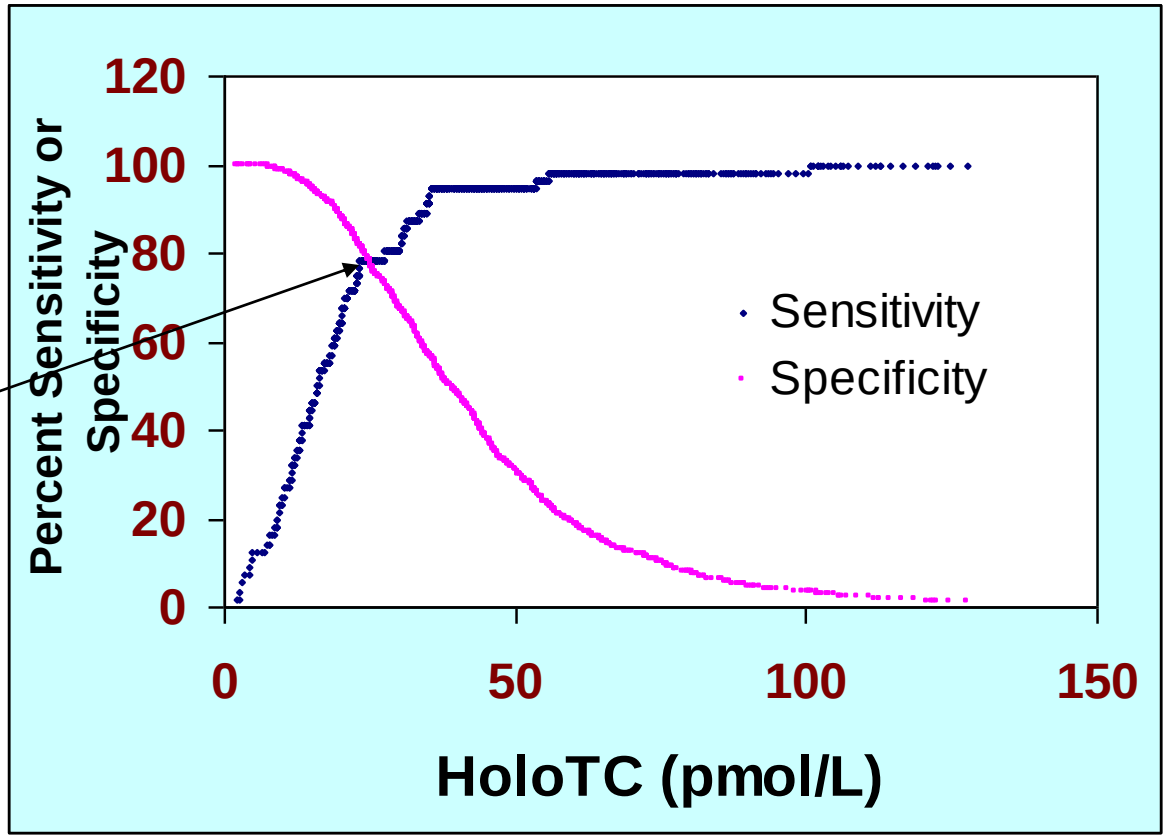


Example of a ROC Curve for holoTC with serum MMA≥0.75 µmol/L as the definition of severe vitamin B12 deficiency



Area under the curve (AUC) is used as a measure of performance

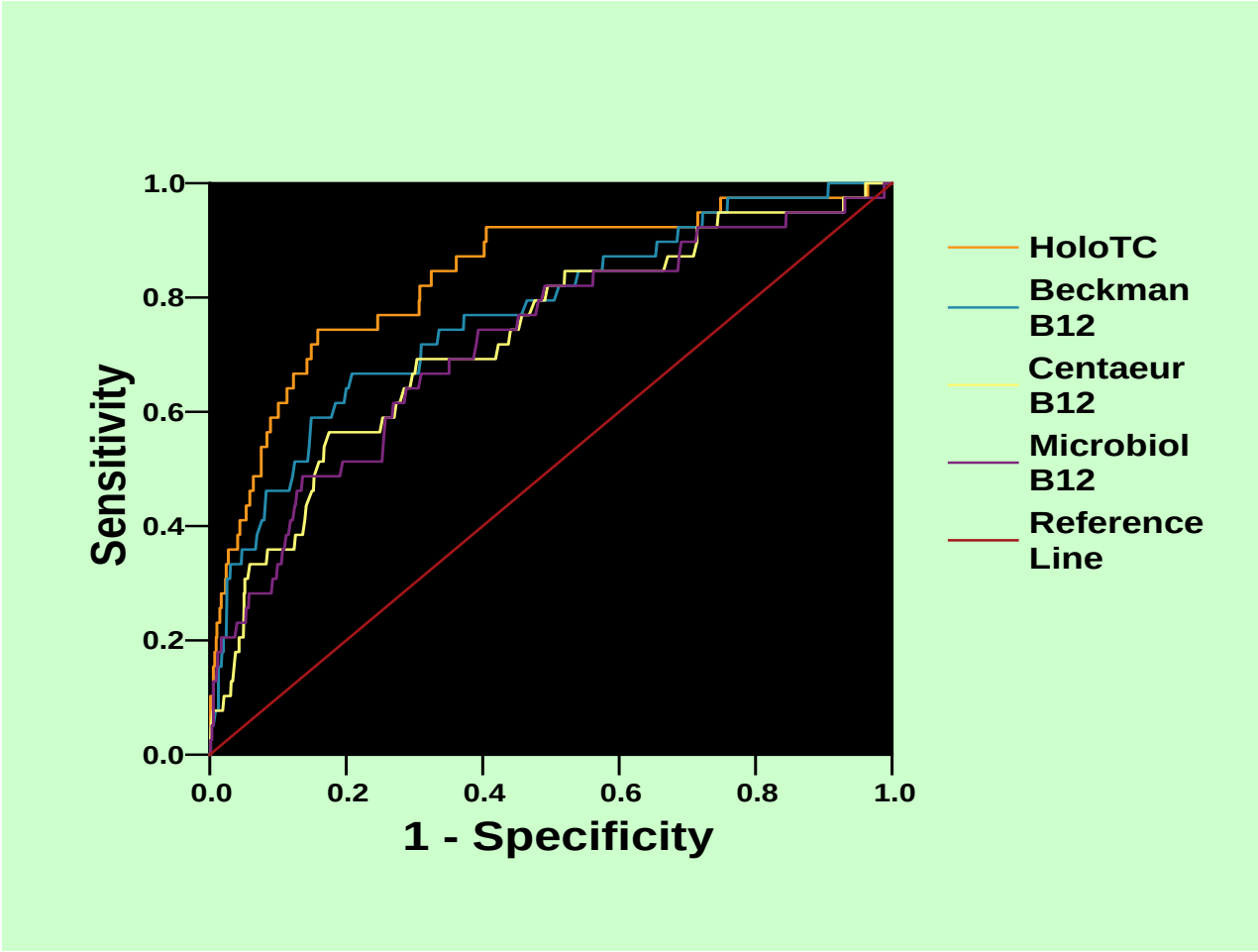
ROC curve derived sensitivity and specificity of holoTC to detect MMA>0.75 µmol/L



Sensitivity – Ability of the test to detect true positives

Specificity – Ability of the test to detect true negatives

ROC Curve with serum MMA≥0.75 as the marker of disease



ROC curve AUCs for detecting B12 deficiency defined as MMA≥0.75 µmol/L in evaluation subsets

	Banbury N=613 AUC (SE)	OHAP N=1052 AUC (SE)	Aarhus N=889 AUC (SE)	Belfast N=915 AUC (SE)
HoloTC RIA	0.91(0.03)	-	0.81 (0.04)	-
HoloTC ELISA-I	-	0.84 (0.04)	0.78 (0.05)	-
HoloTC ELISA-II	-	-	-	0.84 (0.05)
B12 Beckman	0.82 (0.05)			0.76 (0.05)
B12 Centaur	0.84 (0.05)	0.76 (0.04)	0.65 (0.03)	0.72 (0.05)
B12 Micro	-	-	-	0.72 (0.05)

Summary

- HoloTC was superior to assays for total B12, irrespective of which method was used to measure holoTC or total B12.
- Irrespective of whether B12 or holoTC are used, there were more false positives than true positives using MMA as the 'diagnostic gold standard'
- The utility of MMA as a gold-standard has limitations
  - High prevalence of 'unexplained' moderately elevated MMA in older persons
  - Correlated with creatinine and other markers of renal status

Red cell B12 – a surrogate for general tissue status

- Easily obtained
- Relevant to B12 status
  - changes occur in severe B12 deficiency
  - B12 treatment causes an increase in intracellular B12
  - Corresponds to reticulocyte response
- Reflects average B12 status over lifetime of erythrocytes (similar to red cell folate)

Studies on Erythrocyte B12

Study	Method	Mean [SD] pg/mL	95% Reference Range	N Reference population
Harrison 1969	L.Leichmannii	155 [35]	85-225	256
Omer et al 1970	E. gracilis	158 [31]		29
Santini & Millan 1976	L.Leichmannii	185 [54]		13
Tisman et al 1991	Ciba Corning Radioassay	241 [51]		20
Valente et al 2011	L.Leichmannii	134 [48]	48-228	118

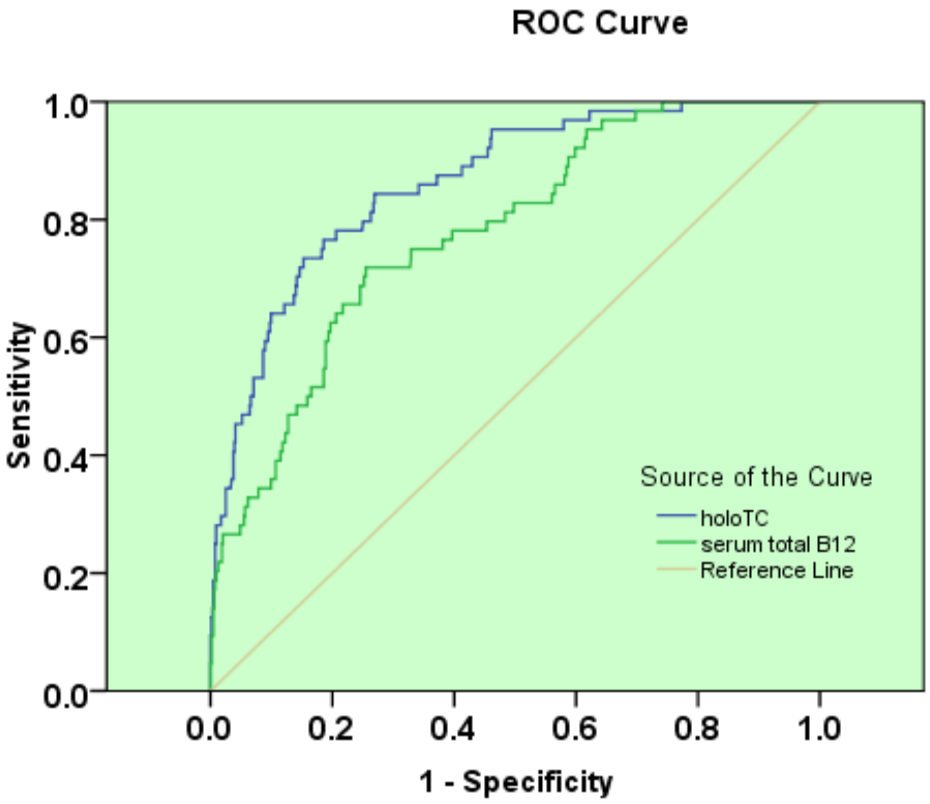
Reference Intervals for vitamin B12 status markers in healthy subjects

	N	Lower limit	Upper limit
holoTC (pmol/L)	119	20.1 (17.8 to 22.7)	124.6 (110.5 to 140.4)
Serum total B12 (pmol/L)	119	123 (111 to 136)	582 (525 to 645)
Red cell B12 (pmol/L)	118	33.0 (24.5 to 41.4)	161.6 (153.1 to 170.1)

Demographic details of the study population

Variable	N	Mean	2.5 to 97.5 percentile	% abnormal (cutoff value)
Age, years	700	81	69-92	
Females, n (%)	490 (70)			
Hematocrit	699	0.386	0.304-0.470	
Hemoglobin (g/dL)	699	12.5	9.4-15.4	
MCV (fL)	699	91.6	79.0-102.9	
Red cell cobalamin (pmol/L)	700	64	21-161	9.6 (<33)
holoTC (pmol/L)	699	47	11-171	8.1 (<20)
Serum total cobalamin (pmol/L)	700	254	83-674	8.0 (<123)
Serum folate (nmol/L)	697	30[a]	7-360	1.7 (<6.8)
Red cell folate (nmol/L)	693	1003	371-3077	1.4 (<340)
MMA (µmol/L)	700	0.347	0.145-1.511	41.7 (>0.36)
tHcy (µmol/L)	700	16.3	9.1-34.0	52.2 (>15.0)
Creatinine (µmol/L)	699	89	51-181	
eGFR(CG) (ml/min)	698	48	18-108	

Performance of holoTC against a disease criterion of MMA>0.75 µmol/L

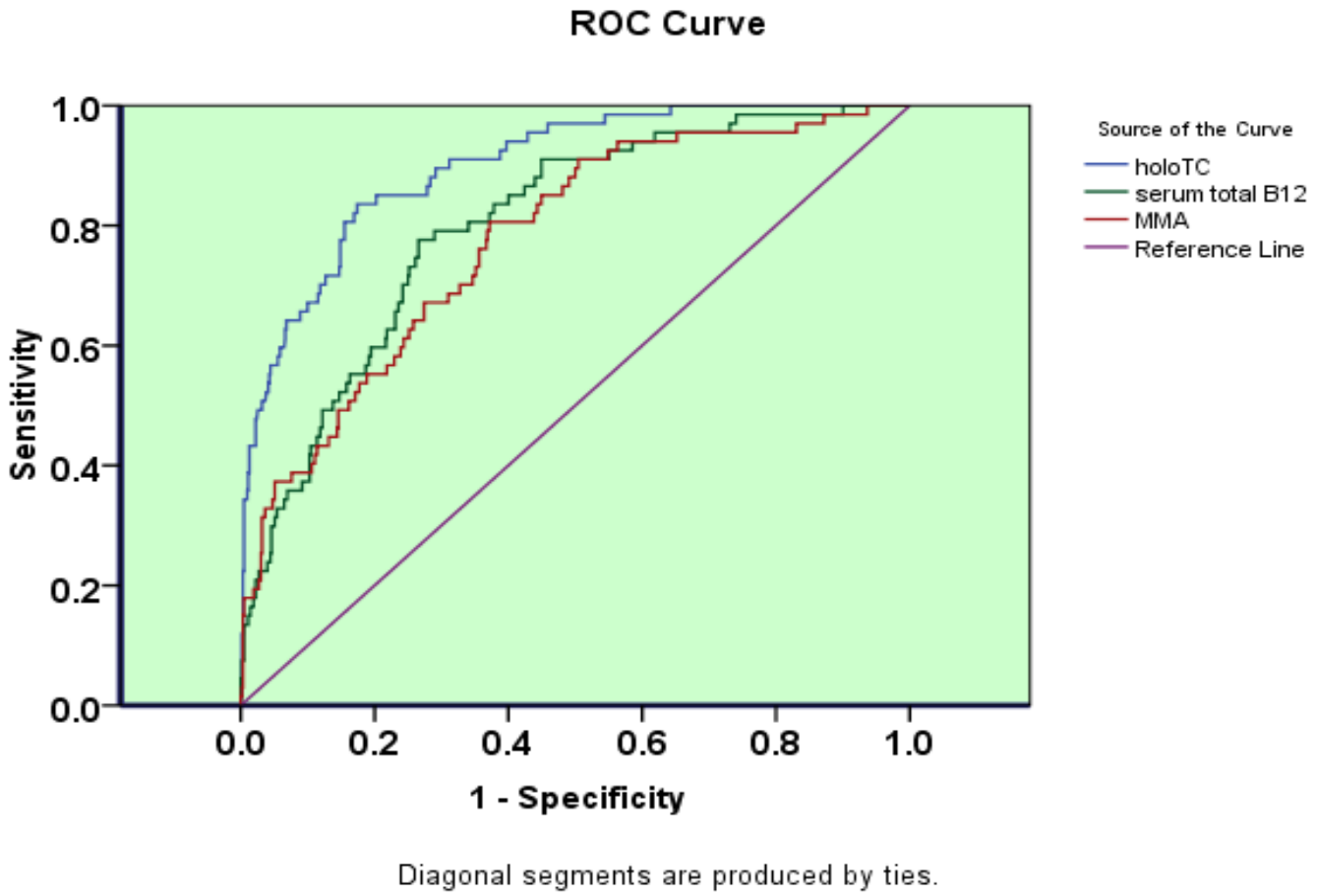


AUC

HoloTC=0.86

Serum B12=0.78

Performance of holoTC, total B12 and MMA in predicting a red cell B12 < 33 pmol/L



AUCs for metabolites using a disease criterion of red cell B12<33 pmol/L

Variable	AUC	95% CI	AUC>0.5, p
HoloTC	0.899	0.862 to 0.936	<0.0001
Serum B12	0.801	0.749 to 0.853	<0.0001
MMA	0.776	0.719 to 0.833	<0.0001
tHcy	0.747	0.688 to 0.806	<0.0001
Red cell folate	0.712	0.647 to 0.778	<0.0001
Serum folate	0.607	0.533 to 0.680	0.002
Hemoglobin	0.582	0.506 to 0.659	0.017
Hematocrit	0.545	0.469 to 0.620	0.122
MCV	0.545	0.468 to 0.622	0.127

Disease Status	Test Result		
	Abnormal	Normal	
Disease	True Positive (a)	False Negative (c)	All people with disease (a+c)
No disease	False Positive (b)	True Negative (d)	All healthy people (b+d)
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Diagnostic performance of status markers at a single cut-off

Marker	cutoff	Sensitivity %	Specificity %	PPV%	NPV%
holoTC pmol/L	<20	55 (43 - 67)	96 (94-97)	56 (45 -70)	95 (93 - 97)
Serum B12 pmol/L	<123	33 (22 - 45)	95 (93 -96)	39 (26 - 53)	93 (91 - 95)
MMA µmol/L	>0.36	81 (69 - 89)	63 (59 - 66)	19 (14 - 24)	97 (95 - 98)
MMA	**>0.75**	**38**	**94**	**37**	**94**

MMA has low specificity

B12 has low sensitivity

Both have low PPV

Three-zone decision thresholds for holoTC, serum cobalamin and MMA calculated for a PPV of 60% and a NPV of 98%.

Marker	Threshold levels	Sens %	Spec %	True +ve	True -ve	False +ve	False -ve	PPV%	NPV %
holoTC (pmol/L)	19.6	50.7	96.4	34	609	23	33	60	95
	29.9	80.6	84.5	54	534	98	13	36	98
Serum B12 (pmol/L)	79	13.4	99.1	9	627	6	58	60	92
	238	86.6	57.3	58	363	270	9	18	98
MMA (µmol/L)	1.402	17.9	98.6	12	625	8	55	60	92
	0.310	88.1	50.9	59	322	311	8	16	98

Grey Zone: holoTC 96 (13.7%); serum B12 313 (44.7%); MMA 349 (49.8%)

Effect of impaired renal function on performance of B12 markers to detect red cell B12<33 pmol/L

eGFR(CG) range	Marker	PPV %	NPV%	Mean conc.
≥60, n=226	holoTC	66 (41-84)	95 (92-98)	45 (41-49)
	Serum B12	31 (12-54)	92 (88-95)	246 (229-264)
	MMA	23 (13-34)	95 (91-98)	0.295 (0.275-0.316
30-59, n=378	holoTC	47 (30-65)	94 (91-96)	47 (44-50)
	Serum B12	44 (26-62)	94 (91-96)	250 (235-266)
	MMA	20 (14-27)	97 (94-99)	0.343 (0.325-0.362)
15-29, n=94	holoTC	78 (40-97)	99 (93-100)	51 (43-60)
	Serum B12	50 (7-93)	93 (86-98)	295 (247-351)
	MMA	12 (5-22)	97 (94-99)	0.531 (0.457-0.616)

Conclusions

- HoloTC is a significantly better marker of low tissue B12 status than other blood indices
- HoloTC is a significantly better marker of impaired metabolic status than other blood indices
- More data are needed on deficiency cut-off levels and strategies to follow-up on 'grey area' results

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