

Ins and outs of Thyroglobulin and Tg-antibodies assays

Eef Lentjes
Clinical Chemist UMCU

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Thyroglobulin

- Glycoprotein 660kDa (2 subunits of 330 kDa each)
- extensive posttranslational modifications: glycosylation (10% of weight), iodination (0.1-2%), sulfation and oxidation
- Plasma T_{1/2}: 30 hr (range from hr to days)
- Thyroid specific: no thyroid → no Tg
- Healthy indiv.: 0.5-50 µg/L (assay dependent)

Synthesis of thyroglobulin

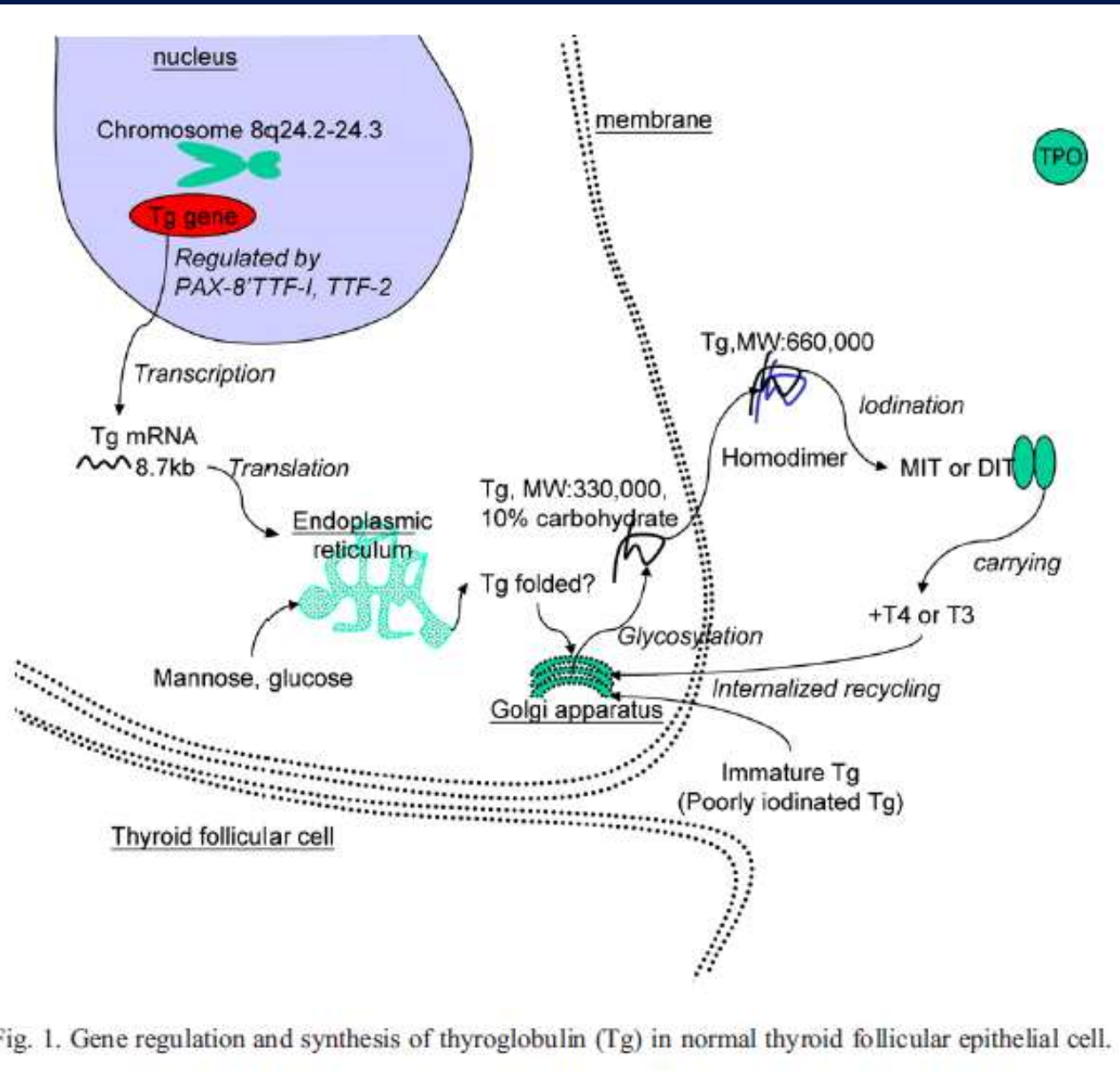
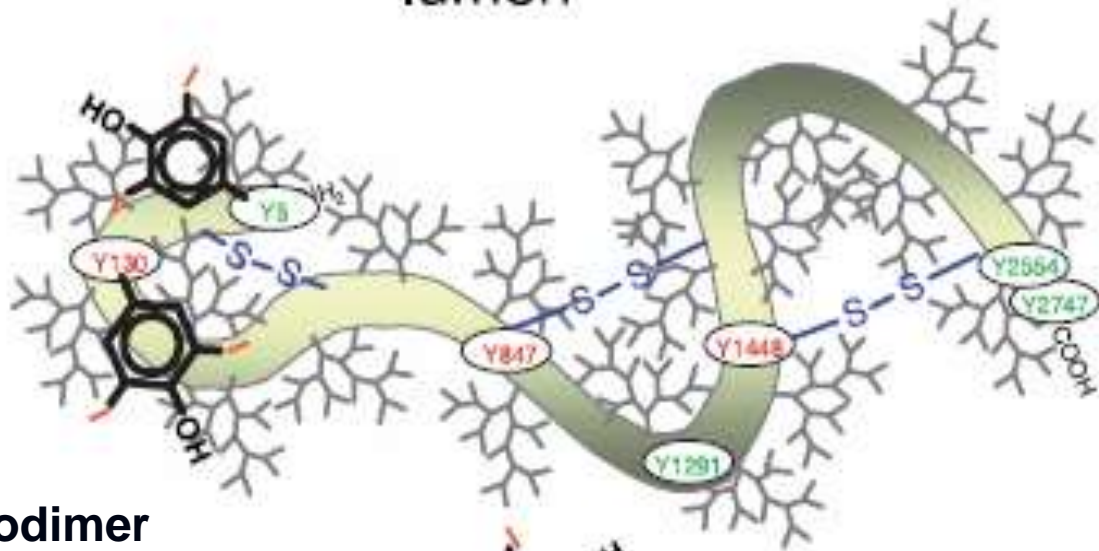
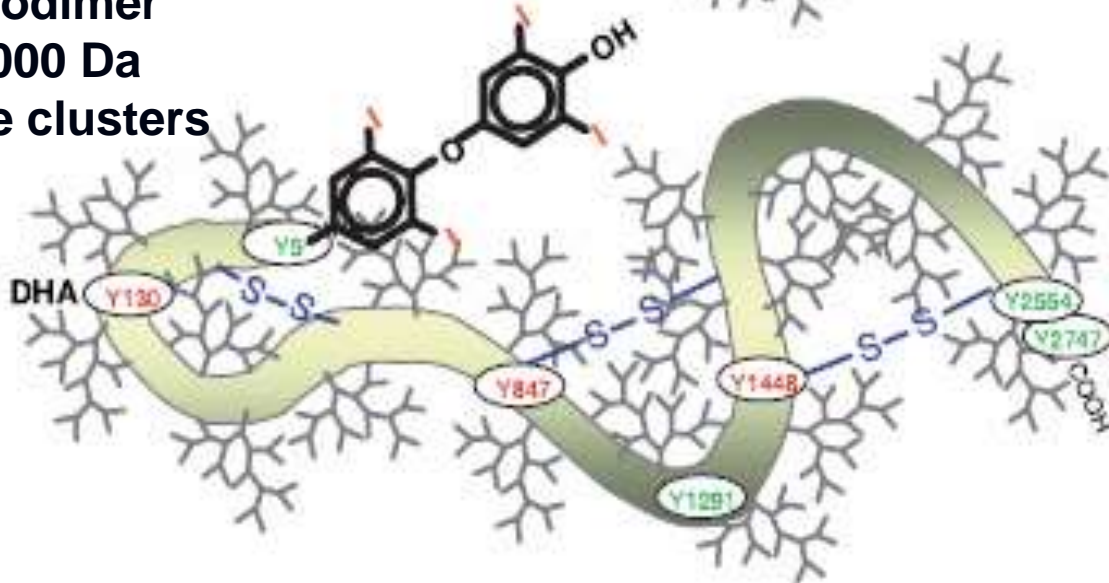


Fig. 1. Gene regulation and synthesis of thyroglobulin (Tg) in normal thyroid follicular epithelial cell.

follicular
lumen



TG: homodimer
Mw 660.000 Da
6 epitope clusters



After iodination

After coupling

Thyroglobuline

reason for TG request:

- follow-up patient with DTC after thyroidectomy and iodine ablation

Important issues:

- **sensitivity**: detectable Tg means persistent or recurrent disease (15%)
- **Standardisation/bias**: important for use of cut-off values in guidelines (2 µg/L after rhTSH stimulation)
- **Interference**: Tg-antibodies, heterophile antibodies

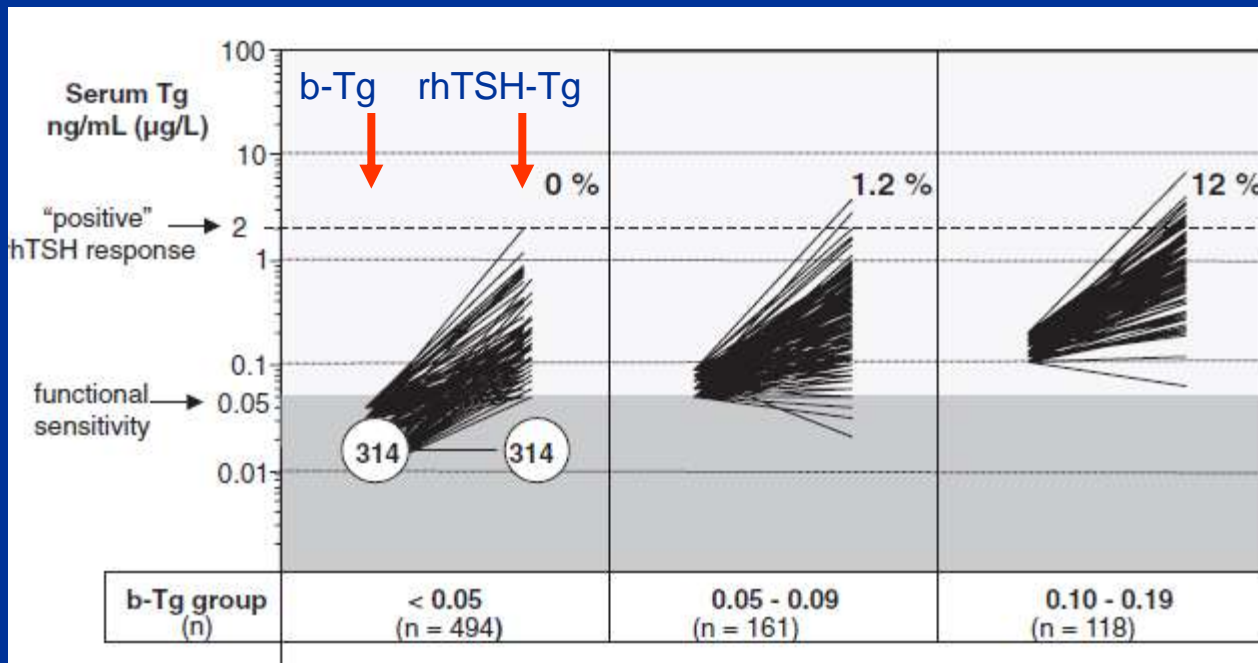
sensitivity

Sensitive Tg

- Available: TG assay with a functional sensitivity (FS: CV=20%) of 0.1 $\mu\text{g/L}$
- Iervasi: Clin Endocrinol 2007:
 - 160 DTC patients tested with two Tg methods:
Access (FS = 0.1 $\mu\text{g/L}$) and Immulite (FS = 0.9 $\mu\text{g/L}$);
 - **Immulite**: few patients with residual tumor were identified (PPV=17%)
 - **Access**: all patients with b-Tg < 0.1 had also a rhTSH-Tg < 2 $\mu\text{g/L}$ and all pat. with Tg > 0.1 had rhTSH-TG > 2 (PPV = 100%, but 10% false pos bTg)
- Smallridge: JCEM 2007
 - 194 DTC patients tested with Access Tg (FS = 0.1 $\mu\text{g/L}$)
 - 80 pat had b-Tg < 0.1 and 2/80 pat. had rhTSH-Tg > 2 (2.5%)

Spencer, 2010

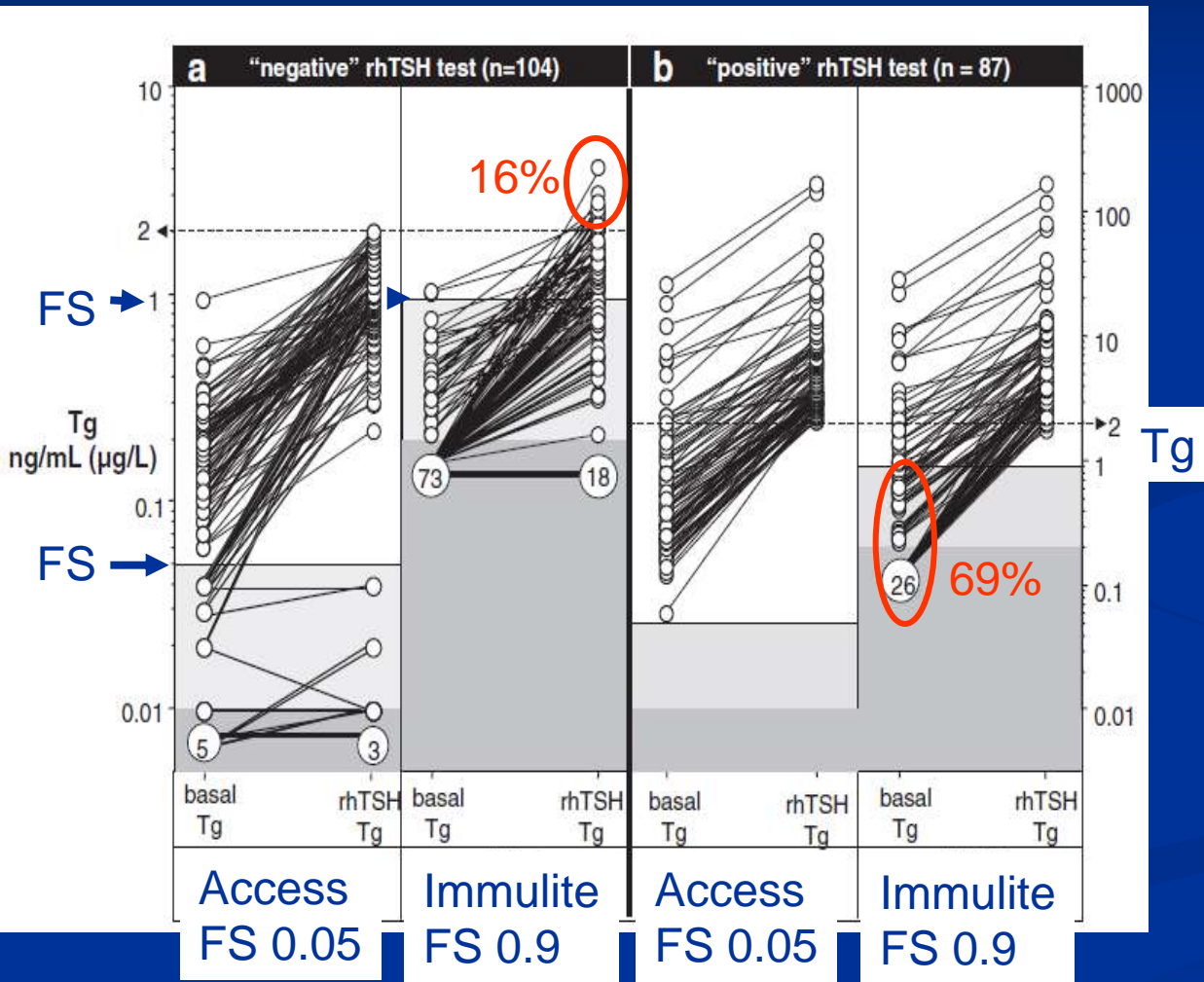
- basal-Tg (TSH <4.5 mU/L) and rhTSH-Tg in specimens from 1029 rhTSH tests on 849 TGAb neg patients
- TG methods: 1) Access FS 0.05 µg/L 2) Immulite FS 0.9 µg/L



Conclusion:

An rhTSH-Tg >2 µg/L is highly unlikely when b-Tg < 0.1 (2/655 pos)

Tg methods: Access (sens) versus Immulite



Immulite:

Negative group:

- 16% have bTg < 0.9 but rhTSH Tg > 2

Positive group

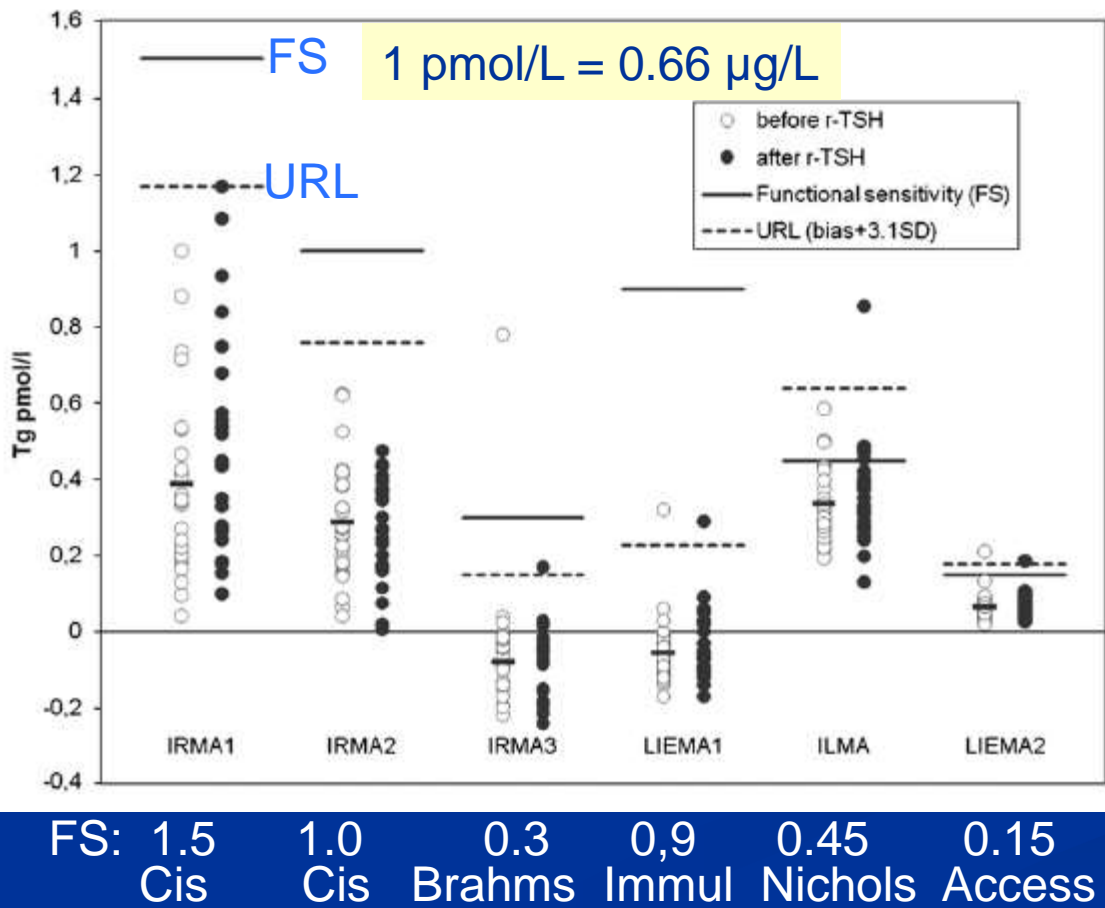
- 69% have bTg < 0.9 but rhTSH Tg > 2
(Acc: 100% bTg boven FS)

Conclusion (for sensitive Tg):
“the routine use of rhTSH-Tg testing appears not to provide any additional diagnostic or prognostic benefit above that of measuring bTg levels alone...”

What about functional sensitivity and bias?

Assay bias may invalidate decision limits and affect comparability of serum thyroglobulin assay methods: An approach to reduce interpretation differences

H. Alec Ross ^{a,b,*}, Romana T. Netea-Maier ^b, Eveline Schakenraad ^b, Bert Bravenboer ^c, Ad R.M.M. Hermus ^b, Fred C.G.J. Sweep ^a



Clinica Chimica Acta 394 (2008) 104–109

28 DTC patients

Tg negative group = no rise in Tg after r-TSH in any method

Conclusion

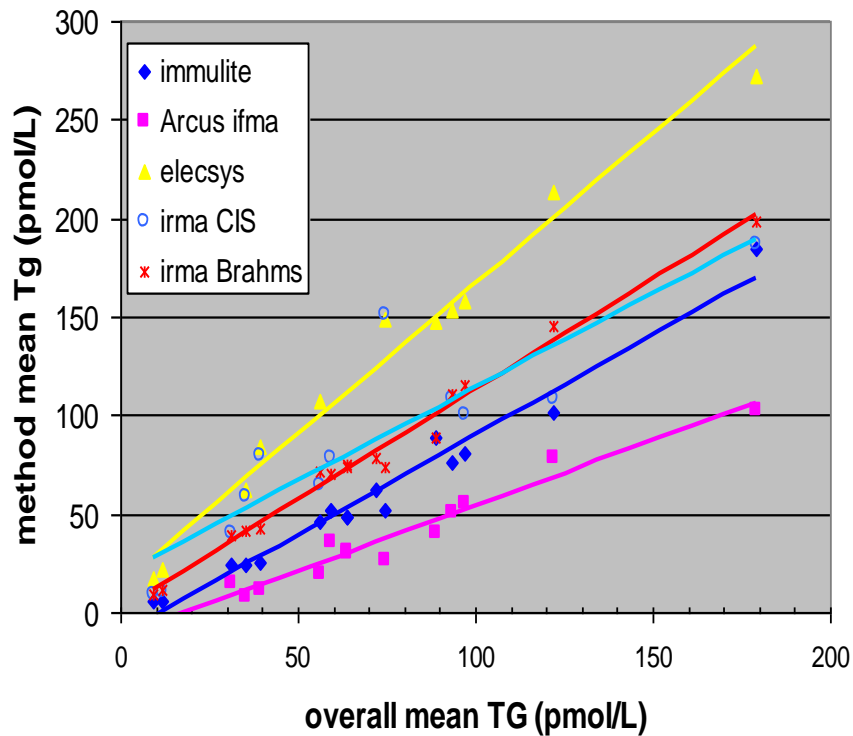
Using the URL instead of FS as a decision limit → much more concordance between methods in predicting outcome of TSH-Tg rise

Standardisation

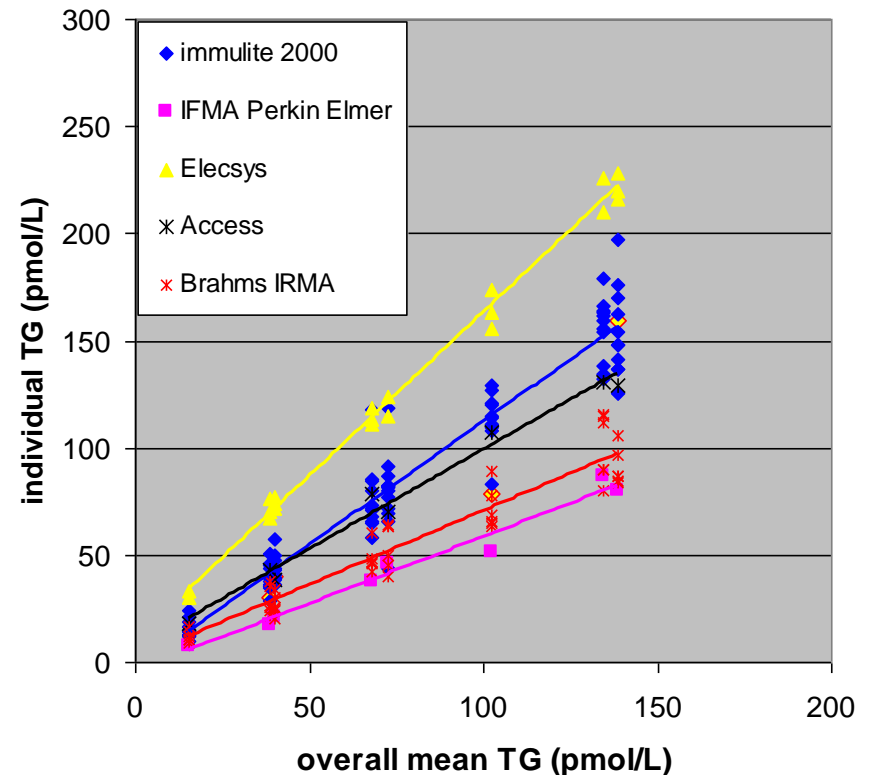
External Quality Assessment Scheme

All methods standardised on CRM 457

thyreoglobulin in SKML 2003 (Tg-ab neg)



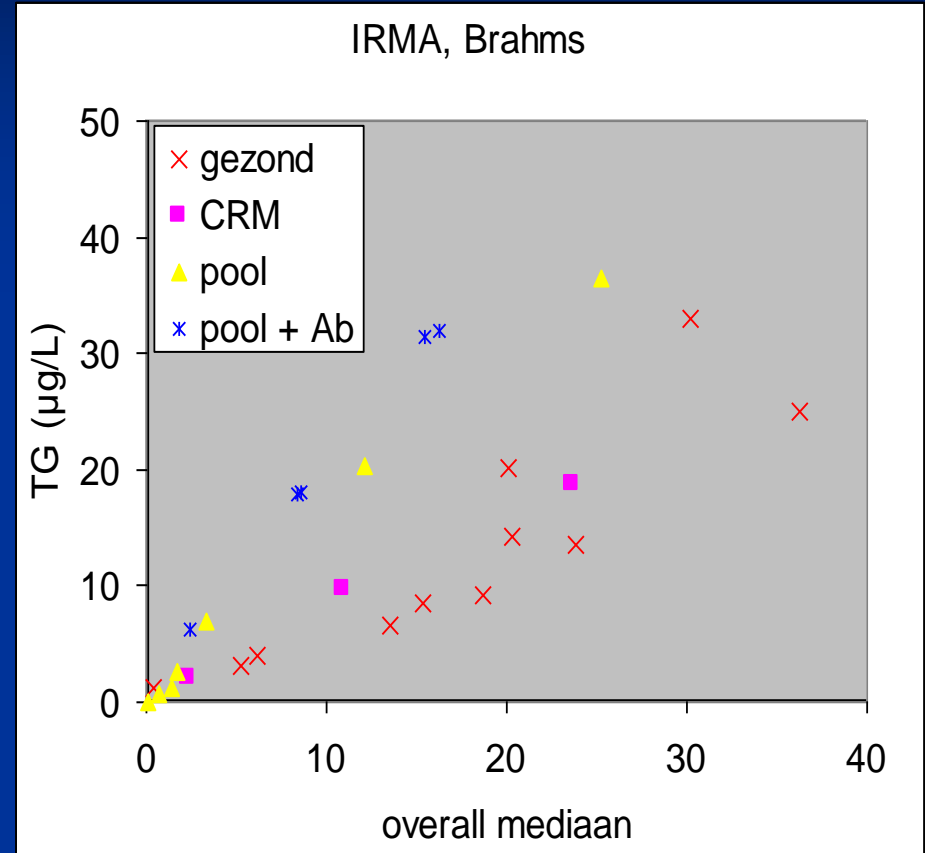
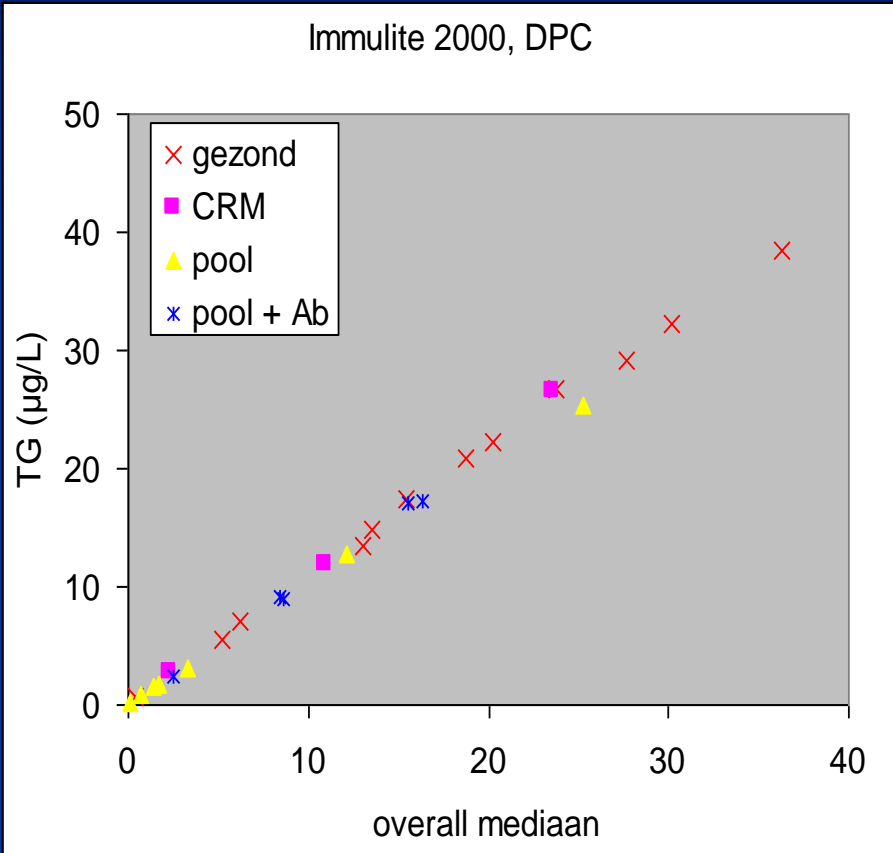
Thyroglobulin in SKML 2010 (Tg-ab neg)



1 pmol/L = 0.66 µg/L

- Large Intermethod variation despite use of CRM 457
- Differences in slope: can we re-standardise methods?
 - Study with 27 samples analysed in 20 laboratories using 6 methods
 - Patient pools, CRM457, individual patient samples
 - Question: is there a sample that is commutable and that can be used as a standard?

Two examples



No commutable sample: neither CRM457, nor pool samples

Next study: only include samples from DTC patients (pool, CRM, indiv pat)

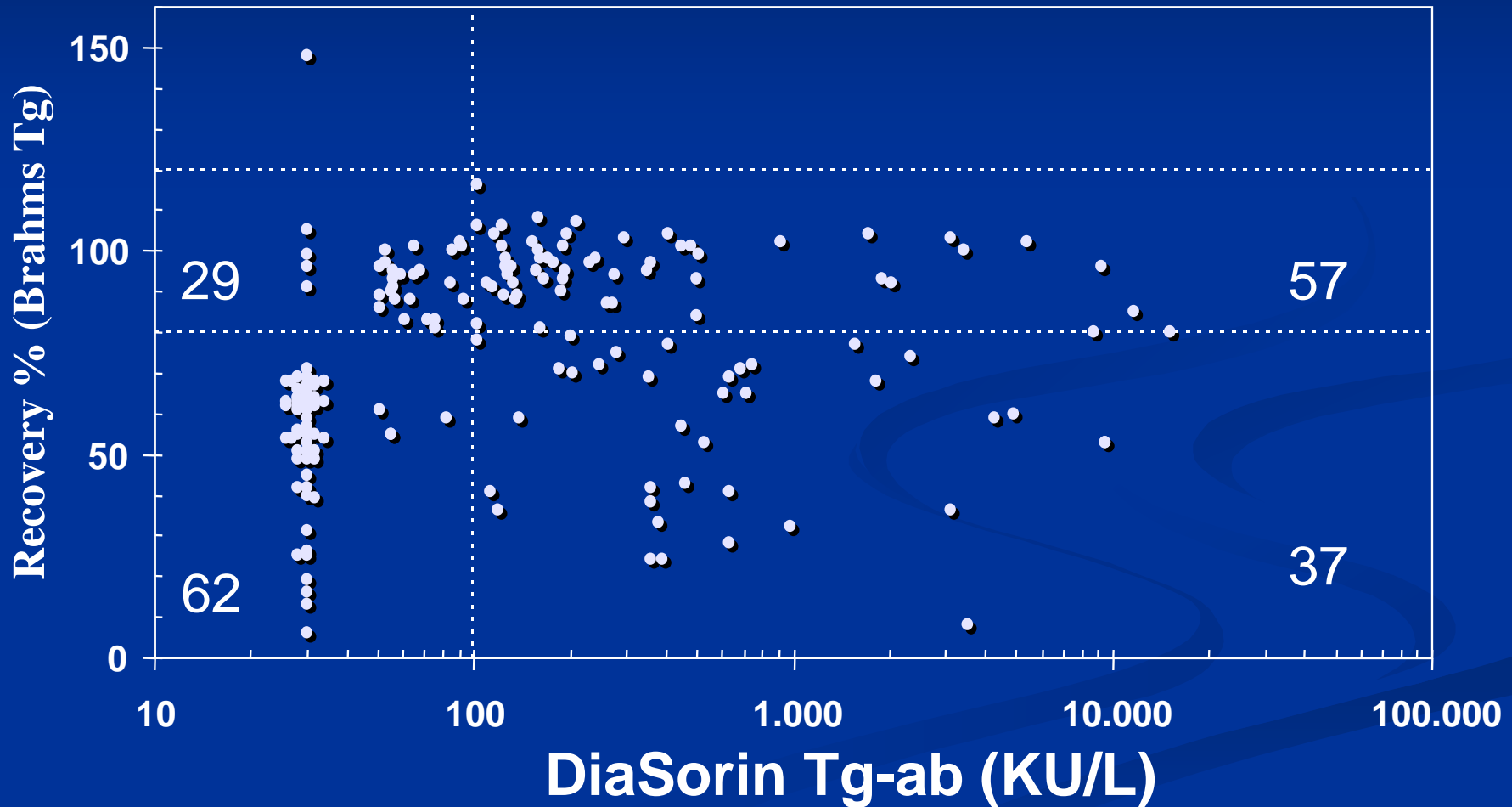
What about Tg-antibody assays

| | A Direct TgAb Methods | | | | | | | | | | | | B % Recoveries | | | |
|----------|-----------------------|-----|-----|-----|-----|-----|------|-----|-----|-----|-----|-----|----------------|--------|--------|--------|
| | ACC | DYN | KRY | DPC | ESO | FLY | FUJ | KRO | ADV | NIB | ELE | TOS | CIS | DEL | KRY | DYN |
| cut-offs | <2 | <30 | <40 | <40 | <3 | <1 | <100 | <1 | <2 | <1 | <40 | <30 | 80-117 | 71-122 | 45-113 | 88-101 |
| 1 | 4 | 0 | 12 | <20 | <1 | <1 | 0 | <1 | <2 | <1 | 16 | <30 | 99 | 102 | 92 | 98 |
| 2 | <2 | 64 | 26 | 39 | <1 | <1 | 0 | <1 | <2 | <1 | 15 | <30 | 101 | 87 | 90 | 90 |
| 3 | <2 | 56 | 29 | 23 | 2 | <1 | 0 | <1 | <2 | <1 | 29 | <30 | 99 | 73 | 79 | 93 |
| 4 | <2 | 28 | 59 | 29 | <1 | <1 | 0 | <1 | <2 | <1 | 35 | <30 | 92 | 101 | 74 | 97 |
| 5 | <2 | 0 | 20 | <20 | <1 | <1 | 0 | <1 | <2 | <1 | 80 | <30 | 103 | 100 | 95 | 98 |
| 6 | <2 | 0 | 19 | 38 | 3 | <1 | 0 | <1 | <2 | <1 | 9 | <30 | 95 | 95 | 87 | 93 |
| 7 | <2 | 50 | 38 | <20 | <1 | <1 | 0 | <1 | <2 | <1 | 21 | <30 | 99 | 97 | 59 | 92 |
| 8 | <2 | 92 | 32 | <20 | <1 | <1 | 0 | <1 | <2 | <1 | 21 | <30 | 93 | 114 | 45 | 110 |
| 9 | <2 | 66 | 33 | <20 | <1 | <1 | 0 | <1 | <2 | <1 | 17 | <30 | 92 | 86 | 99 | 90 |
| 10 | <2 | 39 | 23 | <20 | <1 | <1 | 0 | <1 | <2 | <1 | 24 | <30 | 95 | 91 | 61 | 79 |
| 11 | <2 | 0 | 24 | <20 | <1 | 43 | 0 | <1 | <2 | <1 | 12 | <30 | 100 | 104 | 65 | 97 |
| 12 | <2 | 39 | 23 | <20 | <1 | <1 | 0 | <1 | <2 | <1 | 24 | <30 | 99 | 83 | 87 | 91 |
| 13 | <2 | 0 | 9 | 46 | <1 | <1 | 0 | <1 | <2 | <1 | 19 | <30 | 93 | 96 | 77 | 96 |
| 14 | <2 | 53 | 23 | <20 | <1 | <1 | 40 | <1 | <2 | <1 | 17 | <30 | 73 | 63 | 60 | 93 |
| 15 | 2 | 0 | 24 | <20 | 3 | <1 | 0 | <1 | <2 | <1 | 20 | <30 | 110 | 84 | 83 | 94 |
| 16 | <2 | 48 | 44 | 32 | 1 | <1 | 0 | <1 | <2 | <1 | 14 | <30 | 87 | 70 | 145 | 94 |
| 17 | <2 | 71 | 41 | <20 | <1 | <1 | 0 | <1 | <2 | <1 | 15 | <30 | 86 | 77 | 100 | 91 |
| 18 | <2 | 26 | 34 | <20 | 6 | <1 | 0 | 1 | <2 | <1 | 39 | <30 | 82 | 56 | 65 | 96 |
| 19 | <2 | 26 | 39 | <20 | 4 | <1 | 0 | <1 | <2 | <1 | 82 | <30 | 99 | 53 | 18 | 92 |
| 20 | <2 | 62 | 55 | <20 | <1 | <1 | 0 | <1 | <2 | <1 | 20 | <30 | 103 | 106 | 57 | 97 |
| 21 | <2 | 42 | 58 | <20 | <1 | <1 | 0 | <1 | <2 | <1 | 28 | <30 | 97 | 74 | 70 | 93 |
| 22 | <2 | 0 | 57 | <20 | <1 | <1 | 160 | <1 | <2 | <1 | 15 | <30 | 97 | 105 | 54 | 99 |
| 23 | <2 | 110 | 41 | 47 | <1 | <1 | 0 | <1 | <2 | <1 | 13 | <30 | 95 | 83 | 73 | 94 |
| 24 | <2 | 0 | 99 | <20 | 4 | <1 | 0 | <1 | <2 | <1 | 53 | <30 | 90 | 84 | 72 | 98 |
| 25 | <2 | 42 | 36 | 22 | 5 | <1 | 0 | <1 | <2 | <1 | 74 | <30 | 94 | 77 | 80 | 91 |
| 26 | <2 | 21 | 36 | 38 | 6 | <1 | 0 | 1 | <2 | <1 | 83 | <30 | 86 | 90 | 85 | 98 |
| 27 | <2 | 42 | 128 | 28 | <1 | <1 | 40 | <1 | <2 | <1 | 82 | <30 | 95 | 58 | 110 | 99 |
| 29 | 3 | 160 | 30 | 24 | 8 | <1 | 0 | 1 | <2 | <1 | 38 | <30 | 98 | 37 | 90 | 89 |
| 28 | 3 | 33 | 22 | <20 | 5 | <1 | 0 | <1 | 2 | 5 | 61 | <30 | 80 | 62 | 65 | 88 |
| 29 | <2 | 28 | 40 | 45 | 8 | <1 | 0 | 1 | <2 | <1 | 98 | 46 | 90 | 51 | 55 | 95 |
| 30 | <2 | 130 | 79 | 32 | 14 | <1 | 0 | 2 | <2 | 1 | 100 | 75 | 103 | 26 | 72 | 93 |
| 32 | 3 | 47 | 76 | 62 | 23 | <1 | 40 | 3 | <2 | 2 | 322 | <30 | 100 | 18 | 96 | 90 |
| 33 | 3 | 74 | 160 | 77 | 39 | <1 | 0 | 8 | <2 | 2 | 658 | 52 | 95 | 19 | 80 | 93 |
| 34 | 15 | 0 | 17 | 94 | 47 | 140 | 160 | 4 | 19 | 32 | 57 | <30 | 110 | 55 | 61 | 96 |
| 36 | 106 | 24 | 21 | 55 | 65 | <1 | 160 | 6 | 61 | 45 | 92 | 123 | 103 | 33 | 101 | 97 |
| 35 | 29 | 23 | 22 | 135 | 37 | 95 | 400 | 3 | 25 | 27 | 43 | 257 | 92 | 78 | 77 | 98 |
| 37 | 6 | 44 | 46 | 51 | 19 | <1 | 160 | 3 | 6 | 7 | 83 | 42 | 82 | 47 | 102 | 95 |
| 38 | 20 | 46 | 18 | 325 | 104 | 180 | 400 | 12 | 23 | 52 | 124 | 280 | 50 | 57 | 103 | 92 |
| 39 | 21 | 34 | 45 | 366 | 63 | 150 | 640 | 6 | 24 | 33 | 52 | 351 | 104 | 79 | 112 | 100 |
| 40 | 13 | 220 | 126 | 67 | 55 | 140 | 160 | 7 | 12 | 15 | 207 | 81 | 94 | 95 | 45 | 82 |
| 41 | 70 | 160 | 110 | 289 | 229 | 270 | 1600 | 31 | 112 | 200 | 263 | 191 | 95 | 16 | 81 | 89 |
| 42 | 169 | 45 | 52 | 752 | 173 | 440 | 6400 | 21 | 142 | 168 | 370 | 707 | 91 | 14 | 77 | 93 |

12 TG-ab methods
4 recovery methods

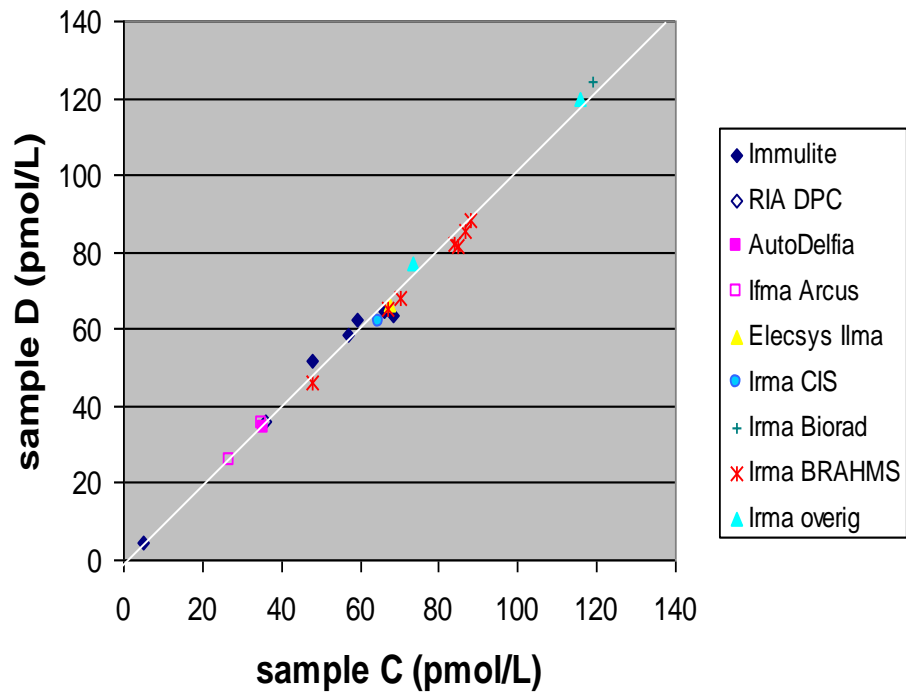
Spencer, 2005;
JCEM 90:5566

Comparison Tg-ab immunoassay vs recovery (185 samples)

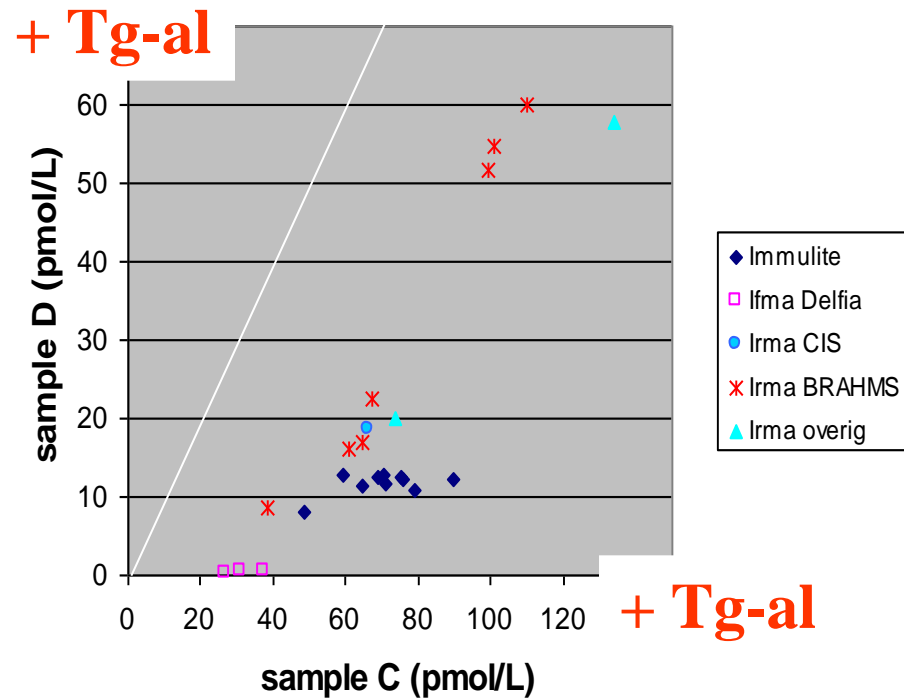


TG in Dutch EQAS +/- antibodies

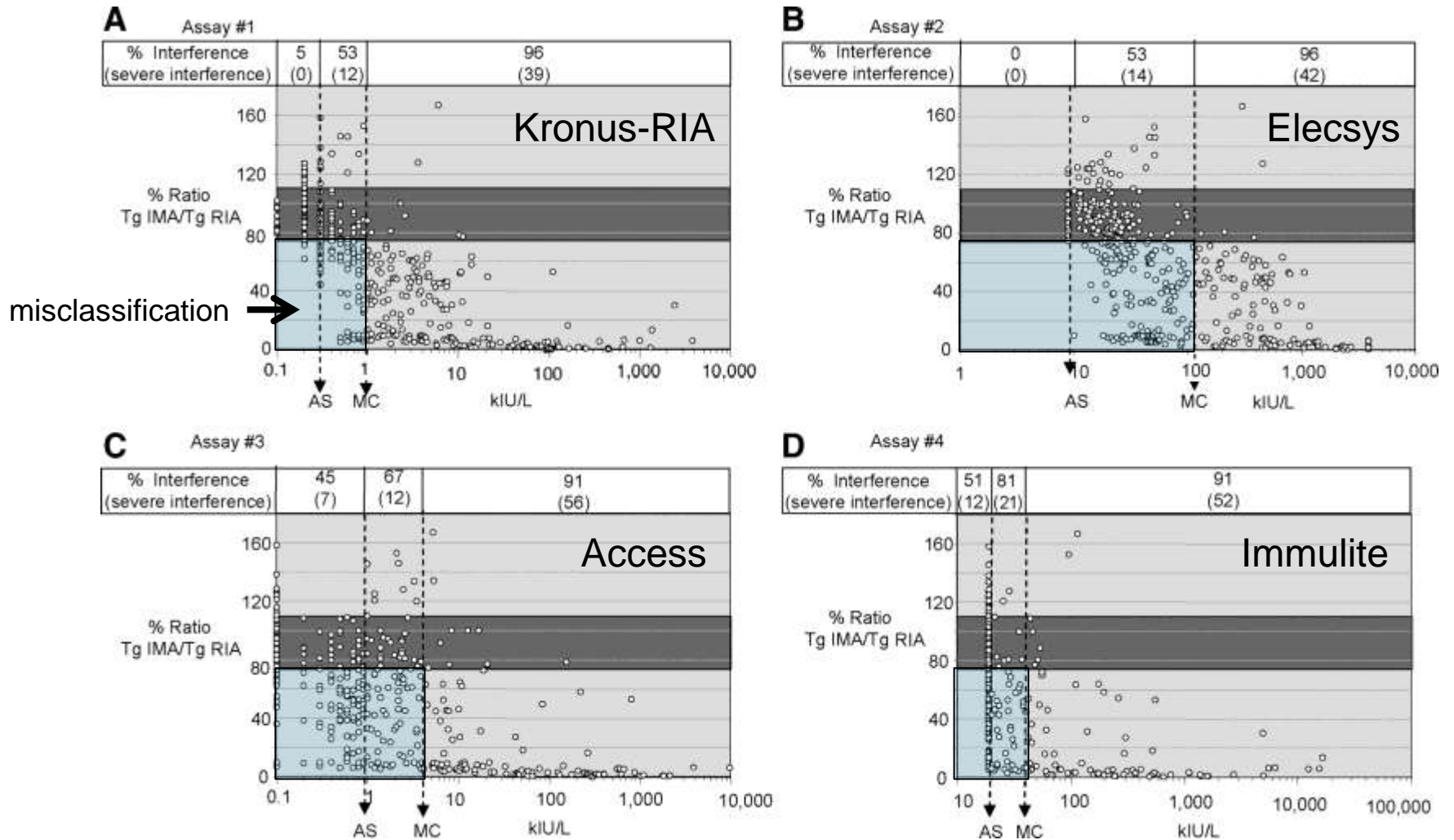
TG, no antibodies



22 laboratories



TgAb concentrations (on abscissae) of 4 TgAb methods and the presence of TgAb interference with serum Tg measurements, as judged from the presence of a low (<75%) serum Tg IMA to Tg RIA ratio.



Spencer C et al. JCEM 2011;96:1283-1291

AS = assay sens. MC = manufacturer cutoff

- Overall: 60% of samples show TG-ab interference in the Tg assay
- In about 20% this is a severe interference
(Tg (Ima) <0.1 but Tg (Ria) ≥ 1 $\mu\text{g/L}$)

Is this a problem?

Undetectable Tg occurs in 20% of those with undetectable Tg-ab.

It is rare for those with undetectable Tg (sens assay) to develop recurrent thyroid cancer (Kloos, JCEM 2010)

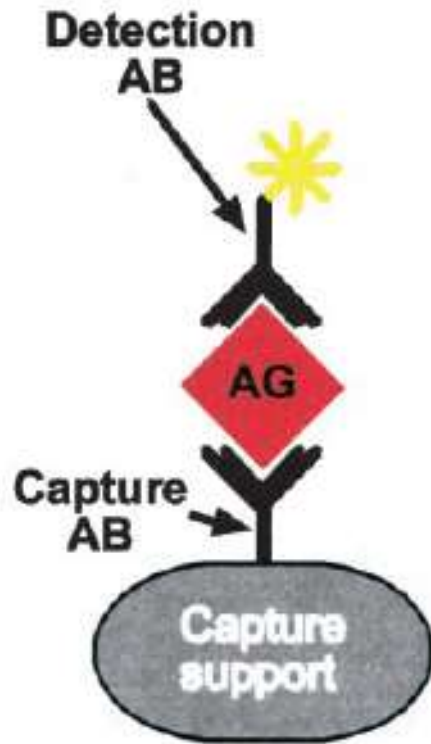
Few patients are likely to be missed

Tg-antibody

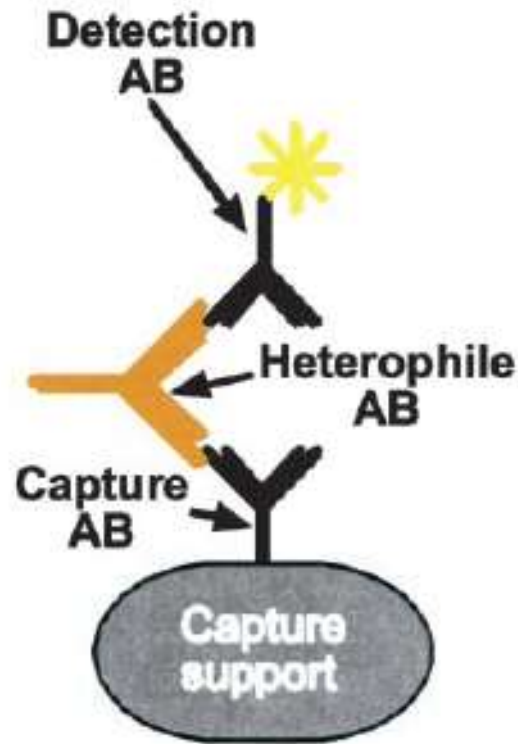
- Tg-Ab methods vary in sensitivity, specificity and absolute values despite standardization against IRP MRC65/93
- Tg-Ab differences probably result from differences in assay specificity for conformational epitopes
- TG-Ab heterogeneity appears to be patient specific (Spencer JCEM 1998)
- Many samples with interfering TG-ab are misclassified as TG-ab negative when using manufacturer-recommended cutoffs

Heterophile antibodies

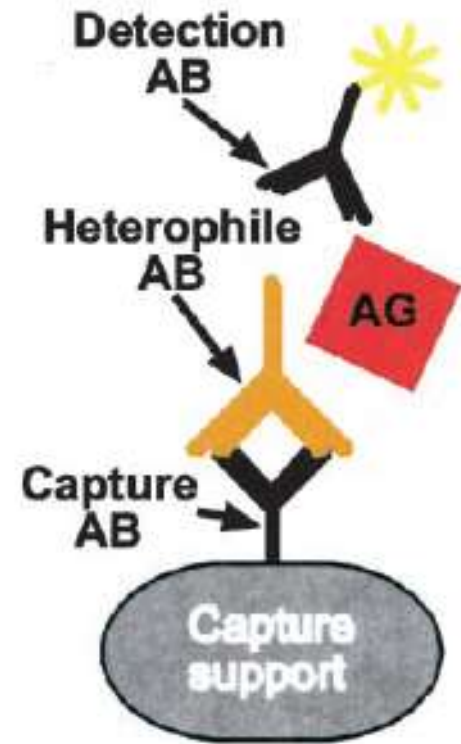
A no interference



B false high/positive



C false low/negative



Preissner, JCEM 2003

- 32 false positive or falsely increased Tg values from 1106 patients (with Tg > 1 µg/L)
 - investigated with Scantibody blocking tubes
- No false negative
- 48 DTC patients: 6 HAb positive (13%)
- Prevalence HAb's: 3%

Persoon clin chem 2006;52(6)1196

Samples from 110 DTC patients

- 1 patient with Tg 8.6 $\mu\text{g/L}$ (Nichols ILMA)
- After blocking tube Tg: 1.2 $\mu\text{g/L}$

Giovanella Clin Chem Lab Med 2009

- 406 samples from DTC pat. :3 FPos and 2 Fneg Tg

| | onT4-Tg | | rhTSH-Tg | |
|---|---------|----------|----------|----------|
| | pre-HBT | post-HBT | pre-HBT | post-HBT |
| 1 | <0.36 | 4.10 | <0.36 | 10.7 |
| 2 | 0.98 | 12.4 | 1.2 | 26.2 |
| 3 | 4.1 | <0.36 | – | – |
| 4 | 1.9 | <0.36 | – | – |
| 5 | 5.7 | 0.8 | – | – |

HBT, heterophile-blocking tubes; Tg, thyroglobulin.

Tg Immunoassay problems

- Interference of Tg-ab's (25% in DTC patient)
- Interference of Hab's (prevalence 1-3%)
- Lack of concordance across platforms

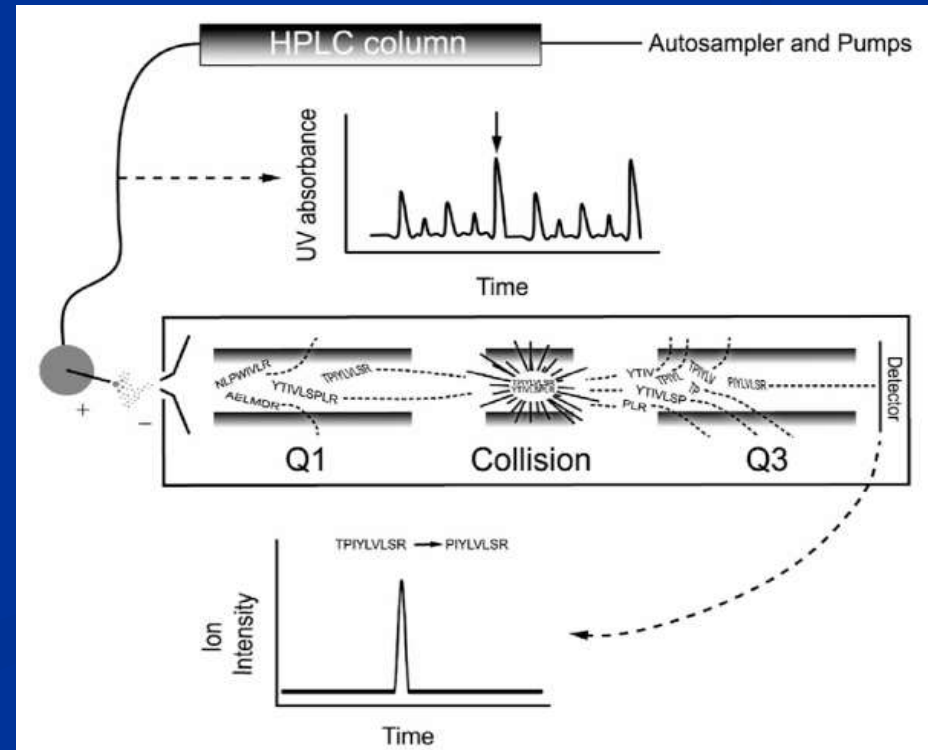
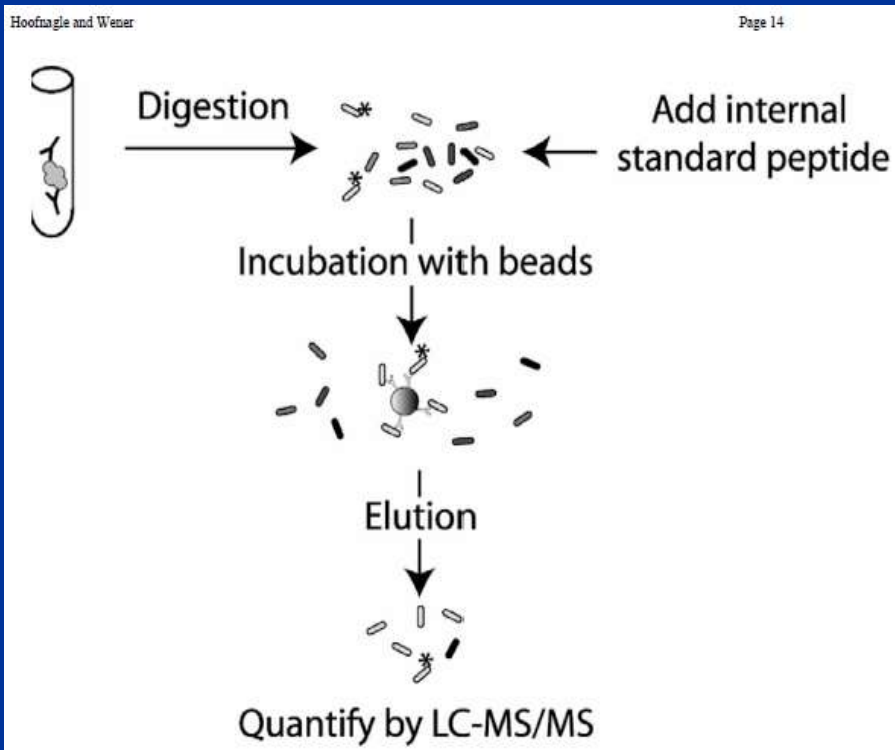
Can we use other methods to detect Tg?

Is LC-MSMS for Tg the solution?

Quantification of Thyroglobulin, a Low-abundance Serum Protein, by Immunoaffinity Peptide Enrichment and Tandem Mass Spectrometry

Andrew N. Hoofnagle^{1,*}, Jessica O. Becker¹, Mark H. Wener¹, and Jay W. Heinecke²

Clin chem 2008



Is LC-MSMS for Tg the solution?

- Laborious method: tryptic digestion (4h + 16h), immunoaffinity peptide enrichment using polyclonal Ab's and LCMSMS
- Total time of analysis: 2 days
- Lower limit of detection: 2.6 µg/L, but functional sens will be higher
- **Problems with LCMSMS**
 - Detecting a peptide does not mean detecting a protein
 - Plasma is a complex mixture → interference of homologous peptides
 - Digestion to completion of large amount of protein (Tg in µg/L en total protein in g/L)
 - Posttranslational modification will affect LCMS results
 - Polymorphisms resulting in changes in the peptides of interest will lead to loss of detection by mass spectrometry

Not a solution yet, but promising

Summary/Conclusions

- The Tg molecule is variable in its presentation → variable reactivity with the antibodies
- Sensitivity: sensitive Tg assays can reduce the rhTSH-Tg tests
- Standardisation: the use of CRM457 was not the solution for TG standardisation. We need a better standard for DTC patients
- Interferences:
 - TG-ab's: large intermethod variation; reduces Tg (metric assay) many samples are misclassified as Tgab negative
 - Hab's can increase or reduce Tg and Tg-Ab results
- Future: LCMSMS?

Thank you