

Short review

Serum thyroglobulin measurement: clinical background and main methodological aspects with clinical impact

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Abstract

It is worldwide recognized that circulating thyroglobulin (Tg) measurement represents a fundamental tool in the follow-up of patients affected by differentiated thyroid cancer (DTC). In the last American and European Consensus Conferences, a surveillance guideline has been extended to the use of thyrotropin (TSH)-stimulated Tg levels for thyroidectomized patients without clinical evidence of residual tumor with Tg below 1 µg/l during TSH suppression. Therefore, sensitivity of the methods is critical to detect small amounts of Tg and/or to observe minimal changes in Tg concentration in the management of DTC patients. It has been proposed that only methods providing the greatest distinction between the lower limit of euthyroid reference range (~ 3.0 µg/l) and the functional sensitivity limit (at least 1 µg/l) of the assay may offer a suitable clinical sensitivity for detecting small amounts of functioning thyroid tissue in TSH-suppressed state (1 g of normal thyroid tissue results in a serum Tg of approximately 1 µg/l when TSH is normal and about 0.5 µg/l when TSH is suppressed). In the last 30 years sensitivity of Tg measurements has been greatly improved, nowadays methods can achieve very good analytical and functional sensitivity to give reliable results also in the very low concentration range (between 0.1 and 1 µg/l). In addition, with the introduction of fully automated assays, results can be readily available to the clinician while patients are still in the ambulatory area. However, despite the large clinical use of Tg measurement, wide differences (by threefold) still remain between results produced in different laboratories due to poor standardization, heterogeneity of circulating Tg, interference from auto-antibodies, differences in the epitope recognition by antibodies used in the assays.

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Keywords: Thyroglobulin; Immunoassay; Differentiated thyroid cancer

Abbreviations: ACC, Access Beckman Coulter; CONS, consensus mean; DTC, differentiated thyroid cancer; ECLIA, elettrochemiluminescentimmunoassay; ELISA, enzyme linked immunoassay; HAMA, human anti-mouse antibodies; ICMA, immunochemiluminometricassay; ILMA, immunochemiluminescentassay; IMA, immunometricassay; IMM2, Immulite one/2000 DPC; CIS, CIS-Schering IRMA; IRMA, immunoradiometricassay; LSN, Liaison DiaSorin; MOD, Modular/Elecsys Roche; NACB, National Academy of Clinical Biochemistry; rh-TSH, recombinant human TSH; RIA, radioimmunoassay; SOR, DiaSorin IRMA; T3, triiodothyronine; T4, thyroxine; TAT, Turn Around Time; Tg, thyroglobulin; TgAb, anti-Tg auto-antibodies; TSH, thyrotropin; US, ultrasonography; WBS, whole-body-scanning.

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1. Introduction

1.1. Background

Thyroglobulin (Tg) is a large glycoprotein comprising two apparently identical polypeptide chains that make up a molecule of approximately 660 kDa stored in the follicular colloid of thyroid gland; it acts as pro-hormone in the intra-thyroid synthesis of thyroxine (T4) and triiodothyronine (T3) and it is produced only by normal thyrocytes or well-differentiated thyroid cancer (DTC) cells. Tg is usually measured in serum, but measurements can also be made in thyroid cyst fluids and materials obtained by fine needle biopsy of thyroid nodules [1]. Serum Tg is elevated in patients with goiter and in most hyperthyroid conditions; a low serum Tg can be also an useful biomarker to confirm the diagnosis of thyrotoxicosis factitia and/or to investigate the ethiology of congenital hypothyroidism [2,3]; Tg measurements can be also useful to confirm a past history of thyroiditis [4].

The primary use of serum Tg measurement is, however, as tumor marker for patients affected by DTC [5–8]; its growing role as a biomarker with a very high reliability is due to the fact that, based on recently published data [9], the frequency of diagnosis of new thyroid cancer appears to be increasing especially in women. The principal aim of the present review is to focus on the new insides on Tg measurement and on the potential clinical impact of more recent immunoassays in following-up patients treated for DTC.

1.2. Tg measurement and DTC: commonly accepted and unsolved key points

Long term survival rate for DTC is more than 90%: 33% of patients develop tumor recurrence depending of initial therapy; 66% of the recurrences occur within the first 10 years and 50% within the first 5 years after therapy; 30% of the recurrences could not be eradicated and 15% of patients die of disease. Mortality rates are lower when recurrences are detected early [6]. Also, more recent data show that a delay in primary treatment has a large impact on outcome, and it thus seems likely than an adverse outcome may result from a delay in the treatment of unrecognized disease that persists after initial management [10].

The spectrum of patients with DTC has changed in the last years and a larger number of DTC are being discovered at an earlier stage; the majority of patients are classified as low risk (stage I < 45 years and stage I or II if > 45 years according to the AJCC/TNM 2002 classification) [11]. Thus, the follow-up should be guided by a protocol with a high negative predictive value to reduce the number of unnecessary investigations and to identify the few individuals with a previously unrecognized risk of recurrence [12].

Because of its tissue specificity and limited tissue distribution, Tg detection in serum represents a very high specific tumor marker for patients treated for DTC. A strong correlation between serum Tg levels and the amount of DTC tissue has

been reported [13–15]. Its main utility is in identifying tumor well before it is apparent by imaging modalities or physical examination [16,17].

Despite the universal application of Tg measurement, no consensus exists on the frequency of Tg determination, the threshold values and whether should be measured during L-T4 treatment alone and/or during thyrotropin (TSH) stimulation; some Authors believe that the pattern of change in Tg values on L-T4 is a better indicator of a change in tumor burden than any single Tg value [18], in fact Tg values on L-T4 is a more stable indicator of tumor mass than Tg measured when the TSH is high (L-T4 withdrawal or recombinant human, rh-TSH administration); 10–15% of patients however who has an undetectable Tg on L-T4 has a detectable Tg after TSH stimulation [19]. According to recent studies, a Tg value < 0.5 µg/l after rh-TSH (rh-TSH) in the absence of anti-Tg auto-antibodies (TgAb) has 98% likelihood of identifying patients completely free of tumor, a large group in which TSH suppression to < 0.1 µg/l and frequent imaging and TSH stimulated Tg testing are unnecessary [10].

1.3. Long-term DTC monitoring during L-T4 suppression

Optimal management of DTC patients includes performance of the appropriate surgical procedure, usually total or near total thyroidectomy, followed by radioiodine ablation of residual tissue and L-T4 treatment to decrease serum TSH to a level that minimizes the risk of stimulating growth of any cancerous thyroid cells. Since DTC may recur at any time for years and L-T4 therapy is life-long, a long-term follow-up by serum Tg measurements and ultrasonography (US) and/or whole-body-scanning (WBS) is necessary [20–22]. Tg measurement is then a part of routinely surveillance and now it is considered the elected biological marker for papillary and follicular thyroid cancer, together referred to DTC [23,24].

1.4. Serum Tg response to TSH stimulation

Some tumors may lack or reduce the ability to secrete Tg; for this reason Tg measured during TSH stimulation is generally considered as a more sensitive index for residual thyroid carcinoma than a basal Tg measurement during L-T4 treatment [25]. The recent availability of rh-TSH makes it possible to stimulate Tg production without the need to induce hypothyroidism by withdrawal of synthetic thyroid hormone supplementation [16,18,25–27]. Well-differentiated tumors typically display a 10-fold stimulation of serum Tg in response to a high TSH [28]; poorly differentiated tumors that do not concentrate iodine may display a blunted response to TSH stimulation. It is still controversial if serum Tg response to rh-TSH might be sufficient by itself to detect occult residual thyroid cancer [18,29–34]; according to some clinicians, *in lieu* of a diagnostic WBS performed 1 year after thyroid ablation, serum Tg measurement after L-T4 withdrawal or rh-TSH stimulation may serve as a guide for the selection of patients who might have persistent cancer [35]. TSH-stimulated serum Tg

may also limit the effects of the lack of sensitivity of methods for serum Tg levels measured during TSH suppression [19]. The conventional accepted cut-off for disease-free patients was assumed 5 µg/l during L-T4 withdrawal; 2 µg/l after rh-TSH stimulation; 1 µg/l during TSH suppressive therapy [25, 36–38].

1.5. Current guidelines for the follow-up of DTC patients

In the two American recent Tg consensus conferences [17, 38], a surveillance guideline has been proposed using TSH-stimulated Tg levels for thyroidectomized and radioiodine-ablated patients without clinical evidence of residual tumor with a serum Tg value below 1 µg/l during TSH suppression. The goal of the consensus report was to formulate from the current literature a cost-efficient post-operative surveillance paradigm for DTC patients who clinically appear to have no evidence of disease after total or near total thyroidectomy and 131-I thyroid remnant ablation. The following seven key points were addressed: (1) The follow-up paradigm should only be applied to those patients who are at low risk of persistent or recurrent disease or cancer death. (2) The physician must have a good working knowledge of the Tg assay that is being used and which should meet the minimum performance standards outlined by the US National Academy of Clinical Biochemistry (NACB) [19]. (3) Undetectable serum Tg level during TSH suppression may be misleading and it is not sufficient to consistently identify patients who are free of residual tumor. (4) Endogenous TSH-stimulated serum Tg levels produced by TSH withdrawal are comparable, in detecting metastases, to rh-TSH stimulated Tg, when a cut-off of 2 µg/l is used. (5) Although serum Tg and WBS have been considered complementary in identifying residual tumor [39,40], an undetectable TSH stimulated Tg alone is usually sufficient to be used alone in the follow-up. (6) Tg measurement, during thyroid hormone suppression, is sufficiently sensitive to avoid further testing in low-risk patients who are clinically free of disease and with an undetectable (<1 µg/l) Tg level during suppression and after rh-TSH stimulation or TSH withdrawal. (7) Many patients do not require annual TSH stimulated serum Tg measurement, information garnered from the prior Tg response to rh-TSH stimulation can form the basis of recommendations for further testing.

Recently, a group of European thyroid cancer specialists gathered at the Institute Roussy in France on March 2003 to assess the implication of pertinent recent studies on the follow-up of DTC and to issue recommendations for a revised follow-up protocol which includes four important elements [12]: (1) In patients with no evidence of disease up to the 6–12 months follow-up, (i.e. low-risk patients), diagnostic WBS adds no information when serum Tg is undetectable and interference from TgAg is absent. (2) The use of rh-TSH protocol represents the gold standard to obtain TSH stimulation for diagnostic follow-up providing greater safety, quality of life and work productivity than does L-T4 withdrawal with its attendant hypothyroidism. (3) Diagnostic radioiodine WBS can be reduced if a neck US, performed by an experienced operator, is available in the follow-up of low-risk patients with no evi-

dence of disease up to the 6–12 months follow-up. (4) The Tg threshold should be determined after rh-TSH administration for each assay method.

2. Tg measurement with clinical impact: methodological aspects

As for every blood determination, the clinical interpretation of the analytical results of Tg measurement strictly depends on the reliability and accuracy of the measurement itself. Theoretically, an ideal biomarker should be highly sensitive, specific and able to be reproduced across different clinical laboratories; the inherent error in the measurement itself (CV%) should be sufficiently low so that small changes in the level of the biomarker reflect true changes in the clinical status of the patient; finally, the assay should be relatively easy to perform and analyse so that the information is readily available to the clinician. As biomarker, Tg measurement should be the most sensitive, accurate and precise as possible [41,42,19,43]; Tg measurement should always be obtained from modern immunometric assay (IMA) with a functional sensitivity of at least 1 µg/l [12].

2.1. Technical problems

Since the first method to measure Tg was described [44,45], particular attention has been given to achieve a satisfactory sensitivity in the low concentration range to discover earlier the presence of tumoral cells in patients on treatment for DTC.

Thus, if on one hand a sensitive method to measure Tg is required, on the other hand serum Tg measurement is one of the most difficult biochemical test in maintaining high precision and reliability. Many analytical problems are related to Tg measurement as are here briefly summarized:

- Standardization of the analytical procedure and assay comparability [46];
- Long-term stability of assay to allow lifetime follow-up (high inter-assay precision over a period of 6–12 months) [19];
- High sensitivity and precision to allow Tg detection in the absence of normal thyroid tissue (in the low range of Tg concentration) [19];
- Working range and high-dose hook effect [19];
- Effect of endogenous TgAb (incidence in DTC is 15–30%) [47];
- Different Tg isoforms released by thyroid tumors that generate the development of antibodies with high specificity for Tg so that the standardization of the corresponding antisera became difficult [41].

All these drawbacks in the analytical performance of the assay may significantly influence the clinical value of Tg evaluation in DTC patients. Physicians should be aware that the diagnostic value of serum Tg is influenced by the laboratory choices to perform this test [48]. For this reason, a patient

should be better followed-up by using the same assay and results should be judged versus cut-off values determined in clinical studies using the same assay and finally the potential for interference by TgAb should be acknowledged [43]. The natural consequence of all the above-mentioned recommendations is the need for an external quality assessment scheme and for an appropriate internal quality control procedure.

2.2. Current status of Tg methods and related difficulties

Currently, IMA gained in popularity over radioimmunoassay (RIA) because they offer the advantage of shorter incubation time, an extended dynamic range for the assay and a more stable labeled antibody. Nowadays, laboratories can choose from a wide range of both isotopic (immunoradiometric assay, IRMA) and non-isotopic (mainly immunochemiluminometric assay, ICMA) methods.

2.2.1. Standardization

Data from a multicenter study conducted almost 10 years ago [49] showed that the between-laboratories variability in samples without TgAb was abnormally high (48 CV% in samples with Tg concentration above 5 $\mu\text{g/l}$ and up to 80–120 CV% in the low concentration range below 5 $\mu\text{g/l}$). These systematic differences between methods were mainly due to both different calibrators and different specificity of the antibodies used (different epitopes recognized by different antibodies). As expected, in samples containing TgAb the CV increased up to 93–116% due to different degree of interference from TgAb in the different methods, that causes underestimation of serum Tg levels [41,50] (see Section 2.2.3). Also the within-methods, between-laboratories, were clearly unsatisfactory (49–117 CV% in the low concentration range and 8–29 CV% in the range 7–73 $\mu\text{g/l}$). The widespread adoption of CRM-457 standard [46] has reduced, but not eliminated, the significant method-to-method variability (Fig. 1). It was hoped that standardization worldwide would facilitate better agreement in the literature from different studies as well as improve the clinical use of Tg determination in monitoring DTC patients. Unfortunately, serum Tg, measured with different methods using the same CRM-457 standard, showed a not appreciable reduction in between-methods variability and Tg results can be different by threefold. According to NACB guidelines [19] these differences, which are greater than the goal for maximum imprecision needed for monitoring an individual, preclude the use of different Tg methods for monitoring DTC patients.

To study these differences between methods, our Institution has organized a quality assurance program which involves at the moment about 90 Italian laboratories. The main advantage for a laboratory is to compare the chosen method to other commercially available methods and the results obtained from other laboratories using the same method. In Fig. 2 is showed the report of samples Tg53a, Tg53b and Tg53c obtained by laboratory code X7 using Access Beckman Coulter (ACC) for Tg measurement in 2005 cycle (unpublished data). The first sample had a Tg concentration of 36.21 $\mu\text{g/l}$ and was TgAb-

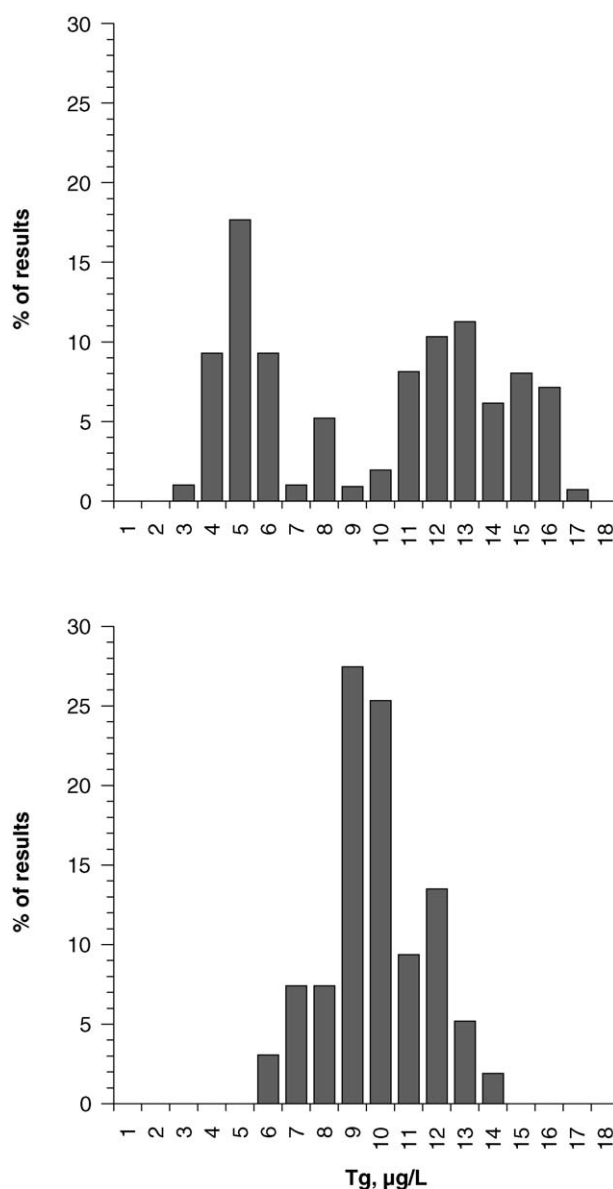


Fig. 1. Frequency distribution histograms of results obtained by participants of an external quality assessment for Tg performed in 1995 assaying pool P007. Upper panel: original results expressed in $\mu\text{g/l}$ of the kit standards (mean 9.2 $\mu\text{g/l}$, CV = 45.7 %). Lower panel: results converted to $\mu\text{g/l}$ of the common standard CRM 457 (mean 9.3 $\mu\text{g/l}$, CV = 18.8 %) using the conversion factor from $\mu\text{g/l}$ of the local standard to $\mu\text{g/l}$ of the common standard [49].

negative (total variability about 20 CV%); the second one had a Tg concentration of 18.15 $\mu\text{g/l}$ and was TgAb-positive (the larger variability, about 70 CV%, in samples with TgAb is attributable to the interference of auto-antibodies in Tg measurement which depend on the method employed); the third one had a Tg concentration of 0.76 $\mu\text{g/l}$ and was TgAb-negative (total variability about 40 CV% in samples with a Tg concentration < 1 $\mu\text{g/l}$ is attributable to the increased differences between methods and less precision in the low concentration range). From results collected in 2004 and 2005 total variability (between-labs, between-methods) for TgAb-negative samples resulted about 30 CV% for samples at Tg

Results processed by Istituto di Fisiologia Clinica CNR, Pisa

Tireoglobulina, ng/mL

Lab. code: X7

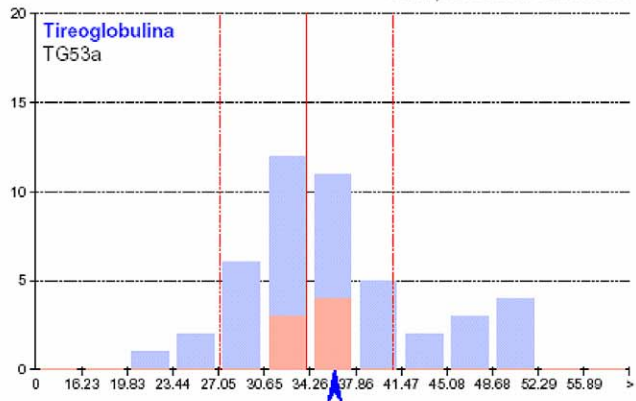
your result | Target | ±2SD

Sample: TG53a (30/09/2005)

Data processed: 03/10/2005

your result is: **36.21** your method is: **ACC**
 -0.8% vs CONS +6.5% vs your method
 z-value vs your method: 0.7 score: **3**

method	n.res	n.outl	mean	CV%	min	max
CONS	46	0	36.49	19.8	20.7	50.49
ACC	7	0	33.99	4.9	31.28	36.21
IMM2	19	0	36.47	9.2	32.4	42.9
MOD	6	0	49.17	3	46.6	50.49
SOR	4	0	29.07	5.9	27.45	31.44
CIS	3	0	33.77	35.3	20.7	44
LSN	3	1	24.82	1.6	24.53	25.1

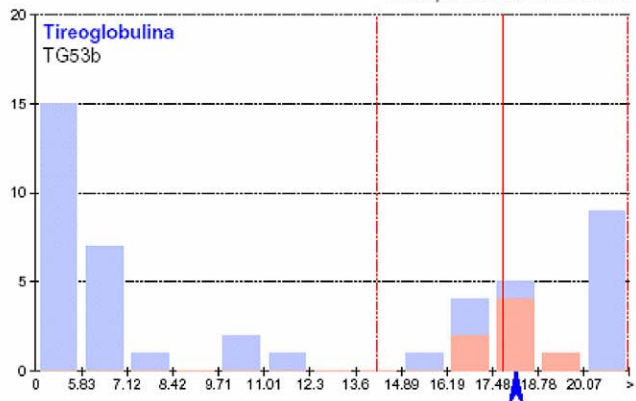


Sample: TG53b (30/09/2005)

Data processed: 03/10/2005

your result is: **18.15** your method is: **ACC**
 +14.1% vs CONS +2.2% vs your method
 z-value vs your method: 0.2 score: **4**

method	n.res	n.outl	mean	CV%	min	max
CONS	46	0	15.91	66.3	2.83	30.02
ACC	7	0	17.75	5.1	16.4	18.9
IMM2	19	2	5.78	7	5.19	6.95
MOD	6	0	28.47	3.3	27.3	30.02
SOR	4	0	17.7	10.8	15.7	20.3
CIS	3	0	20.2	44.7	10.6	28.5
LSN	3	0	5.56	16.8	4.65	6.52



Sample: TG53c (30/09/2005)

Data processed: 03/10/2005

your result is: **0.76** your method is: **ACC**
 +4% vs CONS +4.7% vs your method
 z-value vs your method: 0.2 score: **4**

method	n.res	n.outl	mean	CV%	min	max
CONS	46	4	0.73	43.6	0.06	1.4
ACC	7	0	0.73	7	0.64	0.77
IMM2	19	0	0.75	15.1	0.5	0.97
MOD	6	1	1.63	10.3	1.35	1.8
SOR	4	1	0.26	29.2	0.17	0.3
CIS	3	0	0.82	65.5	0.35	1.4
LSN	3	0	0.2	0	0.2	0.2

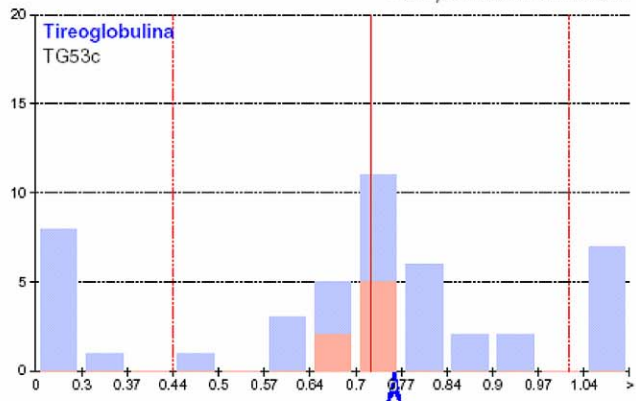


Fig. 2. Report of samples Tg53a, Tg53b and Tg53c obtained by laboratory code X7 using ACC for Tg measurement in 2005 cycle of our external quality assurance program. The report contains: statistics of all results and of results grouped according to the method used, laboratory result and its percent deviation from both consensus mean (CONS) and method mean by the attribution of a score on the basis of Z defined as the ratio between the percent deviation from target (the mean of the method) and the CV% “state of art” (within-method, between-laboratories variability of the mean of the methods). If $|Z| < 0.5$ the score attributed is 4 (excellent), if $0.5 < |Z| < 1$ score = 3 (good), if $1 < |Z| < 2$ score = 2 (sufficient), if $2 < |Z| < 3$ score = 1 (inadequate) and if $|Z| > 3$ score = 0 (outlier).

concentration $< 1 \mu\text{g/l}$, 20 CV% at Tg concentration < 5 and $> 1 \mu\text{g/l}$ and 10 CV% at Tg concentration $> 5 \mu\text{g/l}$.

Modular/Elecsys Roche (MOD) gave higher results as compared to other methods, while Immulite one/2000 DPC (IMM2) is more influenced by TgAb as compared to the

other methods giving lower Tg values. It appears that the clinical cut-off ($1 \mu\text{g/l}$ during suppressive therapy) is not well measurable in terms of analytical performance with the conventionally used assays. Moreover, in a control sample prepared from sera of thyroidectomized patients without clinical evidence of

residual tumor, Tg values reported by the participants ranged from 0.01 $\mu\text{g/l}$ of ACC to 1 $\mu\text{g/l}$ of Liaison DiaSorin (LSN).

2.2.2. Sensitivity and precision

The technical difficulties in Tg measurement depend on both external serum factors, namely the analytical characteristics of the assay such as sensitivity and precision and intrinsic serum factors, namely the presence of endogenous and heterophile antibodies.

The analytical sensitivity (the low detection limit of the assay defined as the value corresponding to a signal 3 SD above the mean of 20 replicates of the zero calibrator) of the assays is critical to detect small amounts of Tg and to observe changes in Tg levels in followed-up patients. It has been proposed that only methods providing the greatest distinction between the lower limit of euthyroid reference range and the functional sensitivity (which is the concentration measurable with a coefficient of variation, $\text{CV} < 20\%$ interpolated on the imprecision profile built from different sera, at different levels of Tg concentration, assayed periodically in a defined period of time) of the assay may offer the most clinical sensitivity for detecting small amounts of thyroid tissue in the TSH-suppressed state [28]. Thus, Tg assays should have a functional sensitivity at least of 1 $\mu\text{g/l}$ with a normal lower Tg limit of 3 $\mu\text{g/l}$.

During the last 30 years the analytical sensitivity of Tg assays has been greatly improved, being between 5 and 10 $\mu\text{g/l}$ with the first RIAs [44,45,51] and 0.08 $\mu\text{g/l}$ (functional sensitivity of 0.2 $\mu\text{g/l}$) recently obtained with a commercial IRMA (DYNOTest, Brhams Diagnostica GmbH, Berlin, Germany) [52] or 0.015 $\mu\text{g/l}$ (functional sensitivity of 0.03 $\mu\text{g/l}$) obtained with a new developed enzyme linked immunoassay (ELISA) (RSR Ltd., Cardiff, UK) [53,54]. Moreover, with the advent of fully automated assays such as IMM2, MOD and ACC, results can be readily available to the clinician in a very short time with limited interventions of the operators. Thus, nowadays methods can achieve good analytical and functional sensitivity and practicability (short turn around time, TAT) to give reliable results also in the very low concentration range and in less than 1 hour. In Table 1 are showed

Table 1
Sensitivities and TAT of some commercially available assays for Tg measurements

Reference	Type of assay ^a	Assay distributor	Analytical sensitivity ($\mu\text{g/l}$)	Functional sensitivity ($\mu\text{g/l}$)	TAT
Manufacturer	ECLIA	Roche, Elecsys	0.10	1.0	18 min
Manufacturer	ICMA	DiaSorin, Liaison	0.20	0.5	30 min
Preissner et al., 2003 [64]	ICMA	Beckman, Access			40 min
Iervasi et al., 2004 [55]			0.01 ^b	0.1 ^c	
Manufacturer	ICMA	DPC, Immulite	0.50	0.9	90 min
Wunderlich et al., 2001 [53]	ELISA	RSD, Ltd.	0.015	0.03	Overnight
Zophel et al., 2003 [54]					
Giovanella et al., 2002 [52]	IRMA	Brahams	0.08	0.2	Overnight
Morgenthaler et al., 2002 [58]	ILMA	Brahams	0.02	0.06	Overnight
Pellegritti et al., 2003 [59]	IRMA	Nichols	0.07	0.5	Overnight

^a See list of abbreviation.

^b Calculated by assaying 20 replicates of the zero calibrator and of a Tg–TgAb free serum (obtained from thyroidectomized patients) in a single run and defined as the Tg value corresponding to a signal 3 SD greater than the mean found for these samples.

^c Calculated by interpolating the concentration correspondent to a 20 CV% on the imprecision profile obtained by assaying 10 serum pools at different concentration of Tg at intervals in a 6-months period of time.

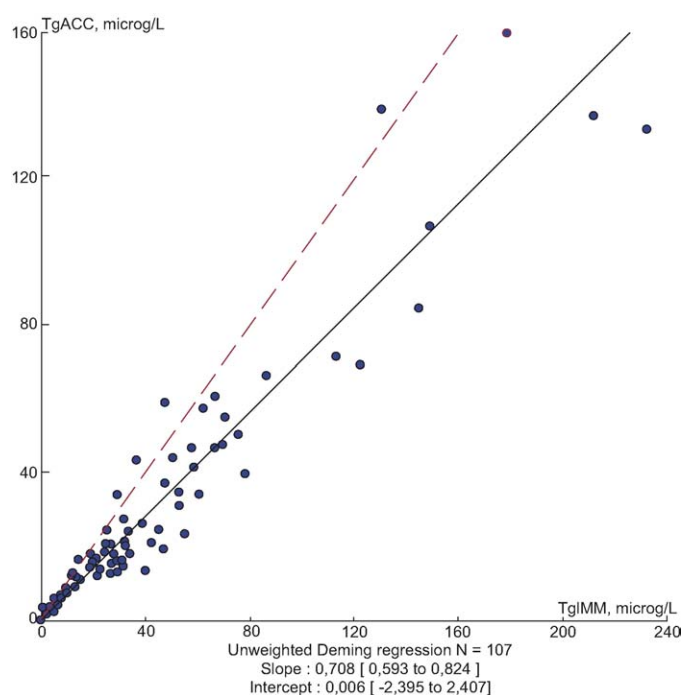


Fig. 3. Deming regression analysis between Tg ACC and Tg IMM2 assays in samples with a Tg concentration $> 0.5 \mu\text{g/l}$, dotted line indicating the identity line and continuous line indicating the regression line. Directly represented by Method Validator freeware software by Phylippe Marquis.

some commercial automated (by Roche, DiaSorin, Beckman and DPC) and manual (radioactive or enzymatic) methods to measure Tg with their sensitivity and TAT. In Fig. 3 Tg ACC and Tg IMM2 results obtained in our laboratory are compared with a good linear regression (in samples with Tg levels $> 0.5 \mu\text{g/l}$, being 0.5 $\mu\text{g/l}$ the detection limit indicated by Immulite manufacturer).

The recent introduction of highly sensitive Tg assays will probably improve the value of Tg measurement in DTC patients. The first illustration has recently been published by our evaluation of a new chemiluminescent immunoassay that showed equally high sensitivity and specificity both during suppressive therapy and after TSH stimulation [55]. With the

development of these methods that allow to an earlier Tg detection, another factor must be taken into account: how to deal with patients with low but measurable Tg levels without proven thyroid cancer? This dilemma was discussed in a recent European consensus paper [12], suggesting that every center should define an institutional cut-off value for Tg levels. Thus, several comments can be made on the optimal strategy of the application of rh-TSH stimulation in the follow-up of DTC thyroid cancer in terms of diagnostic validity, positioning in the light of the development of new, sensitive, Tg assays and the cost-benefit relationship for the patients. Comparison of long-term results in terms of recurrence rate and survival of different follow-up protocols, together with cost-benefit analyses, is necessary to develop a well-founded protocol for treatment and follow-up on patients with DTC [56].

Nevertheless, it is always unclear if higher sensitivity can give clinical advantage. It is well known that, suboptimal Tg assay functional sensitivity compromises the detection of recurrent DTC in the absence of rh-TSH stimulation [57]. Methods with the highest sensitivity would be those displaying the greatest discrimination between their functional sensitivity and the lower reference limit for euthyroid subjects with intact thyroid glands [19,54]. An optimal target for functional sensitivity would be a Tg value approximately 100-fold below the lower reference limit. If more sensitive Tg methods become more widely available, the diagnostic accuracy of Tg measurements made without rh-TSH stimulation will improve and likely reduce the need for rh-TSH [57,58,48]. Some physicians would prefer “less sensitive” assays because it is sometimes difficult to verify the source of very low Tg concentration ($< 0.5 \mu\text{g/l}$) by additional imaging diagnostic, and “confusion” for the physician and the patients may occur. Other people, however, argues in favour of a complete evaluation of patients with detectable serum Tg, irrespective of the concentration. It is possible that very low Tg concentrations do not require immediate treatment. The changes in serum Tg over a certain period or after T4 withdrawal or after rh-TSH are more informative than a single Tg determination. Highly sensitive Tg

assays that can distinguish for example a Tg increase from 0.2 to $0.8 \mu\text{g/l}$ in one patient and confirm a constant low Tg of $0.8 \mu\text{g/l}$ in another patient may be clinically valuable because only the first patient might need to undergo additional diagnoses or treatment. As increasingly sensitive assays become available also the Tg threshold or Tg increase at which clinical intervention should occur must be re-evaluated in clinical studies [58].

In our study we tested analytical and clinical performance of Tg ACC, immunochemiluminometric and automatized assay based on CRM-457 [55]. The detection limit of the assay resulted $0.01 \mu\text{g/l}$ while the value of $0.1 \mu\text{g/l}$ represents the functional sensitivity that we calculated from the imprecision profile at 20 CV% in a 6-months period of time according to NACB guidelines [19] (see the imprecision profile in the Fig. 4). The good sensitivity in the very low concentration range is emphasized by the results of the dilution test showed in Fig. 5. Interesting clinical aspects correlated to higher sensitivity, can be drawn from results of our recent study on the use of Tg ACC measurements during suppressive therapy and after rh-TSH stimulation. Important area of applications of methods with higher sensitivity could be: 1) the identification of a “complete” excision after total thyroidectomy and ^{131}I ablation (i.e. Tg values $< 0.1 \mu\text{g/l}$); 2) the identification of “low-risk” patients at “relatively higher risk” of recurrence, on L-T4 treatment (i.e. Tg values $> 0.1 \mu\text{g/l}$, $< 1 \mu\text{g/l}$); 3) the identification of patients at “very low-risk” of recurrence on L-T4 treatment (i.e. Tg values $< 0.1 \mu\text{g/l}$).

The recent routinely introduction of a rapid and practicable Tg assay with a sensitivity level 10-times higher than the most accepted level ($0.1 \mu\text{g/l}$ instead of $1 \mu\text{g/l}$) might theoretically improve the clinical impact of Tg determination in the follow-up of DTC patients during L-T4 suppressive therapy and will place the need for rh-TSH testing in a new perspective [56].

2.2.3. Antibody interference

If a good analytical sensitivity (i.e. low detection limit $< 0.1 \mu\text{g/l}$) and a good functional sensitivity (inter-assay imprecision

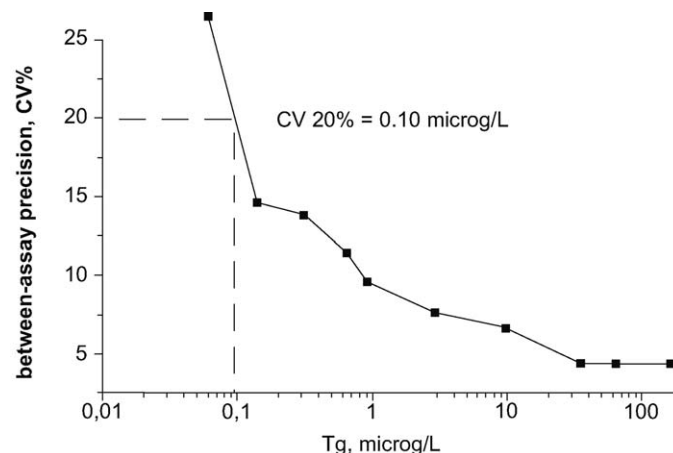


Fig. 4. Imprecision profile for Tg ACC assay: 10 serum pools were assayed during 6 months according to NACB guidelines [19] and the functional sensitivity at 20 CV% was interpolated.

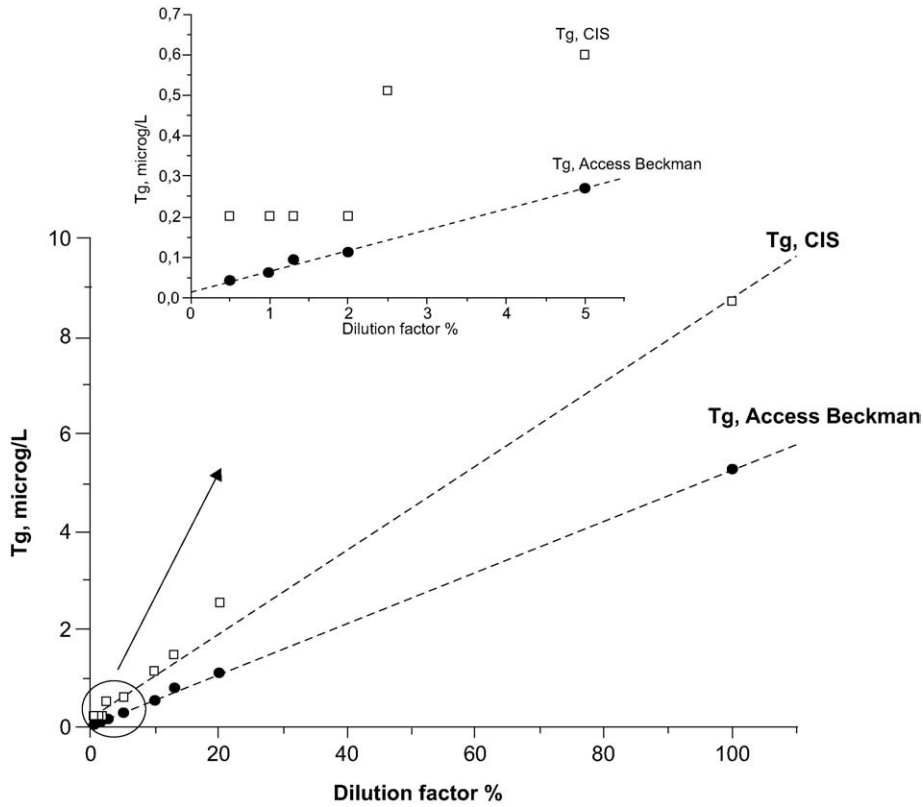


Fig. 5. Dilution test performed with Tg ACC and Tg CIS assays with a serum sample at a concentration of 5 µg/l progressively diluted [55].

Table 2
Effects of factors interfering with the Tg assay on the serum concentration value and on its clinical interpretation

Cause of interference	Serum Tg concentration	Interpretation
TgAb-positive ^a	Undetectable or inappropriately low Tg	Thyroid tissue/recurrence/metastasis can not be excluded
Very high Tg concentrations (Hook effect) ^b	Undetectable or inappropriately low Tg	Thyroid tissue/recurrence/metastasis can not be excluded
No interfering factors	Detectable Tg during L-T4 suppression	Thyroid tissue/recurrence/metastasis is present; no need for TSH stimulation
TgAb-positive	Detectable Tg during L-T4 suppression	Thyroid tissue/recurrence/metastasis is present; no need for TSH stimulation; real Tg value is underestimated
Heterophile antibodies ^c positive	Detectable or undetectable Tg during L-T4 therapy	false-high or false-low measured Tg value; to be suspected when Tg values do not agree with clinical/instrumental data

^a For details see text, page 15 paragraph of Section 2.2.3: Section 2.2.3.1.

^b For details see text, page 18 paragraph of Section 2.3.

^c For details see text, page 17 paragraph of Section 2.2.3: Section 2.2.3.2.

cision at 20 CV% evaluated at interval of 6–12 months), can now be obtained with the more recent methods, it is more difficult to overcome the problems of the interference by endogenous (TgAb) and heterophile antibodies [19], a problem which still affects any commercially available method and might limit clinical interpretation (see Table 2).

2.2.3.1. *Endogenous auto-antibodies.* TgAb interference is the most serious specificity problem affecting Tg measurement [50,60]. No Tg method is free from TgAb interference from TgAb-positive sera, although some methods appear more resistant than others [19] as showed also by our results of the external quality assessment. Non competitive IMA appears to be

more prone to TgAb interference than RIA methods, this results an underestimation of Tg concentration by IMA methods that could mask metastatic disease. In the United States there is a trend for laboratories to restrict the use of IMA methods to TgAb-negative patients while retaining older RIA methods for TgAb-positive patients [19]. It is difficult to predict which TgAb-positive serum sample will interfere with serum Tg measurement because the TgAb concentration does not correlate with the degree of interference and even low concentration of TgAb can interfere with Tg assay. The amount of TgAb interference is not quantifiable since the antibodies have specificity to the epitopes for different antigenic regions on Tg molecule, but it can be minimized by an accurate choice of

antibodies which should be specific to epitopes not involved in the formation of endogenous TgAb. Using sensitive methods TgAb are detectable in serum of 4–27% of healthy individuals, 51% of patients with Graves disease, 97% of patients with Hashimoto thyroiditis and 15–30% in patients with DTC. TgAb are found in 25% of patients with DTC compared to 10% in the general population; thus, the relative risk of TgAb positively in the DTC compared to control group was 2.5% with a 95% confidence interval of 2.0–3.2% [28,41]. For this reason, every laboratory should measure always TgAb in all samples where Tg is measured, as suggested by NACB guidelines, and when TgAb are present the clinicians should consider Tg results less reliable. Therefore, in case of interference with Tg measurement by TgAb, patients should be monitored according to a modified protocol because of the follow-up cannot rely on serum Tg determination [61]. A detectable serum Tg in the presence of TgAb is generally observed in patients with persistent or recurrent disease. In the absence of disease TgAb will progressively decrease and disappear within the first 2 years of follow-up.

Anyway, the differences among the TgAb measurements are also more evident than in Tg measurements and some sera could result negative with one method and positive with another one.

2.2.3.2. Heterophile antibodies. Circulating human antibodies reactive with animal proteins (namely heterophile antibodies) are often unrecognized and unsuspected source of interference for immunological assays, for particular two-site (sandwich) immunoassays. This type of assay interference was first noted in 1971 [62] and has been reported in a wide range of immunoassays (i.e. Tg, TSH, T3, T4, troponin, CEA, Ca125 etc.) [61,63–66]. In some cases, heterophile antibodies arise as result to exposure to a defined antigen (e.g. mouse monoclonal antibody therapeutic agent) but in other cases the antigens are defined. The most common heterophile antibodies are anti-mouse antibodies (HAMA). The true number of people positive for HAMA is not known and estimates vary widely (< 1–80%) [66–71]. In two-site immunoassays, HAMA present in a serum sample can interfere in clinical assays by bridging between the mouse immunoglobulin capture antibody and the mouse immunoglobulin conjugate producing false-positive results. False-negative results are also possible if HAMA reach with one of the assay reagents preventing reaction with the analyte. Clinicians should be aware that if the results of two-site immunoassays, particularly cancer markers, do not fit with the clinical picture, this may be an indication of a heterophile antibodies interference; on the other hand, manufacturers should take steps to minimize this type of interference. Anyway, using heterophile-blocking tubes (HTB, Scantibodies, Santee, CA) according to manufacturer's suggested procedure, it is easy to check the presence of heterophile antibodies in human serum by comparing Tg values obtained before and after serum incubation in HTB tubes if the clinical picture of the patient does not fit to the obtained results [55,65]. Recently, Beckman Coulter has introduced Tg modified reagents in its assay, by adding a chemically polymerized

monoclonal mouse IgG, which provide adequate blocking of heterophile antibody interference, thus obviating the need for HBT pre-treatment of sample [60].

2.3. Hook effect

The hook effect occurs when an excessive amount of analyte overwhelms the binding capacity of the capture antibody; this allows an inappropriate low signal and thus a paradoxically low result for patients with very high serum Tg concentrations. Laboratories should run serum specimens at two dilutions to detect hook. Hook effect is minimized for methods based on two-step assay design.

3. Conclusions

Consolidated clinical practice indicates the serum Tg measurement as a reliable laboratory biomarker for DTC management. The principal application of Tg determination in the medical practice is to early detect DTC recurrence, well before the clinical and instrumental evidence of tumor presence, or to confirm the disease-free status. Very important progress has been done on sensitivity and reliability of Tg immunoassays during last 30 years. The new routinely available methods are practicable and sensitive (i.e. with functional sensitivity 0.1 µg/l or lower) enough to detect small amounts of thyroid functioning tissue also when TSH is in the low range of normal reference interval or even undetectable. Nevertheless, methodological problems still limit its clinical utility; first of all, the interference produced by TgAb.

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