06/09/2022

Methylmalonic acid as indicator of cobalamin deficiency

ASO Lieselot Dedeene

Supervisor: Dr. Apr. Kathleen Croes, Prof. Apr. Nico Callewaert

Vitamine B12 deficiëntie … of niet?

- Vrouw, 27 jaar
- **Spoedopname**
  - Tintelingen kuiten en voeten, progressief verminderde sensibiliteit voeten, verminderd evenwicht, gangstoornissen, paresthesie thv armen, handen,…
  - Subacute polyneuropathie – oorzaak?
- **Technische onderzoeken**
  - CT hersenen: geen bijzonderheden
  - NMR hersenen: geen argumenten voor demyeliniserende pathologie
  - NMR full spine: beeld suggestief voor gecombineerd strenglijden obv vitamine B12 deficiëntie, koperdeficiëntie of infectieuze serologie (cf. neurosyfillis, HIV)
  - Differentiaal diagnoses: Guillain-Barré syndroom, myelitis, atypische presentatie multiple sclerosis
- **Verloop**
  - Eénmalig 1000 mg B12 intramusculair: goed effect op klachten

→ **Normale B12 in serum** kan gepaard gaan met **klinische B12 deficiëntie** met uitgesproken symptomen!

Bepaling	Waarde
SEDS	24 mm/h
RBC	4.16 x10*12/L
HB	14.0 g/dL
HCT	40.2 %
MCV	96.6 fL
MCH	33.7 pg
MCHC	34.8 g/dL
RDW	15.1 %
WBC	6.86 x10*9/L
PLT	259 x10*9/L
MPV	10.6 fL
IG %	0.6 %
LYMF	36.9 %
MONO	9.3 %
NRBC	0.0 /100 WBC
EO_Abs	0.11 x10*9/L
BASO_Abs	0.02 x10*9/L
LYMF_Abs	2.53 x10*9/L
SEGM_abs	3.52 x10*9/L
MONO_Abs	0.64 x10*9/L
IG ABS	0.04 x10*9/L
FE	65.5 µg/dL
B12	353 ng/L
Foliumzuur	12.5 µg/L

Hcy: 41.4 µmol/L (ref: 5-15)
MMA: 31.53 µmol/L (ref. 0.39)

B12 ref < 200 ng/L

Vitamin B12 = cobalamin

Daily B12 requirement

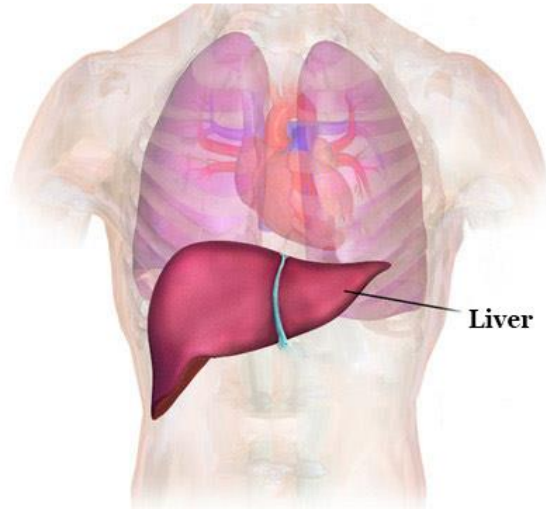
2-3 µg

<<<

Body storage

2-5 mg → 1 mg in liver

~ daily metabolic requirement of 2000 days



Short periods of insufficiency are covered

Persistent **long-term** cause: cobalamin deficiency

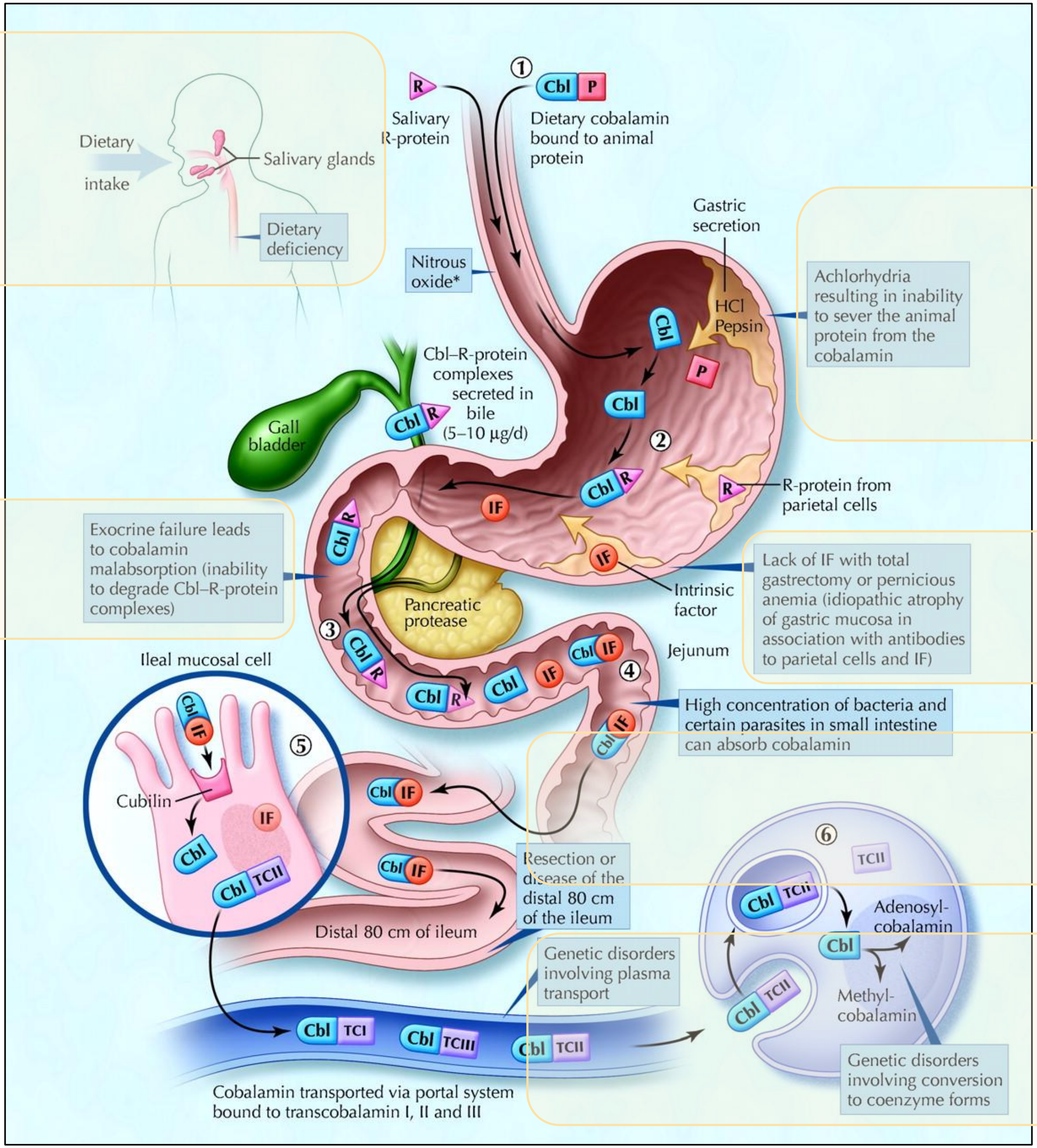
- Chronic alcohol consumption
- Vegetarian or vegan diet
- General malnutrition

- Chronic atrophic gastritis
- PPI, histamine receptor 2 antagonists
- Antacids

- Pancreatic disease
- Pancreatectomy

- Pernicious anaemia

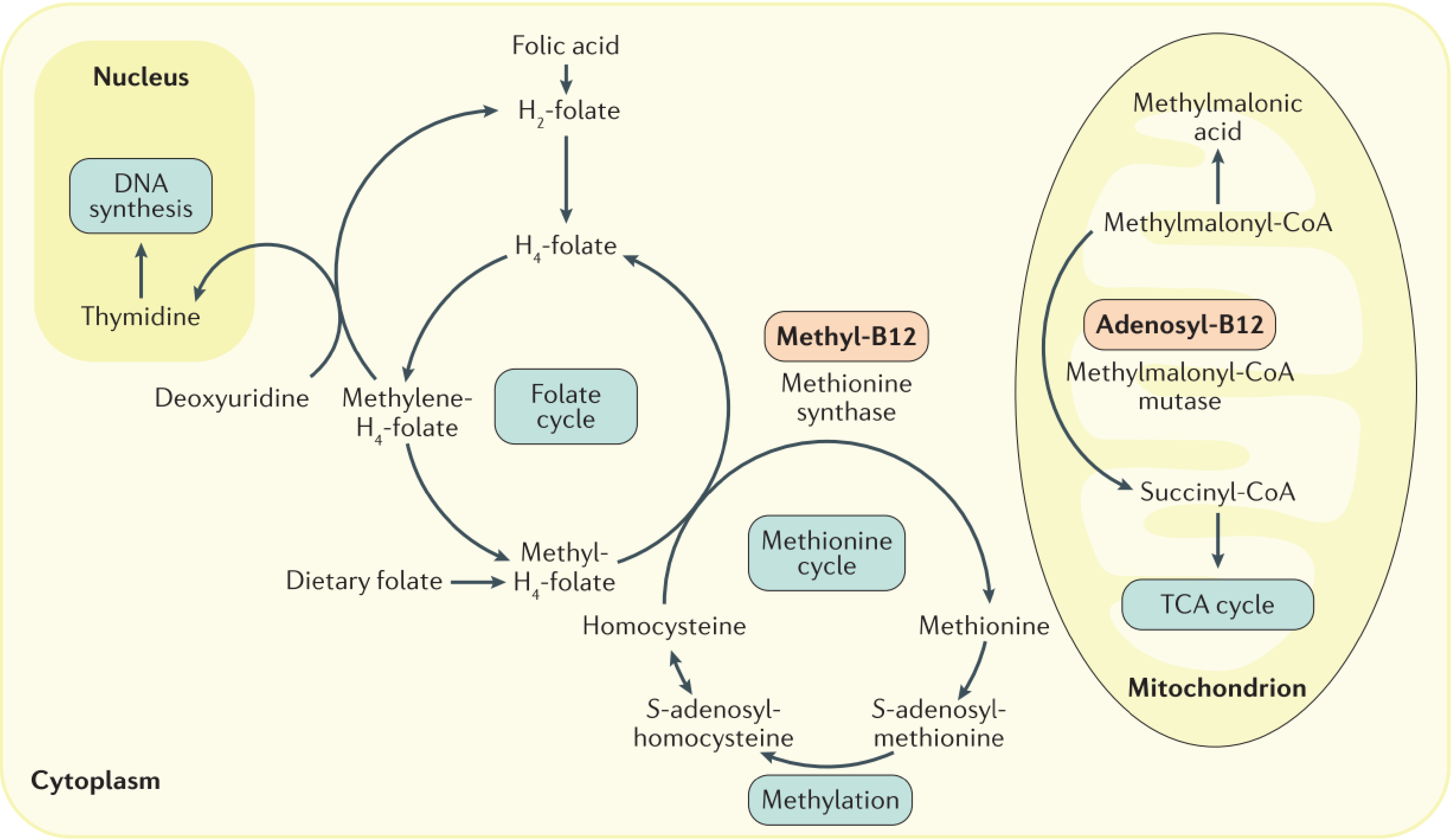
- Inflammatory bowel disease, coeliac disease, ileal resection and bacterial overgrowth



2 transportereiwitten
- Haptocorrin: 80-94%
- Transcobalamin: 6-20% = active!

- Inherited disorders
- Chemical inactivation of B12

B12 metabolism and function



B12 deficiency

Clinical B12 deficiency

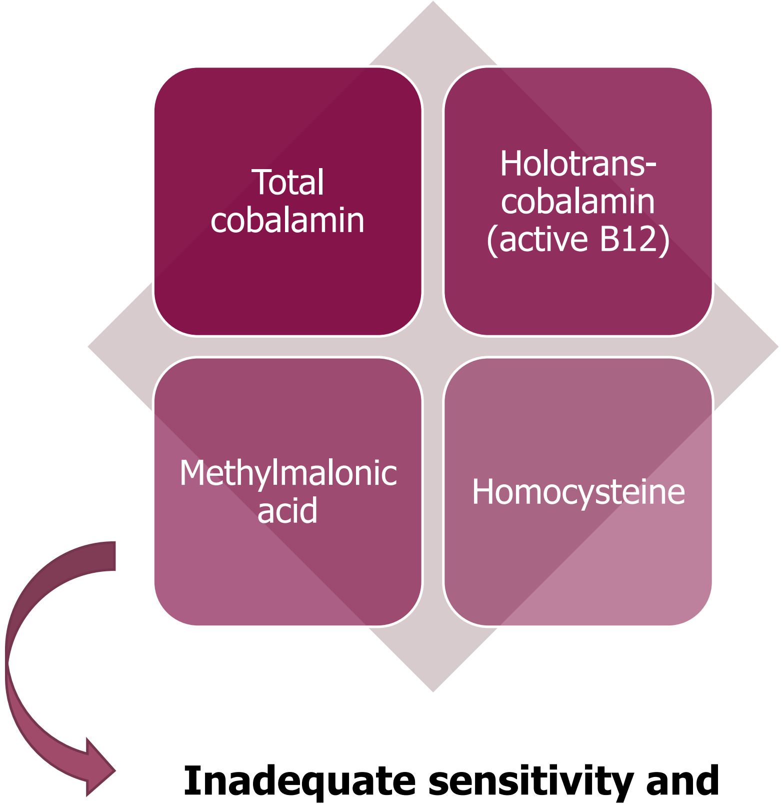
Haematological symptoms: defective DNA synthesis

- Macrocytosis
- Hypersegmentation

Neurological symptoms: demyelination of peripheral and central neurons

- Sensory and motor disturbances
- Spasticity and paralysis
- Cognitive decline
- Potentially **irreversible!**

→ Timely diagnosis and treatment is paramount to prevent irreversible damage!



Inadequate sensitivity and specificity of stand-alone markers
→ combination of min. two markers?

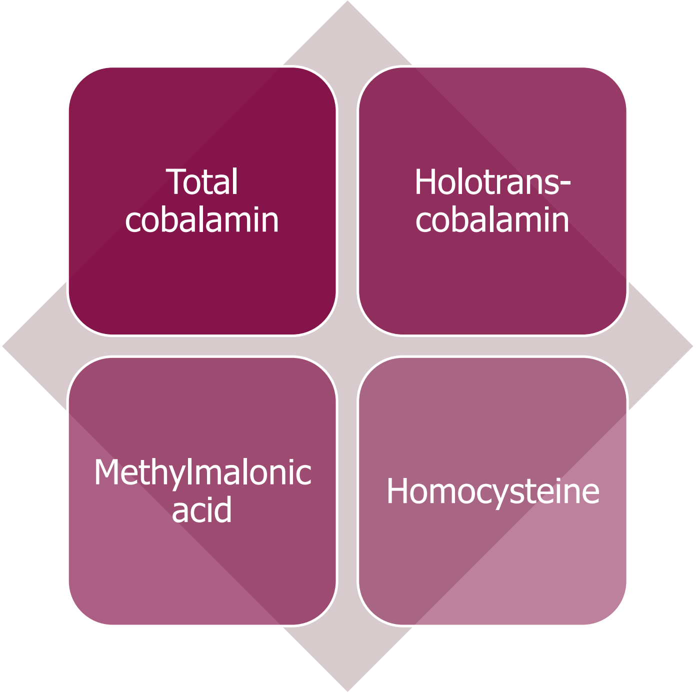
Subclinical B12 deficiency (SCCD)

- Sometimes **non-characteristic symptoms**
- Clinical impact of SCCD and the progression rate towards a clinical deficiency remains to be elucidated!

Research questions

1. **Which biomarkers** can be used in the diagnosis of cobalamin deficiency?

2. **Is it possible to improve the diagnostic process of cobalamin deficiency** by implementing the analysis of methylmalonic acid in the clinical laboratory of AZ Groeninge?



Total cobalamin (total vitamin B12)

Measurement

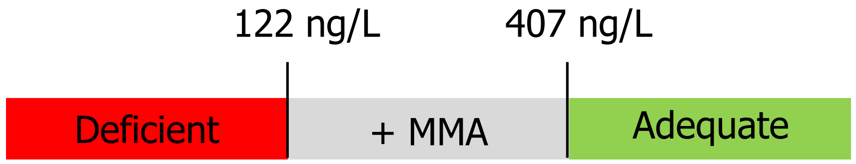
- immobilized nonhuman IF on beads or magnetic particles
- B12 is released from the transporter proteins HC and TC

(+) Advantages

- Highly accessible
- Easy-to-use
- Reimbursed in Belgium

(-) Disadvantages

- Total cobalamin = active (~20%) + INACTIVE fraction (~80%)
- WHO: < 203 ng/L = deficient
- Inadequate sensitivity and specificity around the presumed cut-off level (200 ng/L)



Holotranscobalamin (active B12)

Measurement

- Sandwich immunoassay
- Fraction cobalamin bound to transcobalamin (= the active fraction)

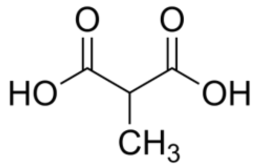
(+) Advantages

- Represents the "**active**" fraction

(-) Disadvantages

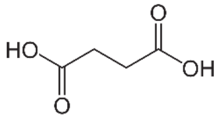
- Influenced by the **daily influx** from the gut/**recent absorption of B12**
- Contradicting findings in literature:
  - Sensitivity & specificity
  - Correlation total cobalamin and holoTC
  - Varying cut-off levels
- Not reimbursed in Belgium

Methylmalonic acid



Measurement

- GC-MS, LC-MS/MS
- Structural isomer succinic acid



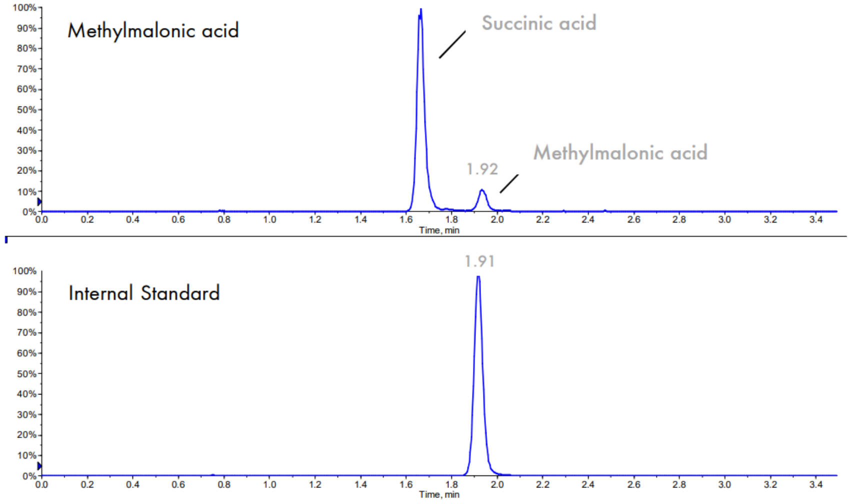
(+) Advantages

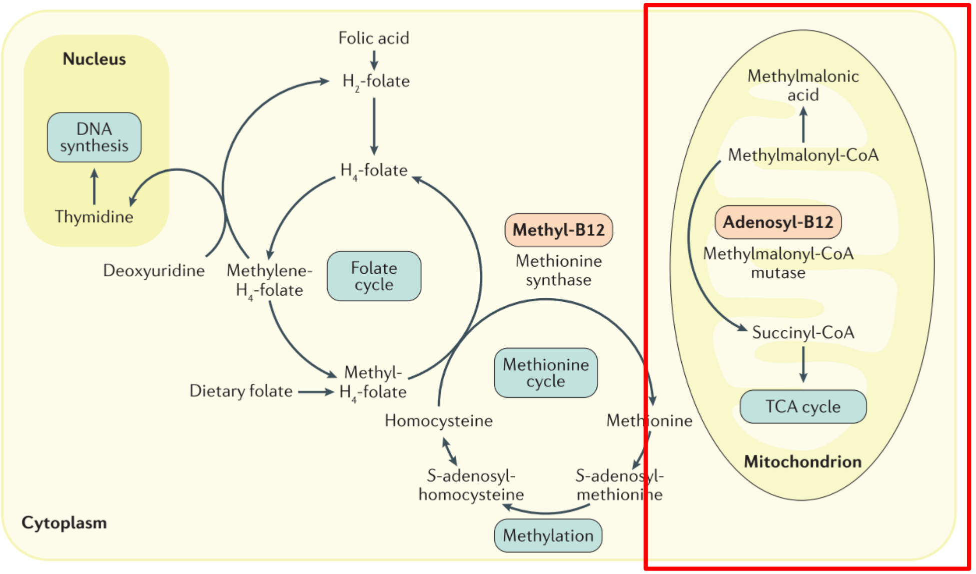
- **Sensitive**, functional marker
- **Specific** marker (<-> homocysteine)

(-) Disadvantages

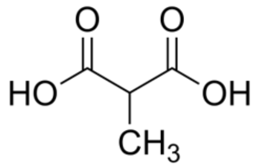
- Increasing **age** → higher MMA levels
- Impaired **renal function** → higher MMA levels
- Other influencing factors
- Not reimbursed in Belgium

different mechanism



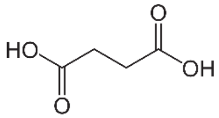


Methylmalonic acid



Measurement

- GC-MS, LC-MS/MS
- Structural isomer succinic acid



(+) Advantages

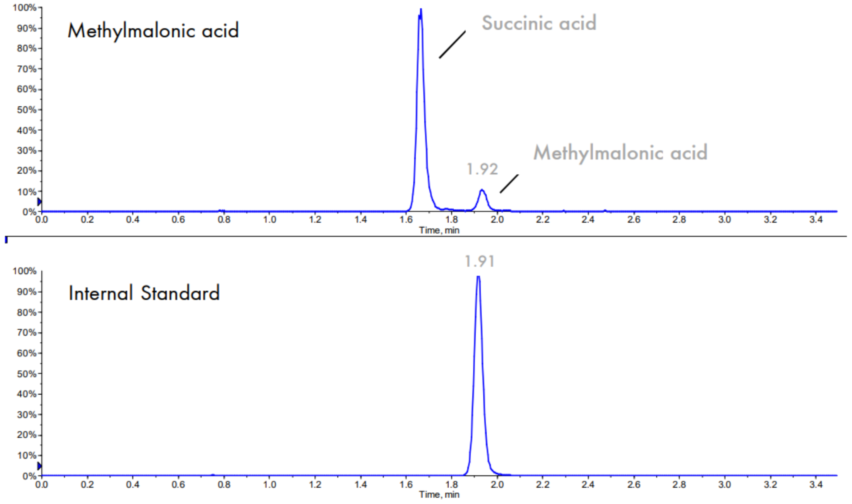
- **Sensitive**, functional marker
- **Specific** marker (<-> homocysteine)

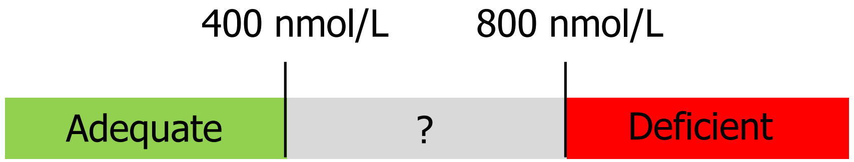
(-) Disadvantages

- Increasing **age** → higher MMA levels
- Impaired **renal function** → higher MMA levels

different mechanism

- Other influencing factors
- Not reimbursed in Belgium





Methylmalonic acid: reference ranges

TABLE 1 Selected published reference intervals for serum or plasma MMA[1]

Authors (reference)	Population	Central 0.95 reference interval, nmol/L	Additional information
Allen et al. (11)	Healthy US middle-aged adults	73–271	Men and women, $n = 50$; 18–65 y
Rasmussen et al. (12)	Healthy Danish middle-aged adults	50–370	Men and women, $n = 58$; 40–68 y (median: 53 y)
Rasmussen et al. (4)	Healthy Danish middle-aged adults before and after vitamin B-12 supplementation for 1 wk (in a few cases for 2 wk)	80–280	Men ($n = 109$) and women ($n = 126$); 20–84 y (men; median: 50 y) and 20–85 y (women; median: 49 y); all but 1 subject had plasma creatinine concentrations within the reference interval for healthy subjects
Joosten et al. (5)	Healthy Belgian, Dutch, and German middle-aged adults	62–247	Men and women, $n = 99$; 19–55 y (mean: 30 y)
	Healthy Dutch elderly living at home	72–476	Men and women, $n = 64$; 65–88 y (mean: 76 y); no participant had creatinine clearance <30 mL/min
	Healthy German elderly after B-vitamin supplementation for 3 wk	55–278	Men and women, $n = 143$; 65–96 y (mean: 75 y); no participant had creatinine clearance <30 mL/min
Lewerin et al. (13)	Swedish elderly with and without B-vitamin supplementation		Men and women, $n = 209$; 70–88 y (women) and 70–93 y (men) (overall median: 76 y)
	Total study group at baseline	110–480	$n = 208$
	Healthy elderly at baseline	120–380	$n = 123$
	Healthy elderly after B-vitamin supplementation for 4 mo	20–340	$n = 78$ (vitamin B-12–replete)
Milman et al. (14)	Healthy Danish pregnant women		Women ($n = 434$) with a normal pregnancy ≥37 wk
	18 weeks of gestation	40–290	$n = 413$
	32 weeks of gestation	50–340	$n = 390$
	39 weeks of gestation	60–360	$n = 250$
	8 wk postpartum	80–350	$n = 160$
Vogiatzoglou et al. (10)	Norwegian middle-aged adults		Men and women, $n = 3684$; 47–49 y
	Unselected	100–320	$n = 3684$
	Vitamin B-12 ≥200 pmol/L	100–300	$n = 3568$
	Vitamin B-12 ≥400 pmol/L	100–280	$n = 1306$ (vitamin B-12–replete)
	Norwegian elderly		Men and women, $n = 3262$; 71–74 y
	Unselected	110–490	$n = 3262$
	Vitamin B-12 ≥200 pmol/L	110–410	$n = 3043$
	Vitamin B-12 ≥400 pmol/L	100–360	$n = 1058$ (vitamin B-12–replete)
Erdogan et al. (6)	Healthy US adults	60–360	Men ($n = 16$) and women ($n = 24$)
	US persons tested for MMA (unknown clinical history)		Males and females ($n = 4944$); highest 10% of results disregarded (potentially unhealthy persons)
	0–10 y	0–510	$n = 28$
	11–20 y	30–260	$n = 39$
	21–30 y	50–330	$n = 165$
	31–40 y	50–400	$n = 287$
	41–50 y	50–400	$n = 545$
	51–60 y	50–420	$n = 813$
	61–70 y	50–440	$n = 918$
	≥71 y	50–480	$n = 2149$

[1] MMA, methylmalonic acid.

Mineva et al, Am J Clin Nutr, 2019

Methylmalonic acid: correction for eGFR

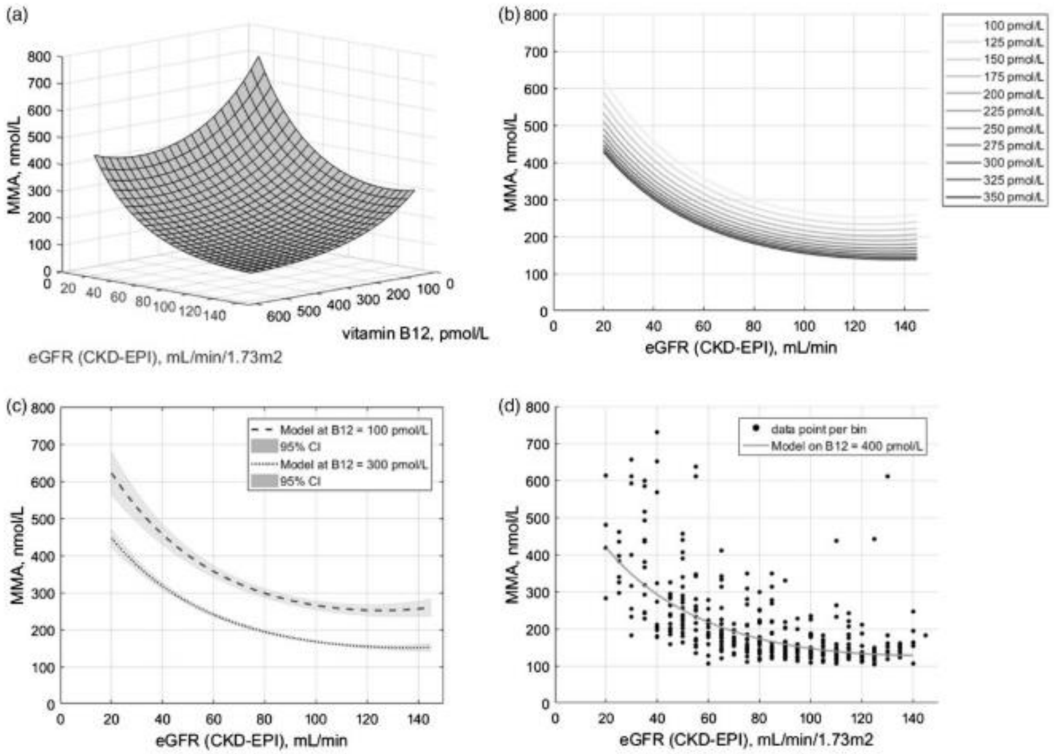


Figure 2. Model. (a) 3D representation of the model plotted against vitamin B12 and eGFR, showing a curved plane (mean adjusted R^2 0.46 ± 0.024 (2SD)). (b) 2D representation of the model, plotted against eGFR at different concentrations of vitamin B12. (c) 95% confidence interval (CI) at different concentrations of vitamin B12 plotted against eGFR. (d) Isolated effect of renal function on MMA plotted together with binned data of only vitamin B12 non-deficient patients, i.e. vitamin B12 >300 pmol/L (n=392). This representation showed the model is in agreement with the data (MAE = 58 nmol/L).
MMA: methylmalonic acid; eGFR: glomerular filtration rate.

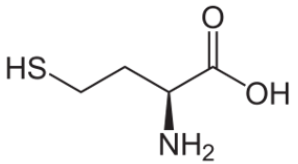
$$MMA_{adj} = MMA_{obs} - \left(\widehat{MMA}(B12,\ eGFR) - \widehat{MMA}(B12,\ 121)\right)$$

$$\widehat{MMA}(B12, eGFR) = \exp(\beta_0 + \beta_1 \cdot B12 + \beta_2 \cdot eGFR + \beta_3 \cdot B12^2 + \beta_4 \cdot B12 \cdot eGFR + \beta_5 \cdot eGFR^2)$$

→ **20%** (Nielsen et al, 2022) **to 40% reduction** (58/144) (Van Loon et al, 2018) in B12 deficiency (MMA > 430 nmol/L)

Van Loon et al, Annals of Clinical Biochemistry, 2018

Homocysteine



Measurement

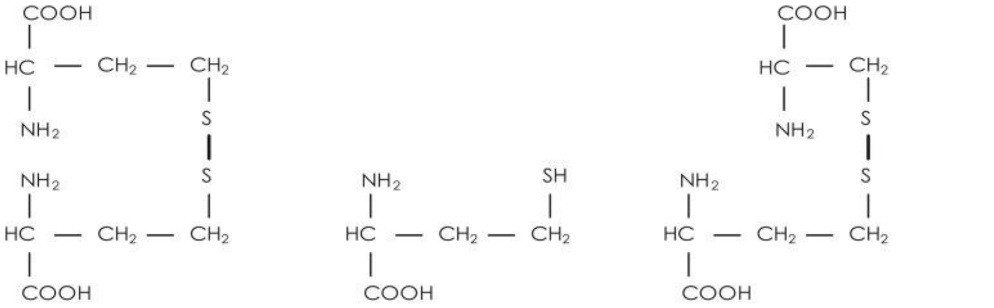
- LC-MS/MS or immunoassays
- Pretreatment with reducing agent (e.g., DTT)

(+) Advantages

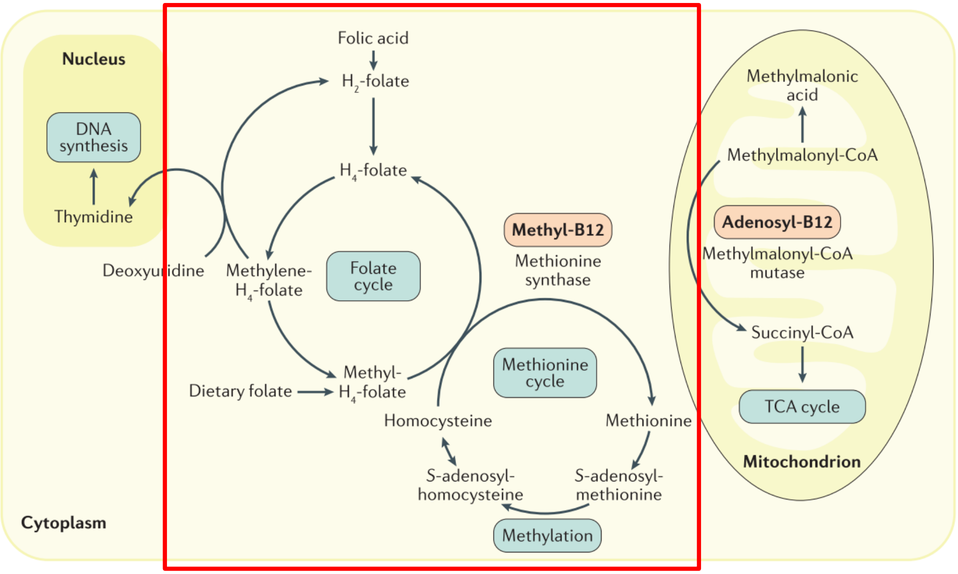
- Sensitive, functional marker

(-) Disadvantages

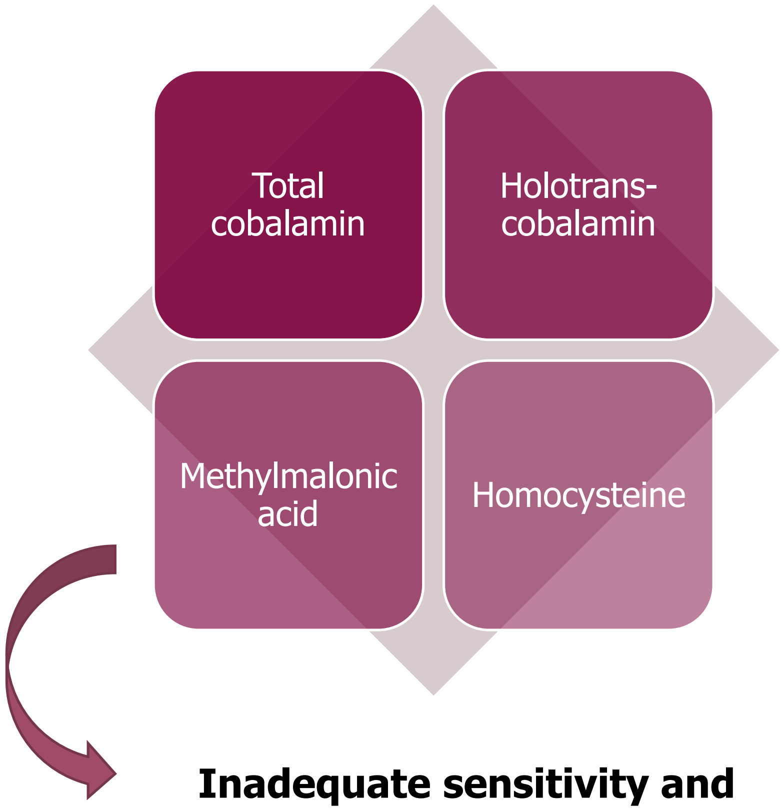
- **Not specific**: folate deficiency
- Sensitive to **pre-analytical** variables: e.g., high protein meal
- RBC: increase of 1 µmol/(L*h)
- **EDTA sample on ice** until centrifugation
- Impaired **renal function and** increasing **age** → higher tHcy levels



homocystine   homocysteine   cysteine-homocysteine



Diagnostic algorithms



Inadequate sensitivity and specificity of stand-alone markers: combination in algorithms?

cB12, the combined indicator of vitamin B12 status

Fedosov, *Metabolism Clinical and Experimental,* **2010**

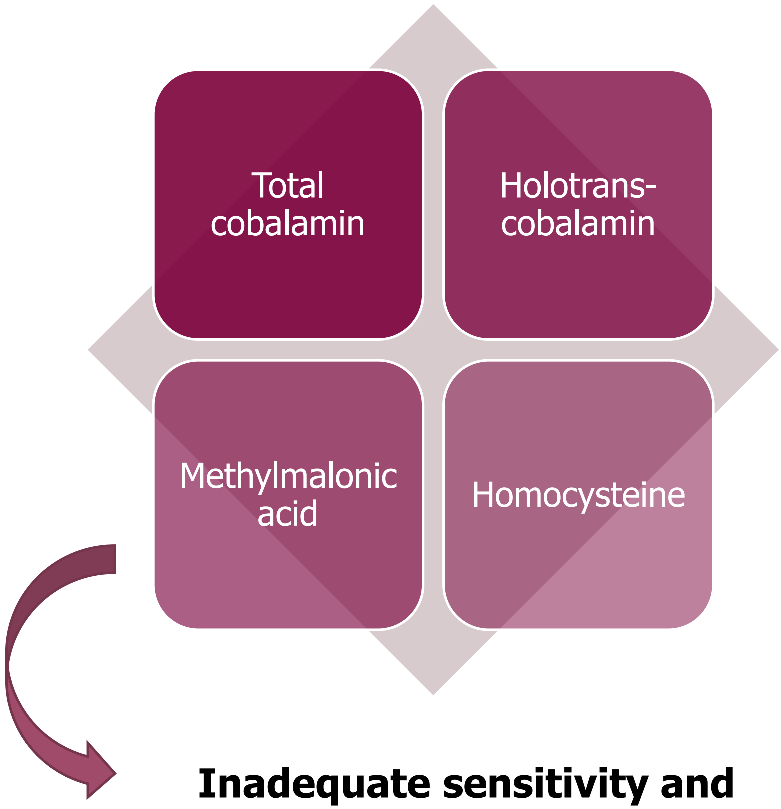
- Different from the classic "if→then" structure
- Only in patients with normal renal function

$$cB_{12} = \log_{10}\left(\frac{holoTC \cdot B_{12}}{MMA \cdot Hcy}\right)_{Test} - \log_{10}\left(\frac{holoTC \cdot B_{12}}{MMA \cdot Hcy}\right)_{Ref} = Test - \frac{3.79}{1+\left(\frac{age}{230}\right)^{2.6}}$$

Update: Fedosov et al., *Clin Chem Lab Med,* **2015**

- Formula if **1-2 biomarkers** are missing
- Hcy **correction for folate** if < 4.4 µg/L
- Can be used as **reference ("gold standard") for B12 deficiency** to evaluate individual markers

Diagnostic algorithms



Inadequate sensitivity and specificity of stand-alone markers: combination or algorithms?

cB12, the combined indicator of vitamin B12 status

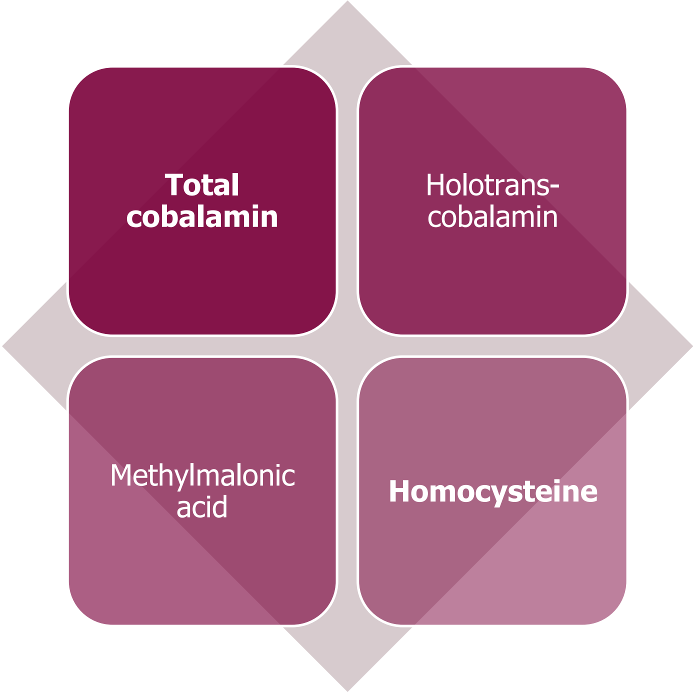
$$cB_{12} = \log_{10}\left(\frac{holoTC \cdot B_{12}}{MMA \cdot Hcy}\right)_{Test} - \log_{10}\left(\frac{holoTC \cdot B_{12}}{MMA \cdot Hcy}\right)_{Ref} = Test - \frac{3.79}{1+\left(\frac{age}{230}\right)^{2.6}}$$

Classification	Biological interpretation	Guidelines
Elevated B12 **cB12 > 1.5**	The pathogenesis of high B12 is not fully understood	Consider potential causes of high B12 levels such as liver disease or current or recent supplementation or treatment
B12 adequacy **cB12: –0.5 - 1.5**	Expected to accomplish all B12 status dependent functions	No action advised unless signs/symptoms present
Low B12 **cB12: –1.5 - –0.5**	Potential subclinical manifestations of B12 deficiency i.e., absence of hematological changes, but **subclinical** neurological impairment	Consider recommending oral supplements
Possible B12 deficiency **cB12: –2.5 - –1.5**	Potential manifestations of **B12 deficiency**	Potentially prescribe oral supplements, assess again in 3–6 months
Probable B12 deficiency **cB12: < –2.5**	It is possible to observe clinical manifestations of **B12 deficiency**. Clinical outcomes are needed to confirm potential clinical deficiency	Consider immediate treatment with IM injections, determine cause with primary consideration for the possibility of pernicious anemia

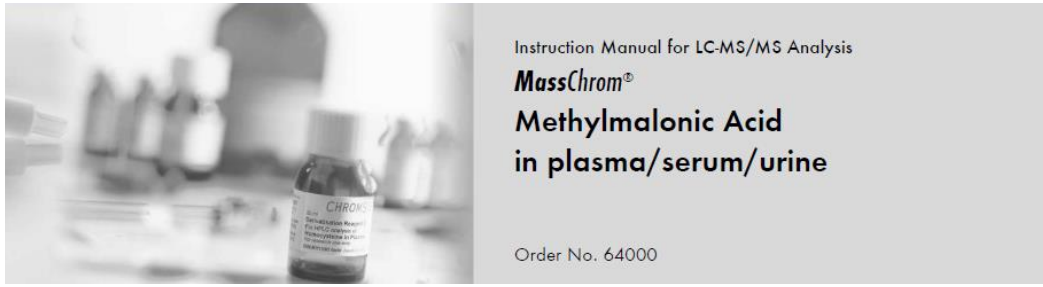
Research questions

1. **Which biomarkers** can be used in the diagnosis of cobalamin deficiency?

2. **Is it possible to improve the diagnostic process of cobalamin deficiency** by implementing the analysis of methylmalonic acid in the clinical laboratory of AZ Groeninge?



Implementation of MMA



Method verification MassChrom reagent kit (Chromsystems)

- Linearity, repeatability, reproducibility, accuracy, total error, selectivity, carry-over, freeze-thaw stability, stability at RT and at 4°C
- **EMA guideline** on bioanalytical method validation

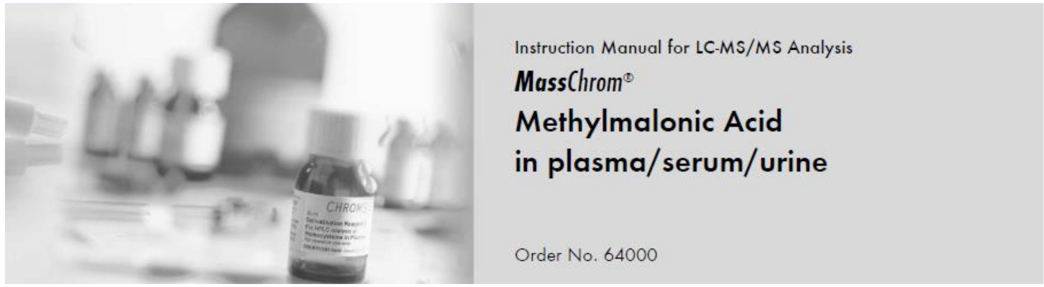
Max. 2 freeze/thaw cycles

min. 7 days

	Repeatability	Reproducibility	Bias	Total error
LLOQ (43.5 nmol/L)	/	14.3%	4.4%	/
QC L1 (174 nmol/L)	1.1%	6.5%	2.9%	13.6%
QC L2 (576 nmol/L)	1.8%	7.1%	-5.7	17.4%
Criteria EMA	15%	15%	15%	(30%)
Biological variation: desirable*	/	3.6%	5.6%	11.5%
Biological variation: minimal*	/	5.4%	8.4%	17.3%

*Lindberg et al, Scandinavian Journal of Clinical and Laboratory Investigation, 2019

Implementation of MMA



Verification of reference values

- CLSI EP28-A3C
- 20 healthy volunteers > 18 years
- Erdogan et al, 2010: **60-360 nmol/L**
- Only 1 person > 360 nmol/L (363 nmol/L)
- **Reference ranges can be transferred**
- **+ grey zone: 360-800 nmol/L (comment on patient's lab report)**

	Healthy volunteers	Reference values
Ratio male:female	10:10	n.a.
Age (years)	33.5 (22-64)	n.a.
MMA (nmol/L)	208 (141-363)	n.a.
Total serum cobalamin (ng/L)	429 (209-590)	> 200
Holotranscobalamin (pmol/L)	66.4 (33.8- > 150)	Kit insert: 37.5 – 188 pmol/L
Homocysteine (µmol/L)	10.4 (6.22-14.7)	< 15
Folate (µg/L)	5.3 (2.2-9.2)	> 3.88
Creatinine (mg/dL)	0.80 (0.66-1.18)	Age-dependent: cf. lab tests guide AZG
eGFR (ml/min/1.73m²)	> 90 (76 - > 90)	n.a.
Haemoglobin (g/dL)	m: 15.8 (14.4-17.3) f: 13.3 (12.3-14.2)	m: 13.7-17.1 f: 11.8-15.5
Number of red blood cells (*10^12/L)	m: 5.1 (4.7-6.0) f: 4.5 (4.1-4.9)	m: 4.3-5.71 f: 3.75-5.11
MCV (fL)	90.2 (80.5-96.7)	84.0-98.3

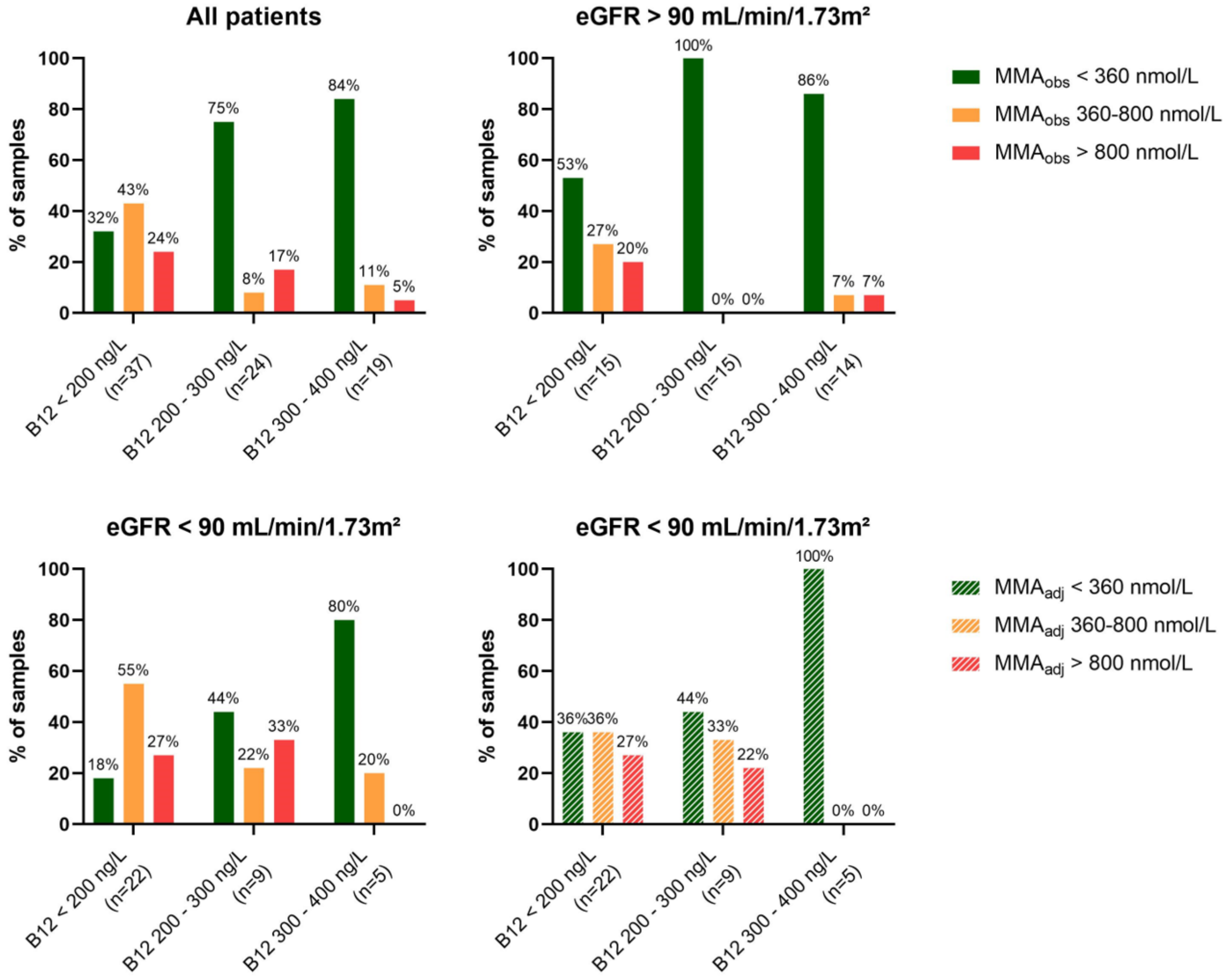
B12 markers in a hospital-specific patient population

Total cobalamin & MMA

- **80 leftover** samples from adult patients

- If **B12 < 200 ng/L**
  - **only 20%** (if eGFR > 90) **to 27%** (if eGFR < 90) of the patients had an MMA concentration **indicative of B12 deficiency**
  - **18%** (if eGFR < 90) to **53%** (if eGFR > 90) of the patients had a **normal MMA**
  - **27%** (if eGFR > 90) to **55%** (if eGFR < 90) of the patients had an MMA concentration within the **grey zone** (360-800 nmol/L)

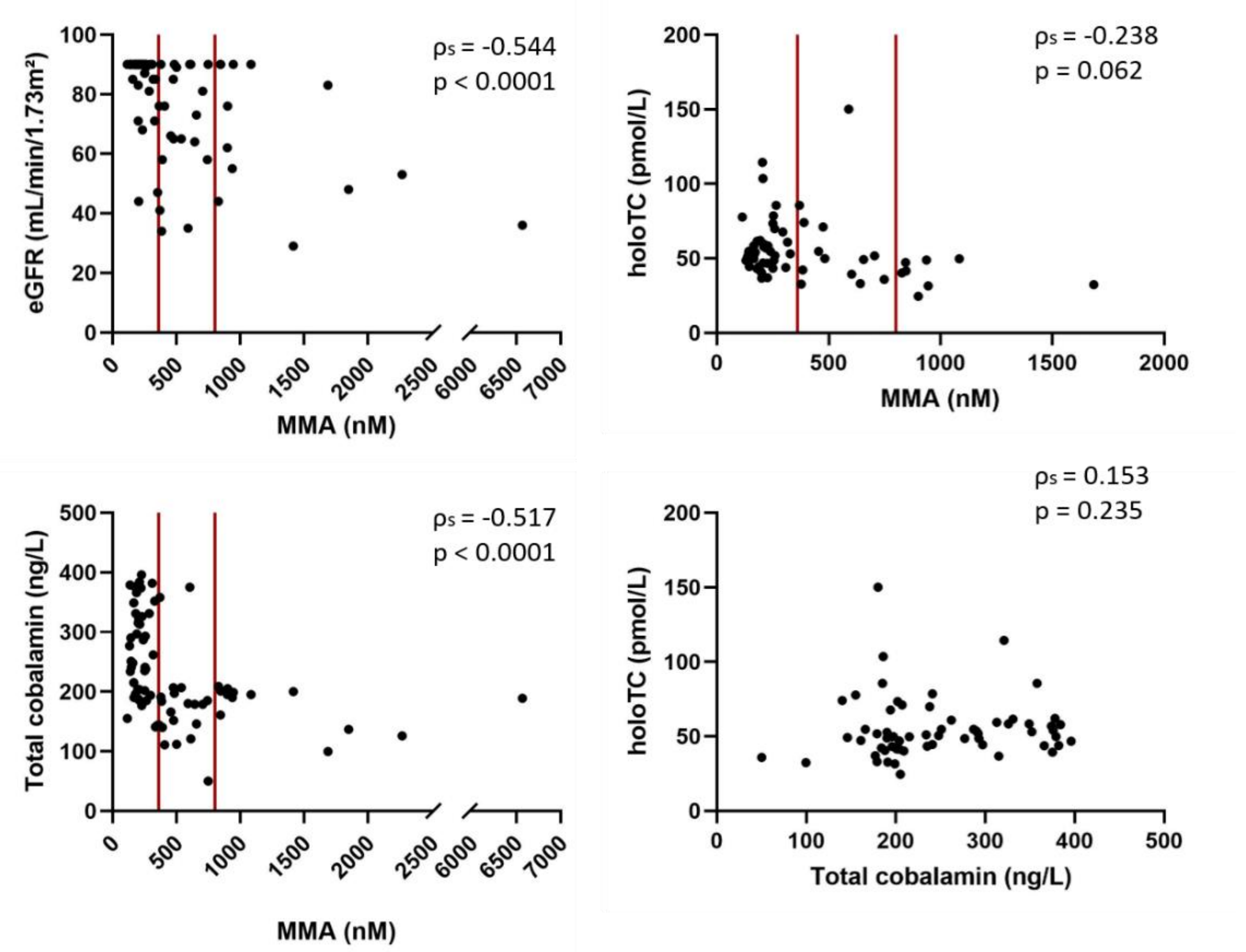
- Correction MMA for eGFR (if < 90) (Van Loon et al, 2018)
  - **After correction, 36%** of the patients with a B12 < 200 ng/L had a normal MMA level **instead of 18% of the patients without correction**
  - **21%** (5/24) of the samples with MMAobs > 360 nmol/L are **reclassified as MMAadj < 360 nmol/L**



B12 markers in a hospital-specific patient population

Correlation analysis

- Spearman's rho correlation analysis
- **Significant** correlation (all patients) between
  - MMA and eGFR
  - MMA and total cobalamin – but…



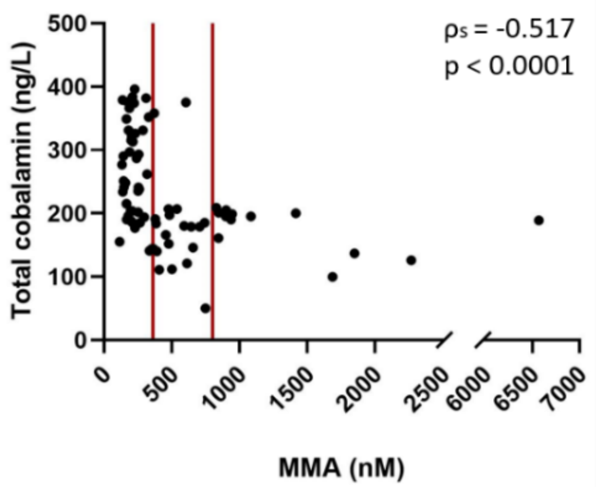
B12 markers in a hospital-specific patient population

Correlation analysis

- Spearman's rho correlation analysis
- **Significant** correlation (all patients) between
  - MMA and eGFR
  - MMA and total cobalamin – but… **not significant if only patients with normal renal function**

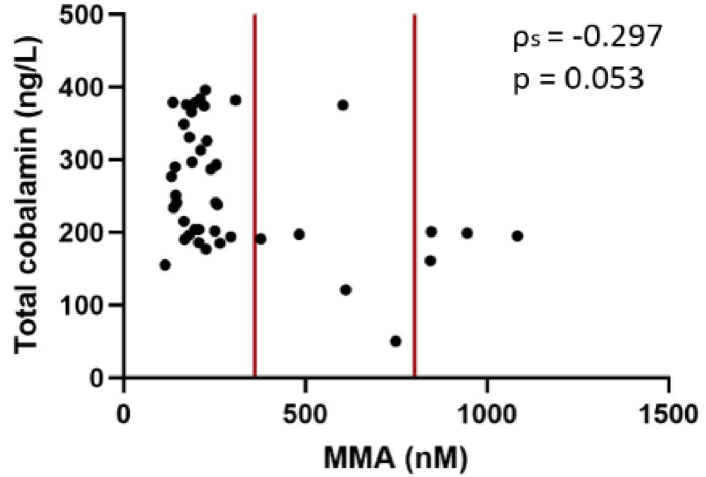
→ correlation driven by mildly increased MMA levels in patients with impaired renal function

All patients



⬌

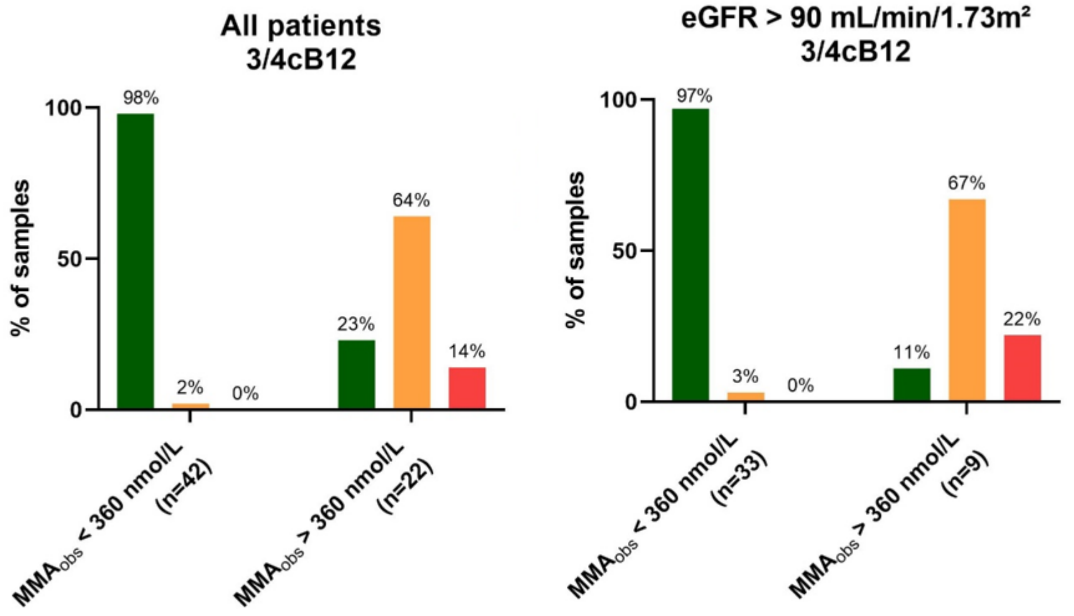
eGFR > 90 mL/min/1.73m²



B12 markers in a hospital-specific patient population

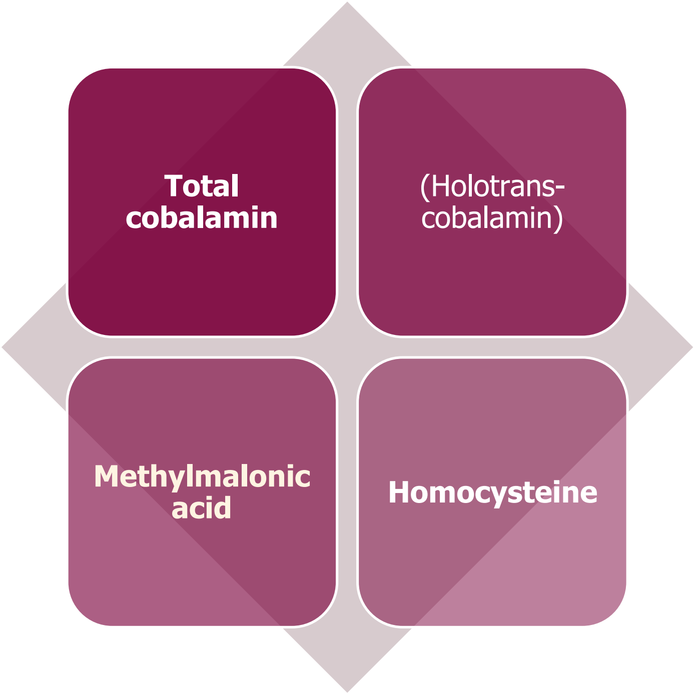
The cB12 score as a reference for B12 deficiency

- 3/4cB12: B12+MMA+holoTC and/or Hcy
- *n* = 64 samples
- When 3/4cB12 is used to define B12 deficiency, the **MMA cut-off of 360 nmol/L**
  - adequately **distinguishes** B12 (sub)clinical deficiency from B12 adequacy
  - closely resembles the **optimal cut-off** level revealed by ROC analysis



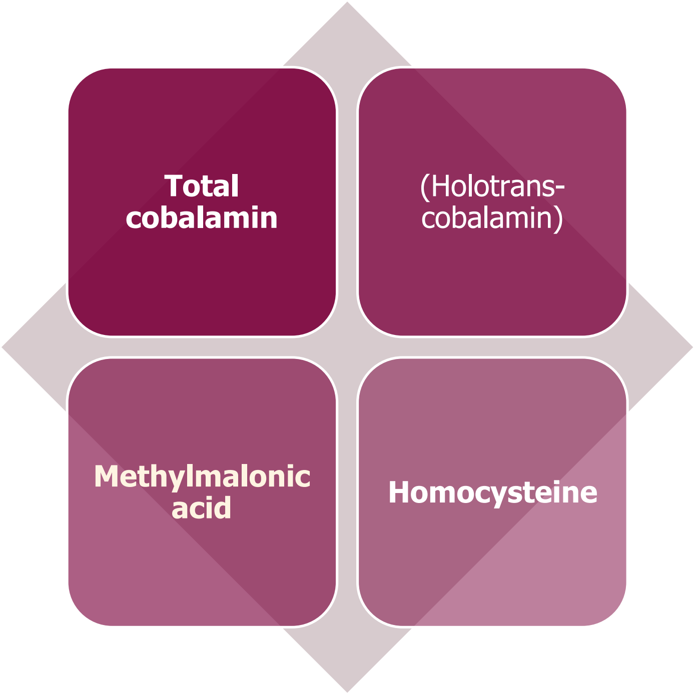
	Number of patients with B12 deficiency	Number of patients with B12 adequacy	Optimal cut-off (nmol/L)	100% sensitivity	100% specificity	AUC
All patients	18	46	373 (94% sens; 91% spec)	> 228	> 622	0.9626
eGFR > 90	9	33	343 (89% sens; 97% spec)	> 228	> 676	0.9630

Implications and conclusions



- ✓ **MMA MassChrom reagent kit** was successfully implemented
- ✓ **Reference values** were verified (60 – 360 nmol/L)
- ✓ **Significant correlation MMA and eGFR**
  → Grey zone 360 – 800 nmol/L
  → eGFR-adjusted MMA may be useful
- ✓ MMA may **elucidate the actual B12 status** and prevent misdiagnosis of B12 deficiency **if B12 < 200 ng/L** (20-27% of the patients)
- ✓ MMA can help to **reliably confirm** the diagnosis of B12 deficiency in patients with **clinical symptoms and B12 > 200 ng/L** (5-17% of the patients)
- ✓ **Thus:** the implementation of an analytical method for MMA in serum will **considerably improve** the diagnostics of cobalamin deficiency in the clinical laboratory of AZ Groeninge

To do’s and actions



- **Informing the clinicians** on the implementation of an analytical method for MMA.
- **Follow-up study of correlations between MMA levels and total cobalamin** concentrations to further explore the **actual prevalence** of B12 deficiency in our lab-specific patient population.
- **Extending the evaluation** of the implemented MMA reference ranges and grey zone, the cB12 score and eGFR-corrected MMA levels by **including additional patients** with normal and impaired renal function

Thank you
for your attention!