



بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ



*Thyroglobulin & Thyroglobulin
antibody*

Challenges in assays & clinical interpretation

Maryam Tohidi

Associate professor of anatomical & clinical pathology

Research Institute for Endocrine Sciences

Shahid Beheshti University of Medical Sciences

January 2022

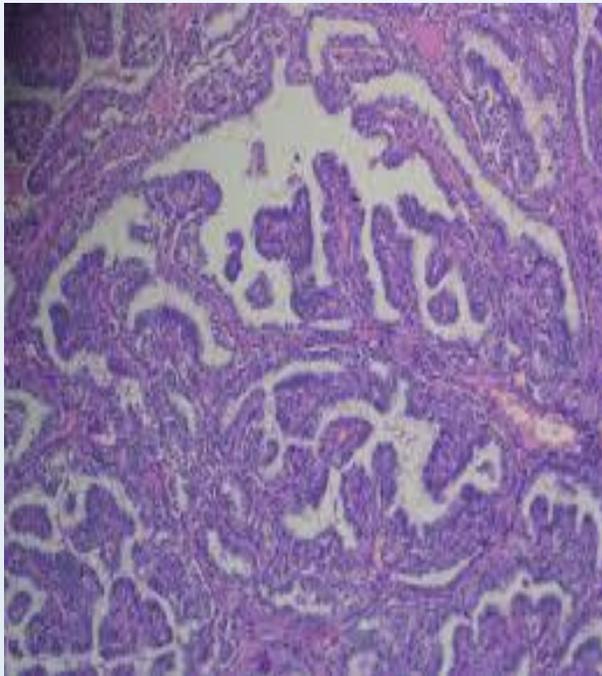
Tehran, Iran

Introduction

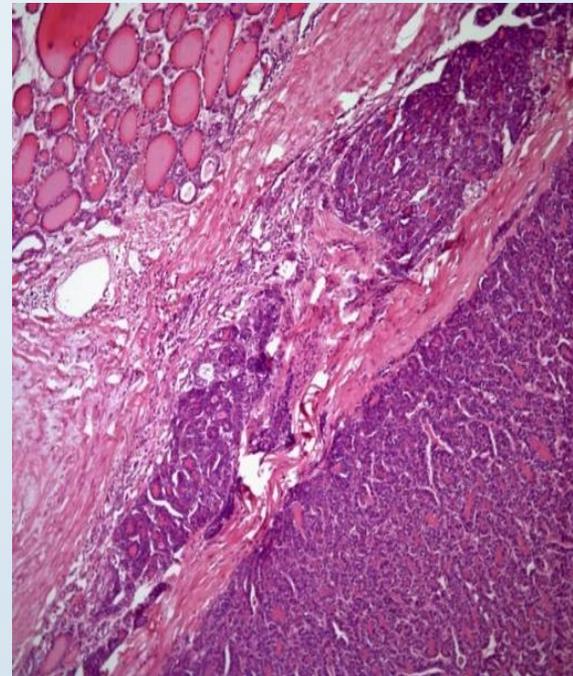
- ✓ Thyroglobulin (Tg): a glycoprotein (*660kDa*) produced exclusively in the thyroid gland
- ✓ Small amounts of Tg are secreted alongside thyroid hormones & present in the serum of healthy subjects.
- ✓ Elevated serum Tg levels in many thyroid diseases (goiter, Graves' disease & thyroiditis)
- ✓ Serum Tg, an insensitive and non-specific test for thyroid cancer
- ✓ Serum Tg test, not recommended for initial evaluation of thyroid nodules

Introduction

- ❖ Serum Tg measurement is mainly used as a tumor marker for management of patients with differentiated thyroid carcinomas (DTC).



Papillary thyroid carcinoma



Follicular thyroid carcinoma

Introduction

- ✓ Measurement of serum Tg (with Tg-Ab), with or without neck ultrasound, are frequently performed as part of the early post-thyroidectomy assessment.
- ✓ For initial follow-up, the recommended interval for measurement of serum Tg is 6–12 months, although, for high-risk patients more frequent Tg measurements may be appropriate.
 - ✓ *Strong recommendation, Moderate-quality evidence*
- ✓ A single rhTSH-stimulated serum Tg <0.5-1.0 ng/mL in the absence of Tg-antibody (Tg-Ab) has an approximately 98–99.5% likelihood of detecting patients completely free of tumor on follow-up.

American Thyroid Association guidelines

Factors affecting post-operative serum Tg & its predictive value

- ✓ Amount of residual thyroid cancer and/or normal thyroid tissue
- ✓ Serum TSH level at the time of Tg measurement
- ✓ Functional sensitivity of the Tg assay
- ✓ Tg cut-off used for analysis
- ✓ Individual risk of having RAI avid loco-regional or distant metastasis
- ✓ The time elapsed since total thyroidectomy
- ✓ Sensitivity of the post-therapy scanning technique (SPECT/CT vs planar imaging)

For post-operative serum Tg evaluation

- ✓ Tg assay should have a functional sensitivity of <1 ng/mL
- ✓ Tg assay should be calibrated against the standard reference material [CRM457 (Recommendation of ATA) or BCR-457]
- ✓ The use of the same Tg assay over time
- ✓ Measurement of TgAb in all samples tested for Tg for identifying samples with potentially falsely low or falsely high Tg due to TgAb interference

Methodologies of Tg assays

I. Immunoassays

- a) Competitive radioimmunoassay (RIA)*
- b) Immunometric assays (IMA)*

II. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays

These methods are different in:

Sensitivity, specificity & susceptibility to interference from TgAb and heterophile Abs.

- ✓ Currently, most laboratories use IMA Tg assay due to practical advantages:
 - ✓ shorter incubation times
 - ✓ automation
 - ✓ higher sensitivity & specificity

Challenges in Tg assays

I. Between-method biases

- ✓ Greater than within-person biological variability of Tg
- ✓ This issue in Tg methods can disrupt the serial monitoring of patients.
- ✓ A study that included 6 Tg immunoassays (*standardized against BCR-457*) showed *about a 2 fold difference* in mean Tg in TgAb-negative euthyroid subjects. (*Spencer CA, Lopresti JS. Nat Clin Pract Endocrinol Metab. 2008;4:223–233.*)
- ✓ Between-method variability (*despite CRM-457 standardization*) probably reflects differences in assay specificity for heterogenous circulating Tg isoforms.

Challenges in Tg assays

II. Functional sensitivity of Tg assay

- ✓ A measure of an assay's precision at low analyte levels
- ✓ Defined as the lowest Tg concentration that can be measured in human serum with a *CV of 20%* over a *6–12-month period* using *at least two different lots of reagents* and *two instrument calibrations*.
- ✓ A change in assay imprecision over time could have a negative impact on patient management.

Challenges in Tg assays

II. Functional sensitivity of Tg assay (cont.)

- ✓ Functional sensitivity of many modern assays is **< 0.1 ng/mL (second generation)**, which may lead to greater confidence of Tg on thyroid hormone therapy instead of performing Tg determination following TSH stimulation.
- ✓ Suboptimal Tg assay functional sensitivity compromises the detection of recurrent DTC in the absence of recombinant human TSH (rhTSH) stimulation.

Challenges in Tg assays

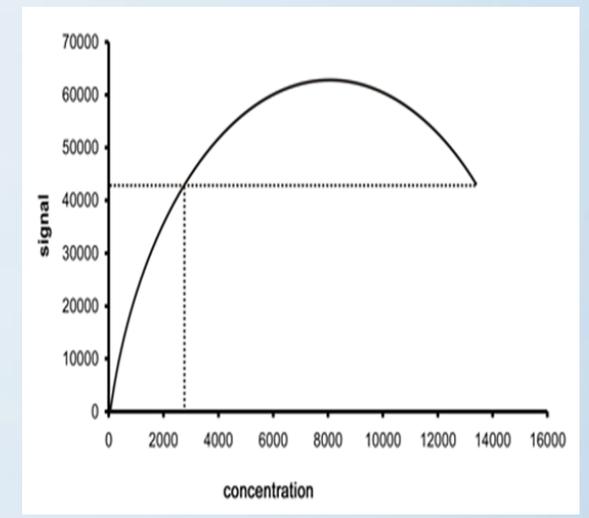
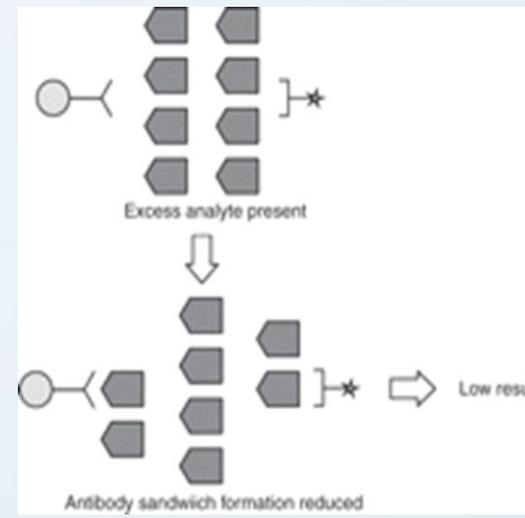
II. Functional sensitivity of Tg assay (cont.)

- ❖ An optimal target for functional sensitivity would be a Tg value approximately 100-fold below the lower reference limit. This target is much lower than would be expected for Tg arising from postoperative thyroid remnants [$\sim 1=2$ ng/ml (g/liter) when TSH is not elevated].

Baloch et al. Guidelines Committee, National Academy of Clinical Biochemistry 2003 Laboratory medicine practice guidelines: laboratory support for the diagnosis and monitoring of thyroid disease. Thyroid 13:3–126.

Challenges in Tg assays

III. Hook effect



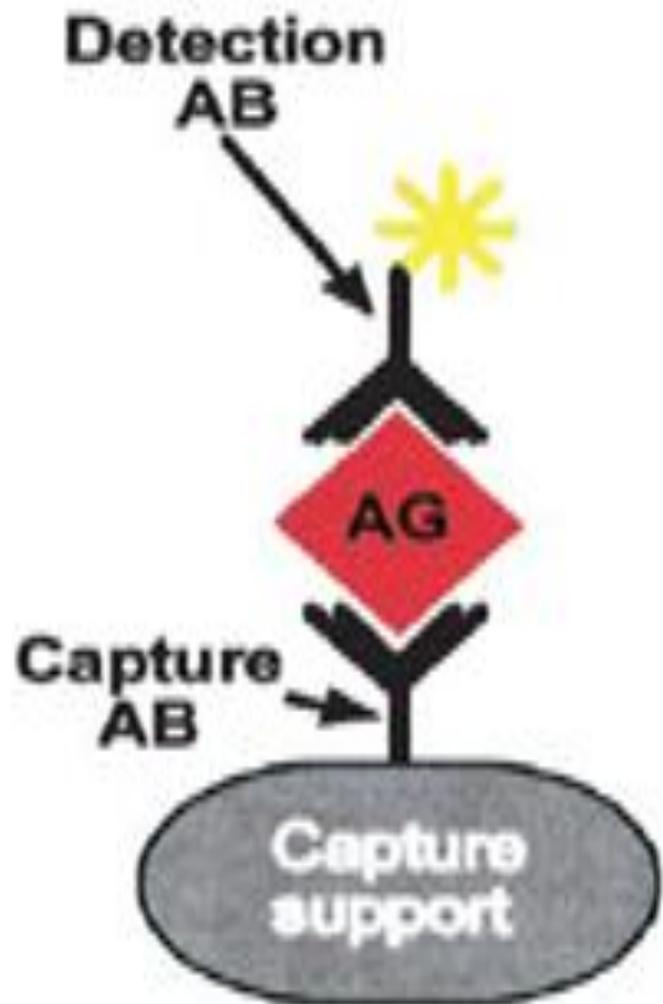
- ✓ may be seen at the upper end of the measurement range
- ✓ **very high antigen concentrations** (in some patients with metastatic disease)
- ✓ Tg concentration exceeds the binding capacity of the capture antibody
- ✓ resulted in **falsely low values**.

Challenges in Tg assays

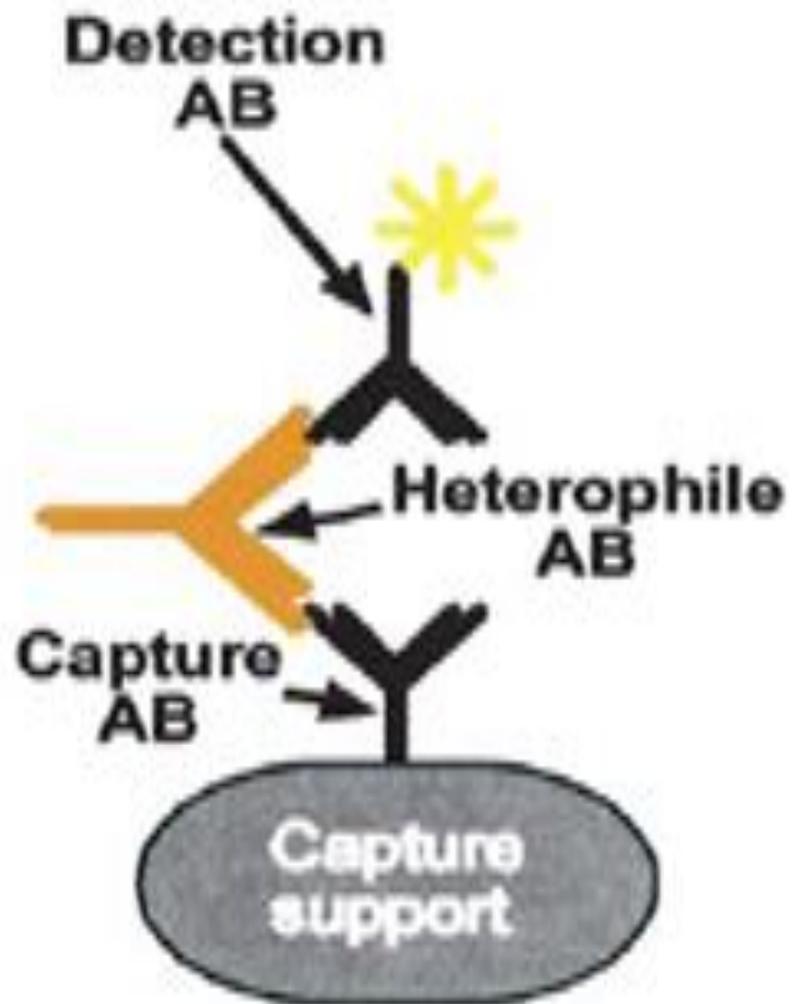
IV. Assay interference by Heterophilic antibody (HAB)

- ✓ HAB are antibodies that can bind to animal antigens.
- ✓ HABs are **usually resulted in overestimated Tg results** by making a bridges between capture & detection antibody in IMA assays.
- ✓ Rarely HAB may also resulted in **falsely low results**.

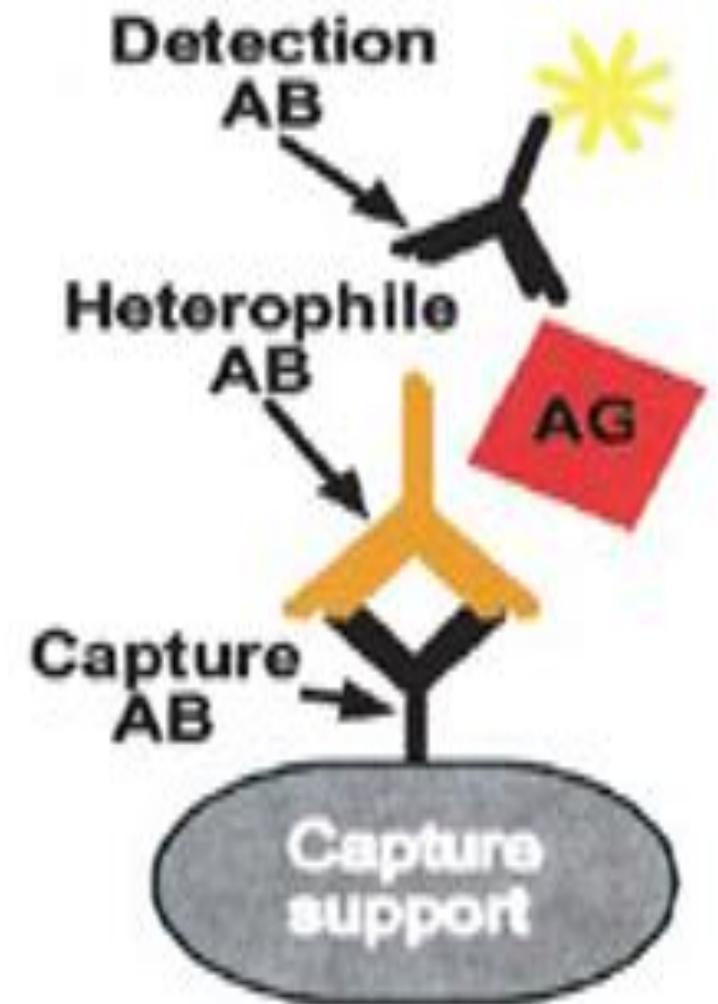
A no interference



B false high/positive



C false low/negative



Challenges in Tg assays

IV. Assay interference by Heterophilic antibody (HAB)

- ✓ HAb interference in Tg IMA is not routinely evaluated.
- ✓ HAB interference should be suspected if Tg results are not appropriate to the clinical findings.
- ✓ Manufacturers have reduced (but not removed) the risk of HAB interference by addition of blockers to assay reagents in modern IMAs.

Evaluation of HAB interference

I. Dilution/Serial dilution & evaluation of linearity

It means a recovery of between 80-120% of the expected value after dilution. Any samples with recovery outside this range after dilution are deemed to exhibit non-linear dilution.

II. Repeat the initial Tg measurement using heterophilic- blocking tubes or reagents which contain a mix of lyophilized mouse antihuman immunoglobulins with high affinity for human anti-animal Abs.

Specific blockers i.e. IgG or IgM directed against human anti-animal immunoglobulins are superior to non-specific blockers (general mixture of animal Igs).

III. Polyethylene glycol precipitation (PEG 6000)

IV. Tg measurement by an alternative IMA or RIA method

V. Using LC-MS/MS (Netzel BC, et al. Usefulness of a thyroglobulin liquid chromatography tandem mass spectrometry assay for evaluation of suspected heterophile interference. Clin Chem. 2014;60:1016–1018.) 17

Phantoms in the Assay Tube: Heterophile Antibody Interferences in Serum Thyroglobulin Assays

(The Journal of Clinical Endocrinology & Metabolism. 2003, 88(7):3069–3074)

Aim: Evaluated the prevalence of HAB interference in a commonly used automated immunoassay in 1106 consecutive specimens with Tg values greater than 1 ng/ml.

Conclusions:

- ✓ HAB interference is relatively prevalent (1.5–3%) in a commonly used automated Tg assay and can lead to clinically significant artifacts.
- ✓ HAB interference should be suspected if Tg results are not appropriate to the clinical picture, unless a Tg assay is confirmed to be free of HAB interference or uses additional blocking steps.

Challenges in Tg assays

V. Assay interference by Tg autoantibody (Tg-Ab)

TgAb are present in:

- ◆ approximately 10% of the general population
 - ◆ up to 20-30% of patients with DTC
-
- ✓ Circulating Tg-Ab interferes with serum Tg assays in a *qualitative*, *quantitative*, and *method-dependent* fashion.
 - ✓ TgAb interference is variable between patients.
 - ✓ The severity of interference does not correlate with TgAb concentration.

Challenges in Tg assays

V. Assay interference by Tg –Ab (Cont.)

- ✓ IMAs are prone to interference from Tg Abs, which commonly is the source of **falsely low serum Tg result**.
- ✓ Although, the RIA methods appeared to be more resistant to Tg-Ab interference, but these methods have the potential to either under- or overestimate Tg depending on the affinity and specificity of the antibody reagents & Tg & Tg-Ab concentrations.
- ✓ LC-MS/MS

Thyroglobulin (Tg) testing revisited: Tg assays, TgAb assays and correlation of results with clinical outcomes

Brian C. Netzel, Stefan K. G. Grebe, B. Gisella Carranza Leon, M. Regina Castro, Penelope M. Clark, Andrew N. Hoofnagle, Carole A. Spencer, Adina F. Turcu, and Alicia Algeciras-Schimnich

- ✓ Using Tg and Tg-Ab admixtures
- ✓ At Tg concentrations of 1 mg/L, Tg-RIA showed overestimation of Tg ranging from 2- to 11-fold in the presence of Tg-Ab.
- ✓ In the most clinically relevant TgAb concentrations (100–200 IU/mL) the observed overestimation of the Tg-RIA was between 2- and 5-fold at a Tg concentration of 1 mg/L.

Conclusion: The effect of Tg-Ab on Tg-RIAs is Tg-Ab and Tg concentration dependent.

J Clin Endocrinol Metab. 2015 Aug;100(8):E1074-83. doi: 10.1210/jc.2015-1967.

Challenges in Tg assays

V. Assay interference by Tg -Ab (Cont.)

- ✓ Tg-Ab interference is more difficult to detect and overcome.
- ✓ The prevalence of Tg-Ab in DTC patients is approximately 2-3 times compared to the general population.

- ✓ The difference in Tg-Ab prevalence in DTC populations may be due to:
 1. Different anti-Tg assays
 2. Difference in the frequency of chronic lymphocytic thyroiditis

Assessment of Tg-Ab interference in Tg-assay

- I. Repeat of Tg measurement by another method
- II. Tg-Ab measurement to assess Tg-Ab interference
- III. Thyroglobulin (Tg) Recovery Testing
- IV. Mass spectrometry assays

I. Repeat of Tg measurement by another method

- ❖ A reliable hallmark of Tg-Ab interference is the presence of RIA/IMA discordance.
- ✓ However, inter-method comparisons are impractical because today RIAs for Tg are not widely used.
- ✓ RIAs for Tg may be less sensitive (functional sensitivity of 0.5 ng/mL) than IMAs in detecting small amounts of residual tumor.

II. Tg-Ab measurement

There are some limitations/challenges:

- I. Tg-Ab methods are highly variable & Tg-Ab detection is strongly method dependent.
- II. Tg-Ab concentrations do not correlate with the severity of interference.
- III. Tg-Ab positivity does not point toward interference per se, substances other than TgAbs can interfere with Tg assay.
- IV. Tg-Ab assays may be affected by HAB.
- V. Reference range (often determined for chronic lymphocytic thyroiditis)

Limitations/Challenges in Tg-Ab assays (Cont.)

- ✓ Spencer et al. (*J Clin Endocrinol Metab* 2005;90:556–75) have reported that the use of the cut-off s determined for identifying patients with autoimmune thyroid disease, rather than the detection limit, can cause misclassification of patients as antibody-negative.
- ✓ Spencer et al. (*Clin Endocrinol Metab.* 2011;96:1283–1291) proposed the use of the LoD (lowest concentration of analyte that can be detected with a stated uncertainty) for a given assay as the cutoff for Tg-Ab positivity when evaluating Tg assay interference.
- ✓ Other studies have suggested the use of the functional sensitivity or limit of quantification (LoQ) for the evaluation of TgAb interference.
- ✓ Using the LoQ instead of the LoD as a lower limit for Tg-Ab detection would provide a higher degree of certainty that the measured Tg-Ab concentration represents a potentially relevant interference.

Netzel BC et al., J Clin Endocrinol Metab. 2015;100: E1074–E1083 & *Spencer C et al. Curr Opin Endocrinol Diabetes Obes.* 2014;21:394–404.

Example: A commercial kit for Tg-Ab

- Measuring range: 10.0-4000 IU/mL (defined by the lower detection limit and the maximum of the master curve).
 - Values below the lower detection limit are reported as < 10.0 IU/mL.
 - Values above the measuring range are reported as > 4000 IU/mL.
 - Lower limits of measurement
 - Lower detection limit: < 10.0 IU/mL
- ❖ This should also be determined by individual laboratories.

Waddah Katrangi, Stephan K.G. Grebe and Alicia Algeciras-Schimnich*

Analytical and clinical performance of thyroglobulin autoantibody assays in thyroid cancer follow-up

- ✓ Aim: To evaluate the concordance between 4 commonly used Tg-Ab assays using the LoQ of the respective assays
- ✓ positive and negative concordance ranged from moderate to substantial (kappa values 0.41 to 0.71)
- ✓ The same group demonstrated that discordance between assays was significant for classification of TgAb-positive cases.

Original Article

Causes of discordance between thyroglobulin antibody assays

Alan John Pickett, Meinir Jones and Carol Evans

Department of Medical Biochemistry & Immunology, University Hospital of Wales, Heath Park, Cardiff CF14 4XN, UK

Corresponding author: Dr Carol Evans. Email: Carol.evans9@wales.nhs.uk

***Ann Clin Biochem* 2012; 49: 463–467. DOI: [10.1258/acb.2012.012008](https://doi.org/10.1258/acb.2012.012008)**

Limitations/Challenges in Tg-Ab assays (Cont.)

Discordance between Tg-Ab assays has been attributed to a high variability in their characteristics & analytical performance including:

- ✓ Variation in limit of detection
- ✓ Different functional sensitivity
- ✓ Heterogeneity of Tg-Ab
- ✓ Differences in the specificity of circulating Tg-Ab for Tg antigen
- ✓ Tg interference
- ✓ Differences in assay reagents (including the particular preparations of Tg employed) and the standards used by the various methods.

Important points about Tg-Ab measurement to assess Tg-Ab interference

- ✓ Tg-Ab assay could miss a proportion of interfering Tg-Abs.
- ✓ It is proposed that in thyroid cancer patients with surgically proven Hashimoto thyroiditis, measurement of Tg-Ab by an alternate assay might be considered if the routine Tg-Ab assay is negative.
- ✓ Tg-Ab assays should not be used interchangeably & in different laboratories.
- ✓ Clinicians, should be aware that Tg-Ab results should not be compared between different assays.

III. Thyroglobulin (Tg) Recovery Testing

- ❖ Tg in sera with (Tg2) and without (Tg1) added Tg (aTg) are measured and calculated the percentage recovery from the following formula:

$$\frac{\text{Tg2} - \text{Tg1}}{[\text{aTg}]} \times 100$$

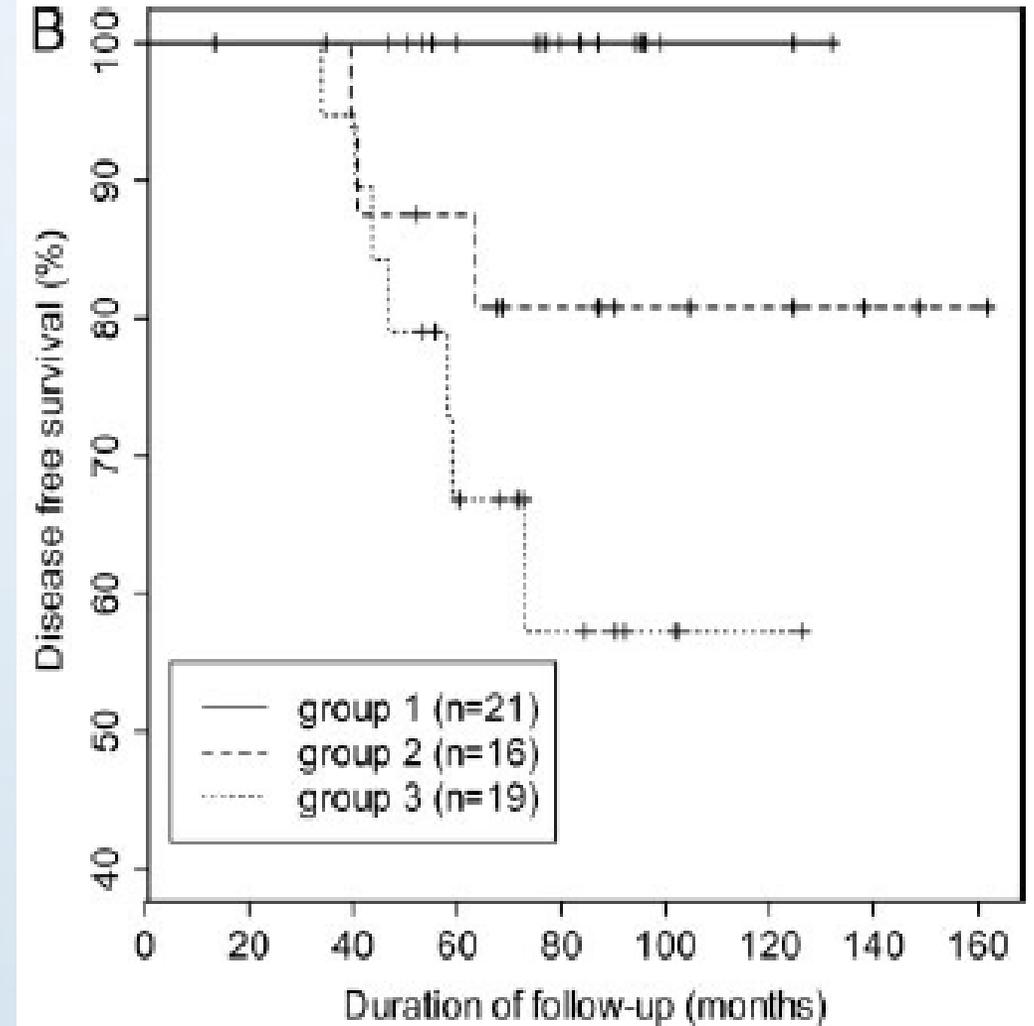
- ❖ Tg- recovery < 80% might reflect some form of interference, e.g., by autoantibodies not recognized by the Tg-Ab assay or from substances other than Tg-Abs.
- ❖ Some studies & professional guidelines indicated that the Tg recovery test is not reliable enough indicate that all samples preferably be pre-screened for Tg-Ab by sensitive IMA methods prior to Tg assay.

Thyroglobulin (Tg) Recovery Testing with Quantitative Tg Antibody Measurement for Determining Interference in Serum Tg Assays in Differentiated Thyroid Carcinoma
(*Clinical Chemistry* 52, No. 6, 2006, 1196-99)

- ❖ TgR assay has **value complementary** to that of quantitative TgAb measurement in the detection of interference in Tg measurements, **in particular in sera with low Tg-Ab titers**.
- ❖ This method can detect interference from Tg-Abs not detected by direct Tg-Ab measurement or from other interfering substances, such as heterophilic antibodies.

Tg-Ab as surrogate tumor marker For DCT

- ✓ Tg-Ab levels decline progressively and become undetectable during the first few post-thyroidectomy years.
- ✓ Patients with decreasing Tg-Ab levels are at a lower risk of recurrent or persistent disease than patients with stable or rising TgAb concentrations.



Tg-Ab as surrogate tumor marker For DCT (Cont.)

- ✓ Serial quantitative Tg-Ab measurements are of use for the follow-up of differentiated thyroid cancer (DTC).
- ✓ Using TgAb as a tumor marker is only possible when the same assay is used longitudinally.

IV. Mass spectrometry assays

- ✓ Tg by LC-MS/MS (Tg-MS) as a solution for accurate Tg quantitation in the presence of Tg-Ab.
- ✓ Excellent agreement and correlation with commonly used Tg-IMA in the absence of Tg-Ab (difference < 10%).
- ✓ For most TgAb-positive samples: ***Tg-MS > Tg-IMA***
- ✓ The degree of IMA underestimation varies significantly depending on the assay
- ✓ Tg-MS assays have suboptimal analytical sensitivity (0.5-0.6 ng/mL) that may affect clinical performance of Tg-MS assays.

Tg measurement in needle washout fluid after FNAB

- ✓ First proposed by Pacini (1992)
- ✓ A growing trend to measure Tg in the needle washout fluid following fine-needle aspirate biopsy (FNAB) of suspicious lymph nodes on cases of suspected metastatic DTC.
- ✓ As an adjunct to cytological examination
- ✓ Compare favorably with cytology and often allows for the accurate diagnosis of cases in which cytology is non-diagnostic.

Tg measurement in needle washout fluid after FNAB

- ✓ Sensitivity of 95% & specificity of 94% *(a systematic review and meta-analysis of diagnostic accuracy including 24 studies & 2865 LN FNAB. J Clin Endocrinol Metab. 2014;99:1970–1982)*
- ✓ Significant heterogeneity in clinical sensitivity and specificity was attributed to:
 - ◆ differences in patient selection
 - ◆ collection technique & media (normal saline, phosphate buffered saline (PBS) or Tg-free serum)
 - ◆ analytical methods
 - ◆ the cutoff used for classification in patients with and without thyroid gland
- ✓ Serum TgAb interference in Tg measurement in FNA wash out sample *(Hyun Joo Shin et al. PLoS One. 2015 Jun 29;10(6):e0131096)*

Messages

- ✓ Ideally, serum Tg and Tg-Abs should be assessed longitudinally in the same laboratory and using the same assay for a given patient.
(Strong recommendation, High-quality evidence)
- ✓ Despite improvements in standardization of Tg assays, there is still a 2-fold difference between some assays, leading to the recommendation that measurements in individual patients over time be performed with the same assay.

Messages

- ✓ The presence of Tg-Abs should be suspected when the surgical pathology indicates the presence of background Hashimoto thyroiditis.
- ✓ Tg-Ab should be quantitatively assessed with every measurement of serum Tg.
- ✓ TgR assay has value complementary to that of quantitative Tg-Ab measurement in the detection of interference in Tg measurements, in particular in sera with low Tg-Ab titers.
- ✓ HAB interference should be suspected if Tg results are not appropriate to the clinical picture.

Messages

- ❖ Laboratories must keep clinicians informed about any changes in the TgAb assay and suggest to measure patient samples at least once with both assays to establish an individual baseline level if a change in assay occurs (re-baseline).
- ❖ Tg measurement in the needle washout fluid following FNAB of suspicious lymph nodes is an useful tool, as an adjunct to cytological examination on cases of suspected metastatic DTC, especially when FNAB is non-diagnostic.

