Thyroglobulin and Thyroglobulin Antibodies

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5.1 Introduction

Thyroglobulin (Tg) is a large glycoprotein that in healthy thyroid tissue is stored in the follicular colloid of the thyroid gland where it acts as a substrate for the synthesis of thyroid hormones. As it is produced by normal or well-differentiated malignant thyrocytes only, its tissue-specific origin makes it highly useful as a tumour marker [1]. Tg is released into the bloodstream together with thyroid hormones both upon physiological and pathophysiological stimulation but also upon destruction of the thyroid gland [2] (Table 5.1).

The advent of Tg measurement in the early 1980s greatly improved the follow-up of DTC, and due to the gradual improvements in the sensitivity and precision of Tg assays, the measurement of serum Tg has thus become the cornerstone in the follow-up algorithms for management of thyroid carcinomas after successful treatment [3, 4]. However, until recently, optimal sensitivity of Tg assays for the detection of smaller disease foci required stimulation of endogenous Tg production by high serum TSH concentrations, obtained after expensive exogenous injections with recombinant human TSH or after withdrawal of the

Table 5.1 Causes of increased Tg levels into the bloodstream

TSH receptor stimulation by TSH, TSH receptor antibodies and human chorionic gonadotropic hormone (hCG)
Increased proliferation (e.g. *benign and malignant nodules*, *goitre*)
Iodine deficiency
Destructive thyroiditis (e.g. *subacute*, *postpartum and silent thyroiditis*, *hashitoxicosis*)
Thyroid surgery
Radioactive iodine therapy
Fine needle aspiration cytology and core needle biopsy
Thermal ablation of thyroid nodules
Serious manipulation of the thyroid gland (e.g. *anterior neck trauma*, *strangulation*)

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patient's levothyroxine (LT4) replacement therapy, resulting in profound hypothyroidism [5–8].

Over the years, the sensitivity and precision of Tg assays have improved by multiple orders of magnitude, and nowadays new highly sensitive Tg assays are available. In fact, such assays are sufficiently sensitive to obviate the need for TSH stimulation in most patients with DTC [9–12].

Therefore, the increasing adoption of these assays in clinical practice has considerable implications, such as a reduction of costs of DTC follow-up and avoidance of hypothyroidism [13].

However, measuring Tg is technically challenging, and in addition, criteria adopted to define assay sensitivity by different manufacturers, laboratories and clinicians may diverge considerably. In the present chapter biological basis, advances and challenges in Tg measurement techniques and their impact on clinical management of DTC patients are reviewed.

5.2 Thyroglobulin: Biochemistry and Physiopathology

The thyroid gland is responsible for the production of thyroid hormones, mainly the prohormone thyroxine (T4), which contains four iodide molecules [14]. The gland consists of thyroid follicles; i.e. epithelial cells that border a lumen with their apical membranes and are in contact with the blood circulation through their basal membranes, respectively. Thyroidal proteins involved in thyroid hormone synthesis are the thyroid-stimulating hormone receptor (TSH-R); i.e. a seven-transmembrane receptor located in the basal membrane. Upon stimulation of the TSH-R, several processes, including Tg synthesis, are upregulated in the thyroid cells to favour thyroid hormonogenesis [15]. The most abundant protein in the thyroid gland is Tg which functions as a scaffold protein for thyroid hormonogenesis and as a storage protein for thyroid hormones and iodide. Initial transcription of the Tg gene (>300 kb) is regulated by thyroid-specific transcription factors (TTF-1 and TTF-2) and Pax8 [16]. After translation of the mRNA, the post-translational route of Tg starts with the signal peptide directing the uptake in the endoplasmic reticulum (ER), where the first mannose and glucose residues are added and the Tg protein is folded. Therefore Tg is directed to the Golgi apparatus, where glycosylation proceeds. At this point, Tg molecules have a molecular weight of 300,000, contain 10% carbohydrate structures and are routed through to the follicular lumen of the thyroid cells where homodimers with a molecular weight of 660,000 are formed [17]. The sodium iodide symporter (NIS), located in the basal membrane, and pendrin, located in the apical membrane, are responsible for the iodide supply and transport within the gland. Iodination of specific tyrosine residues in Tg and coupling of these iodinated residues to form thyroid hormones are done by the thyroid peroxidase (TPO) anchored in the apical membrane and on one or more thyroid oxidases (Tox) that provide the H_2O_2 [18]. Before the secretion of thyroid hormones, Tg is taken up by the follicular cells through a process involving endocytosis and phagocytosis, and thyroid hormones (mainly T4) are released by proteolytic enzymes into the bloodstream [19]. For a long time, it was assumed that no Tg secretion or leakage from the healthy thyroid could occur. However, when more sensitive methods to measure Tg became available, low concentrations of circulating Tg were demonstrated in virtually all healthy subjects [20–22].

As previously mentioned different benign and malignant thyroid diseases release significant amounts of Tg into the blood (i.e. Graves' disease, goitre, destructive thyroiditis, differentiated thyroid carcinoma) with a wide overlap between them. As a consequence, serum Tg measurement cannot be used to diagnose and differentiate different thyroid diseases, and Tg is mainly useful as a tumour marker only after thyroidectomy (ideally followed by thyroid remnant radioiodine ablation).

However, in rare cases of patients with proven distant metastases, high Tg levels may serve as a useful tool to identify an unknown primary thyroid cancer. Finally, serum Tg may be measured to aid in the differential diagnosis of congenital hypothyroidism and *thyrotoxicosis factitia*.

Interestingly, serum Tg levels recently proved to be a useful complementary tool in the challenging management of thyroid nodules with indeterminate cytology reading [23].

5.3 Thyroglobulin Measurement: Methods and Analytical Performance

Immunoassay has been the main analytical technique used for the measurement of serum Tg, first by competitive immunoassay and later by immunometric (reagent excess) assays. More recently, also mass spectrometric methods have been developed. Each new assay format has been developed to attempt to overcome the major analytical challenges in measuring Tg, a heterogeneous analyte of large molecular weight, in the presence of interfering antibodies. It is notable, however, that there is no reference method system including a reference method procedure available for Tg, and use of the BCR® 457 certified reference material (formerly CRM 457) has not completely eliminated the notable differences in results obtained by different methods [24]. Issues of commutability of the BCR® 457 material and the need for harmonization have yet to be addressed [25].

5.3.1 Standardization and Harmonization

Tg is a large (660 kDa), highly glycosylated dimeric molecule that is heterogeneous in serum due to differential splicing of Tg mRNA as well as carbohydrate and iodide heterogeneity. In addition, biosynthesis of the mature Tg molecule may become deregulated in thyroid tumour cells resulting in differences in the structure of circulating Tg protein. These changes can lead to exposure or masking of epitopes and hence differences in Tg immunoreactivity. Different Tg assays employ a number of antibodies against Tg, with varying specificity for different epitopes. Potentially this introduces variability in the measurement of different Tg isoforms and ultimately to differences in Tg concentration reported by the assays [26, 27]. Early international collaborative studies showed that serum Tg concentrations varied by as much as 40–60% between methods [28, 29]. The introduction and use of the BCR® 457 have significantly reduced inter-method variability to about 30% but have not completely eliminated it [30]. Consequently, any change in Tg assay has the potential to disrupt serial monitoring and prompt inappropriate clinical decisions. For longitudinal consistency of clinical care, consecutive measurements of Tg concentrations should be performed in the same laboratory using the same assay each time. If an assay change is unavoidable, a new baseline of the individual patient's serum Tg concentrations should be established through parallel Tg measurements using both the old and the new assay [26, 27, 30]. Furthermore, internal and external quality control programmes, including samples at low and very low Tg concentrations, are of pivotal importance for checking the precision, reproducibility (internal quality control) and accuracy (e.g. lack of bias of analytical results) of assays to ensure optimal patient care. Laboratories providing Tg measurement are required to participate in a certified national or international programme of quality assurance [31].

5.3.2 Analytical Performance

Laboratory specialists are familiar with the necessary experiments that must be performed in order to verify assay performance, namely, assessment of linearity, measuring range, trueness (measurement bias), comparability through patient comparison studies and limit of detection/limit of quantitation/functional sensitivity. It is worth considering some particular points with regard to serum Tg [32]. Commercial assays may be provided with an assay diluent and specify a dilution value (e.g. 1 in 10 v/v). Laboratories may wish to consider whether this covers the range of concentrations that is required from a clinical perspective (i.e. monitoring of metastatic disease). The concentration at which the high-dose hook effect has been excluded should also be

determined. The feasibility of using an in-house human serum pool as diluent (with undetectable TgAb as measured by a suitable assay and Tg concentration less than 0.1 μg/L) may need to be investigated and the linearity over a wider concentration range determined. Estimation of recovery of added Tg has been proposed as a method of assessing whether there is interference by endogenous antibodies (i.e. Tg antibodies, TgAb and heterophilic antibodies, HAb), although this is not advocated by current guidelines (see section on Interferences). Nevertheless, determination of quantitative recovery should be performed as part of the method validation of the assay [32]. Studies have shown that the measured recovery is dependent on the protocol used—in particular the Tg concentration, source of Tg (degree of iodination) and incubation time. Assessment of recovery using a source of Tg independent of the kit calibrators is suggested, though manufacturers may recommend a protocol if recovery is being determined in the context of assessing assay interference [26].

5.3.2.1 Analytical Sensitivity

The growing recognition of the clinical need for improved precision of assays at low Tg concentrations has been paralleled by improvements in assay sensitivity with the original radioimmunoassays (RIAs) reporting down to 2–5 μg/L, the first immunometric assays (IMA) down to 1 μg/L and, more recently, IMAs with limits of about 0.1 μg/L. However, these broad comparisons are limited because of differences in bias between different assays and because different experimental and statistical methods were used to determine the sensitivity of the assays. In the first instance, analytical sensitivity has often been determined by repeat analysis of the zero calibrator and determination of the apparent concentration equal to the zero plus 2 or 3 standard deviations of the signal for immunometric assays (minus for competitive assays), which is known as the limit of the blank (LOB). In the majority of cases, the measured sensitivity will be below the concentration of the lowest concentration calibrator. Although of limited use in understanding the precision of low concentration samples, the LOB can be useful when optimizing conditions during assay development. The limit of detection (LOD) is defined as the lowest analyte concentration that can be distinguished from the LOB using replicate analysis of a sample of known low concentration. Lastly, the limit of quantitation (LOQ) is similar to the functional sensitivity but does have an additional requirement for predefined goals for bias and imprecision and is increasingly used as a measure of sensitivity for both immunometric and mass spectrometric assays. The relationship between these estimates of sensitivity is LOB < LOD ≤ LOQ. Manufacturers should quote LOB, LOD and LOQ as determined by regulatory authorities and national guidelines (e.g. those of the Clinical and Laboratory Standards Institute EP17-A2) [33, 34]. Functional sensitivity (FS) was introduced as a measure of analytical sensitivity and was originally described for assessing the sensitivity of TSH assays. The NACB protocol [4] indicates that FS may be determined from between batch precision of Tg measurement:

- In patients serum pools
- In the same test mode (singleton or duplicate) as the patient samples
- Over the clinically relevant concentration range
- Over two different lots of reagents and calibrators
- Over a period of >6 months

The patient pools should be TgAb negative. The protocol specifies three different concentration ranges for the patient pools. From the calculated precision profile, a cut-off value corresponding to a CV of 20% (somewhat arbitrarily) is taken as the FS (Table 5.2).

The difference in FS between Tg assays has created a "generational" nomenclature system with each subsequent generation exhibiting a substantial improvement (i.e. tenfold). It should be recognized, however, that there are limitations to this approach when determining the sensitivity of an assay (i.e. potential matrix effects) and differences in the statistical approach to the calculation of the precision profile, principally to

Table 5.2 Definitions of different parameters describing analytical sensitivity

Parameter	Definition	Protocol
Limit of blank	Highest measurement that is likely to be observed for a blank sample [$mean_{blank}$ + 1.645(SD_{blank})]	IFCC
Limit of detection	Lowest amount of that can be detected, but not quantified as an exact value LOB + 1.645 ($SD_{low\ concentration\ sample}$)	IFCC
Limit of quantitation	Lowest amount of analyte in a sample that can be quantitatively determined with stated total error, understated experimental condition	IFCC
Functional sensitivity	Lowest amount of analyte that can be quantitatively determined with an inter-assay coefficient of variation <20%	NACB

do with the identification of outliers and the confidence intervals of the profile. In facts confidence intervals of the precision profile may vary significantly over the concentration range indicating the confidence with which the calculated FS can be viewed. In addition, the published literature is often not helpful since details of the procedure used to generate the precision profile are rarely provided (e.g. whether within or between batch precision was used, how many samples were analysed and whether samples were analysed in singleton or duplicate). Therefore, with such sparse data, the relationship between imprecision and concentration can only be poorly estimated [35]. Finally, given that assay performance can vary with time, operator, reagent lot, calibration, equipment maintenance and other factors monitoring of sensitivity whether as FS or LOQ should be ongoing, and laboratories should determine their own FS/LOQ rather than just quoting manufacturers' data [36]. Consequent to above arguments, it becomes clear that in comparing the performance of different Tg assays and in order to provide clinicians with realistic interpretations of Tg results, it is necessary to know exactly how "sensitivity" has been determined such that like can be compared with like. Some examples of these approaches are illustrated in Table 5.3.

5.3.2.2 Reference Range

Valid estimations of a reference range require sizable groups of subjects; all of whom must be correctly identified according to the absence of disease by methods other than the diagnostic tests being evaluated [37]. Selection criteria of reference population are critical as, for example, Tg reference values are geographically sensitive, since serum Tg is influenced by iodide availability and intake [38]. According to the National Academy of Clinical Biochemistry, the reference range could be evaluated in healthy non-smokers with thyroid-stimulating hormone (TSH) within the normal reference range for the population, with no personal or familial history of thyroid disease, no palpable or visible thyroid gland nor positive antibodies against Tg (TgAb) or thyroperoxidase (TPOAb). The Tg reference range should be expressed as median ± 2 standard deviations obtained after log-transformation of data [39, 40]. Reference ranges of Tg in widely employed Tg assays are summarized in Table 5.4.

The tissue-specific origin of Tg biosynthesis dictates that Tg in serum will be absent in DTC patients treated by thyroid ablation (i.e. expected Tg values theoretically correspond to zero). As a consequence, the clinical impact of Tg reference range in thyroid healthy subjects is limited. However, a reliable reference range is useful to properly evaluate the assay performance, especially at low Tg concentrations. In fact, assays which provide the greatest distinction between the lower limit of the euthyroid reference range and the analytical limit of the assay offer the most clinical sensitivity for detecting small amounts of thyroid tissue even in the TSH-suppressed state [4]. Subnormal to undetectable serum Tg levels (despite a negative TgAb test results) are occasionally encountered in clinical practice in DTC patients with clear disease foci or significant thyroid remnants [41–43]. Such false-negative results may erroneously suggest complete biochemical response and may occur when the spatial conformation of Tg is changed, leading to decreased immunoreactivity or when the ability

Table 5.3 Analytical sensitivity of some commercially available Tg assays as reported in the literature or quoted by manufacturers

Assay	Manufacturer	Methodology	Parameters (ug/L)
sTg KRYPTOR	BRAHMS	TRACE	LOB = 0.02 (M), LOD = 0.04 (M) LOQ = 0.1 (M), total allowable error of ≤40%
Tg II COBAS	ROCHE	ECLIA	LOB 0.02 (M), LOD 0.04 (M) LOQ 0.1 (M), total allowable error ≤30% FS 0.1 inter-assay CV = 20%
Tg Access	Beckmann	CLIA	LOD 0.1 (M) FS 0.1 precision profile from ten pools at a CV = 20% FS 0.05 according to NACB
Dynotest Tg Plus	BRAHMS	IRMA	LOD 0.16 (M) FS 0.4 (M) Values corrected for difference in BCR® 457 standardization (i.e. correction factor: x 2)
eIASON	Iason GmbH	ELISA	FS 0.2 (M)

Legend: *LOD* limit of detection, *LOQ* limit of quantification, *FS* functional sensitivity, *M* manufacturers, *TRACE* time-resolved amplified cryptate emission, *ECLIA* electrochemiluminescent immunoassay, *CLIA* chemiluminescent immunoassay, *IRMA* immunoradiometric assay

Table 5.4 Tg reference ranges in different assays

Assay	Manufacturer	Methodology	Reference range (μg/L)
RIA Tg-plus	BRAHMS	IRMA	2.00–51
sTg KRYPTOR	BRAHMS	TRACE	2.40–48
Tg II COBAS	ROCHE	ECLIA	3.50–77
Tg IMMULITE 2000	DPC	CLIA	1.60–60
Tg Access	Beckmann	CLIA	1.59–50

Legend: *IRMA* immunoradiometric assay, *TRACE* time-resolved amplified cryptate emission, *ECLIA* electrochemiluminescent immunoassay, *CLIA* chemiluminescent immunoassay

to secrete Tg is lost by cancer cells. In other cases false-negative Tg results may occur in the presence of an undetected TgAb interference [44].

As preoperative Tg below the lower reference limit may be detected in these cases, a measurement of Tg and TgAb before thyroidectomy was proposed in all DTC patients. This strategy provides "baseline" Tg and TgAb concentrations which serves as benchmark for the subsequent follow-up and could theoretically allow assessment of the reliability of post-surgery Tg and TgAb measurements [4, 44]. However, this simple concept is not widely accepted in clinical practice [despite pre-therapy measurements are recommended for other tumour marker such as calcitonin in patients with medullary thyroid carcinoma.

5.4 The Relationship Between Thyrotropin and Thyroglobulin

Like the physiological thyroid secretion, tumoural secretion of Tg mostly displays a TSH dependency as follicular-derived tumour

cells mostly preserve TSH receptors. As a consequence, Tg concentrations measured under maximum TSH stimulation (i.e. stim-Tg) exceed Tg values under TSH suppression (i.e. on Tg) by one order of magnitude. In clinical practice TSH stimulation is obtained by withdrawing levothyroxine (~4 weeks) or by administering recombinant human TSH (rhTSH). The protocol for Tg stimulation approved by regulators in every country where rhTSH is marketed consists of an intramuscular injection of 0.9 mg of rhTSH in the buttock, followed by a second injection of rhTSH 0.9 mg 24 h later. A serum stim-Tg is obtained 72 h after the second rhTSH injection [45]. A significant correlation was found between peak of Tg after hormone withdrawal and administration of rhTSH; however, rhTSH-stimulated Tg levels are usually (significantly) lower than off-Tg ones [46]. Several explanations may be offered for the Tg increments after thyroid hormone withdrawal being higher than those after rhTSH. Tg synthesis and secretion are more continuous and prolonged during endogenous TSH stimulation, and Tg clearance rate may be lower, compared with exogenous stimulation. Whatever the cause, this finding poses relevant problems in the interpretation of the serum Tg results obtained by different stimulation protocols. The TSH level is necessary to achieve adequate Tg stimulation after thyroid hormone withdrawal has not been determined, and the commonly used cut-off is derived from the level thought necessary for radioactive iodine imaging. Valle and colleagues [47] reported that TSH and Tg levels continuously rise throughout 4 weeks of thyroid hormone withdrawal, and the minimal TSH cut-off of >30 μUI/mL may be inadequate to detect many patients that eventually demonstrated a stimulated Tg ≥2 μg/L. A TSH cut-off of >80–100 μUI/mL was more reliable to detect these patients, suggesting that consistent methods and intensity of stimulation are necessary for adequate comparisons when monitoring patients with DTC. Interestingly, when the Tg was undetectable (<0.2 μg/L) at a TSH level > 20 μUI/mL, their final Tg did not stimulate to ≥1–2 μg/L during 4 weeks of thyroid hormone withdrawal with 91% and 100% certainty, respectively. Similar problems when using rhTSH stimulation were also reported regarding body surface area, lean body mass and age, as these factors may affect the TSH concentration that is reached after rhTSH administration [48–50]. All in all, consistency in the method and manner in which stimulated Tg is performed is needed, and differences should be acknowledged when consistency is not possible. Additionally, in contrast with clinical guidelines and current practice, the same cut-off thresholds should not be used for different stimulation methods. In fact, when Tg measurements were obtained using both stimulation methods in the same patients, it was observed that Tg levels after rhTSH stimulation were fourfold lower than after withdrawal of thyroxine replacement, respectively [51]. In practice, clinicians should be prompted to recognize the dynamic variables of TSH, weeks of thyroid hormone withdrawal and method of Tg stimulation when they compare different stimulated Tg results in a DTC patient. Overall, by considering the principle factors influencing serum Tg concentrations (i.e. thyroid tissue mass, injury and TSH), it is evident that the trend in basal Tg, measured when TSH is suppressed, should reflect changes in thyroid tissue mass and thus provide more accurate clinical information than stimulated Tg testing [4]. A follow-up strategy centred on unstimulated Tg is now possible by employing highly sensitive Tg assays. Using an assay with a functional sensitivity of 0.4 μg/L, Giovanella et al. measured unstimulated serum Tg in 117 low-risk DTC patients: the negative predictive value of a Tg level < 0.4 μg/L was 96% and increased to 99% when combined with neck US. In this study, rhTSH-stimulated Tg measurement only detected one additional recurrence in 104 patients with an undetectable unstimulated Tg [10, 13]. After that, a number of studies were performed to investigate the performance of basal highly sensitive Tg measurement in the follow-up of DTC patients [12, 44, 52–57]. Recently, Giovanella et al. reviewed and meta-analysed data from nine studies including 3178 DTC patients and confirmed the very high negative predictive value (98–100%) of an undetectable basal highly sensitive Tg (e.g. <0.1 μg/L). Importantly, these assays also have an adequate

sensitivity for detection of recurrent disease (88–98%) [58]. All in all, the negative predictive value of a rhTSH-stimulated Tg value below 1–2 μg/L is comparable to a basal high-sensitive Tg value below 0.10–0.20 μg/L. Therefore highly sensitive assays obviate the need of TSH stimulation in DTC patients with undetectable basal Tg levels. Additionally, although the low frequency of DTC recurrences impacts the ability to study positive predictive values (PPVs), the PPV of an rhTSH-stimulated thyroglobulin above 1–2 μg/L appears comparable to a basal highly sensitive Tg above 0.10–0.20μg/L [59, 60]. In addition, the trend in basal hsTg, measured when TSH is lowered/suppressed at constant level, should reflect changes in thyroid tissue mass and thus provide a sensitive parameter for disease [4, 44]. This is also supported by a growing number of studies showing the prognostic utility of monitoring the basal hsTg trend and thyroglobulin doubling time [61–66].

5.5 Thyroglobulin Antibodies and Other Interferences on Thyroglobulin Measurement

Together with thyroperoxidase antibodies, TgAb are important pathogenic markers of thyroid autoimmune disease, present in approximately 10% of most female populations, depending on, e.g. the iodine intake [2, 67]. In DTC, on the other hand, TgAb are detected in 15–40% of patients, i.e. roughly twice or more as often as in the general population. It has also been noted that the frequency of a previous or current history of thyroid autoimmunity is higher than expected in DTC patients [68]. Epitope recognition patterns of TgAb were recently shown to be restricted to immunodominant clusters in 58% of patients with different thyroid cancer, whereas the rest were either broadly heterogeneous (16%) or nonreactive (26%). However, median Tg recovery did not differ between sera with restricted and unrestricted specificities (69% vs 80%; $p > 0.05$). Tg recovery in these sera was inversely correlated with the total number of epitopes recognized by sera ($r = -0.66$; $p < 0.001$). TgAb with both restricted and broad specificities were present in patients with differentiated thyroid cancer. TgAb interference was related to the number of epitopes recognized by sera rather than the pattern of epitope recognition [69]. In an earlier study, Ruf et al. showed that Tg epitope specificity of thyroid cancer TgAb was similar to that of thyroid healthy subjects with low TgAb concentrations but different in patients with overt thyroid autoimmune thyroid diseases such as Graves' disease and Hashimoto's thyroiditis [70]. Anyway, regardless of whether the presence of TgAb is due to true autoimmune disease or not, the possibility of compromising serum Tg measurements as tumour marker in DTC is not negligible. The initially established radioimmunoassays for measurement of serum Tg used double antibody techniques, which could result in either falsely high or falsely low serum Tg quantification, depending on the nature of the second antibody in the assay. The influence of the presence of TgAb in serum, however, will always be unidirectional resulting in a false lowering of the Tg concentration when using current immunometric assays. In these cases, Tg contained in a patient's serum is "sandwiched" between capture and detection antibody. TgAb can prevent binding of capture or detection antibody (or both) by blocking access to their respective epitopes on Tg, thereby resulting in false low Tg measurements [71] (Fig. 5.1).

5.5.1 Screening for TgAb Interferences in Tg Measurement

5.5.1.1 Semiquantitative and Indirect Methods

Semiquantitative assays make use of percentage of spiked labelled Tg that can be precipitated as immunocomplexes, gelatin agglutination and immunofluorescent methodologies with serial sample dilution. These methods are not used in current practice due to their low sensitivity and specificity, manual format and technical com-

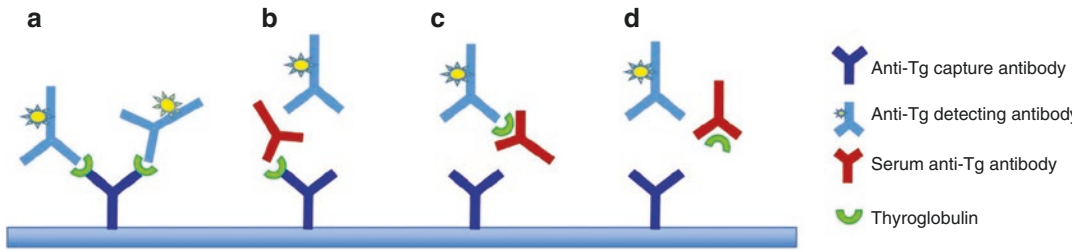

Fig. 5.1 Schematic representation of TgAb interference in Tg immunoassay. Legend (**a**) Normal interaction between anti-Tg capture antibody, Tg and anti-Tg labelled antibody; (**b**) TgAb binds Tg preventing interactions between anti-Tg labelled antibody and the complex Tg-anti-Tg capture antibody; (**c**) TgAb binds Tg preventing interactions between anti-Tg capture antibody and the complex Tg-anti-Tg labelled antibody; (**d**) TgAb sequesters Tg, preventing the binding with the capture and the labelled antibody

plexity. Indirect assays are based on comparing observed recoveries with expected recoveries of defined amounts of exogenous Tg spiked into patient samples (i.e. recovery test) and were widely adopted in clinical practice. However, recoveries may be influenced by exogenous Tg as multiple Tg isoforms have been found in both serum and the tissue-derived Tg preparations typically used for recovery testing. Conventional recovery testing with serum buffers containing 40–50 μg/L of Tg is considered undisturbed by most manufacturers if recovery rates are >70–80%; that is, a Tg concentration of 10–15 μg/L can be missed without finding a pathologic recovery. Given such a wide reference range, only strong interferences will be shown when using a conventional recovery buffer containing 40–50 μg/L Tg. In earlier days, when the lower detection limit of Tg assays was approximately 5 μg/L, this low sensitivity of recovery measurement might have been adequate. In modern clinical practice, a level of less than 1 μg/L is considered relevant, and as a consequence, conventional recovery testing is no longer adequate, and its use in clinical practice is discouraged [72–74]. A new development in recovery testing is the introduction of the so-called mini-recovery, in which recovery measurement is performed by adding serum with a low (i.e. 1–5 μg/L) Tg concentration. Theoretically, this mini-recovery test should be able to identify a detection loss of about 1 μg/L Tg, which, as already described, represents a clinically relevant limit. Preliminary clinical data are promising, but the performance of this mini-recovery has not yet been investigated extensively in DTC patients [75–77].

5.5.1.2 Thyroglobulin Antibodies Immunoassay

The determination of TgAb started 50 years ago with little sensitive qualitative techniques such as complement fixation, indirect immunofluorescence tests, passive hemagglutination, particle agglutination and immunodiffusion [78]. Later, in the 1980s, more sensitive competitive RIAs became available; they detected TgAb as a function of ^{125}I-Tg binding and reported results in kIU/L relative to the International Reference Preparation (IRP) and Medical Research Council (MRC) 65/93 [72]. Over the past decade, most of the laboratories have preferred non-isotopic, competitive or noncompetitive, automated IMA methods. They are currently considered the "gold standard", and their use is recommended by both clinical and laboratory guidelines [4, 8, 44, 79]. The main analytical performances of the current TgAb automated IMA methods are described in Table 5.3. They employ defined Tg-derived antigens in controlled concentrations, and most of them claim to be standardized against the IRP MRC 65/93 reporting results in kIU/L (= IU/mL). However, despite IRP standardization, several studies demonstrated the persistence of marked differences between TgAb IMAs: high variability of LOD, LOQ, FS and TgAb results for the same specimen measured by different methods (variation up to 100-fold) [78, 80–90]. The sources of TgAb inter-method variability are

manifold. First, the IRP MRC 65/93 dates back about 60 years, and it is made up of a pool of human plasma samples containing TgAb with Tg epitopic specificities which are more representative of thyroid autoimmunity than DTC [69, 91, 92]. In addition, some methods use their own internal standards being not standardized directly with IRP MRC 65/93 (Table 5.1), and so the difference between the epitope specificity of the reference preparation and of the secondary standards could result in widely discrepant numeric TgAb values for the same serum specimen (Table 5.3) [84, 86]. Another source of variability is the heterogeneous Tg immunoreactivity: differential splicing of Tg mRNA, various post-translational modifications (glycosylation, sialic acid content, iodination and sulfation) and alterations of biosynthesis regulation in thyroid tumour cells lead to exposure or masking of epitopes with resulting differences in Tg immunologic structure [93]. If the variations of Tg during the follow-up of DTC patients affect TgAb, characteristics are a matter which merits to be studied [94]. The assay discordance could also be assigned to the distinct specificity of patient's circulating TgAb for Tg. It seems that each patient has a typical IgG subclass and specificity for recognizing the Tg assay reagents [95]. Latrofa et al. [92], using recombinant human monoclonal TgAb, demonstrated the existence of two different models of TgAb epitopes: autoimmune thyroid disease (AITD) model and non-AITD model; the first displayed a more restricted epitope pattern with higher inter-method concordance; the non-AITD model had heterogeneous epitope pattern with consequent discrepant results in different assays. Interestingly, papillary thyroid carcinoma with lymphocytic thyroiditis resembled a model similar to that AITD with less epitope heterogeneity [94]. TgAb heterogeneity has two main implications: first, the ratios between TgAb measurements made with different assays remain constant during the serial monitoring of individual patients [73]. Secondly, even when different assays correspond well with each other in most patients, they might give discrepant results in certain individuals, probably due to dissimilarities in the Tg-derived antigens used in the assays, which might be recognized differently by distinct patient's TgAb [71, 96]. The determination and the interpretation of the cut-off of positivity are another important point to be addressed; in fact, the analysis of several studies showed a relevant discrepancy between the upper reference limits (URLs), according to the method employed [84, 85, 88]. A comparative study with most of the currently marketed immunometric automated methods in order to determine the experimental URL (e-URL) at the 97.5th percentile for each TgAb method was conducted according to the CLSI standard C28-A3c (submitted data) [97–99]. A panel of 120 sera obtained from healthy subjects were tested for TgAb. A wide variability of the e-URLs was found between methods but also, within the same method, between the manufacturer's URL (m-URL) and the e-URL (Table 5.5).

Particularly, with the exception of the Architect i1000 and the Maglumi 2000 Plus, e-URLs were lower than those claimed in the package inserts. These discrepancies could be related to the lack of strict criteria in the selection of the subjects for the reference group; in fact, there could be racial differences as most of the studies, sponsored by manufacturers, were performed in the geographical area of the production line and consequently difficult to reproduce in other settings. Moreover, the use of non-stringent criteria in the choice of subjects could have led to the enrolment of individuals with subclinical AITD and so high levels of TgAb, causing the raise of the URL. All in all, the differences between m-URL and e-URL highlight the need for individual laboratories to confirm the appropriateness of the reference intervals according to the method they use and the patient population they serve [85, 99–102]. Theoretically, a simple relationship exists between Tg and TgAb and the higher the TgAb concentration, the higher the Tg concentration that can be concealed by TgAb. Effectively, a logarithmic relationship was reported between TgAb concentrations (measured by IMAs) and surrogate measures of TgAb interference in Tg assays, such as the prevalence of undetectable Tg concentrations in patient populations or abnormal Tg spike recovery. Interference rates in two studies, for example,

Table 5.5 Analytical performance characteristics of the main current TgAb automated immunoassays

Manufacturer	Platform	Method	tracer/enzyme	Assay	Imprecision (%): *intra-; inter-; total*	LOD[d] (kIU/L)	LOQ[d] (kIU/L)	Assay range (kIU/L)
Abbott Diagnostics	ARCHITECT	CLIA	Acridinium esters	NC	1.7–6.6[b]; nd; 2.7–8.2[b]	0.07	0.31	0.07–100,000
Beckman Coulter	Access	CLIA	Lumi-phos 530/alkaline phosphatase	NC	3.6–5.7; 1.7–5.2; 4.8–7.7	0.9	nd	0.9–25,000
bioMerieux	VIDAS 3	ELFA	4MUP/Alkaline phosphatase	NC	2.6–9.5; 5.5–16.0; nd	6.2	6.4	6.4–8000
Thermo Fisher Scientific BRAHMS	Kryptor[a]	TRACE	Europium cryptate/XL 665	C	1.5–3.5; 6.8–20.0; nd	10	33	10–850
DiaSorin	Liaison	CLIA	Isoluminol derivatives	NC	2.3–3.2; 4.4–8.9; nd	5	10	5–5000
Fujirebio	Lumipulse G	CLEIA	AMPPD/ Alkaline phosphatase	NC	1.8–4.6; nd; 2.5–5.3[c]	5.152	5.152	5.152–3,000,000
Phadia AB, Thermo Fisher Scientific	Phadia 250	FIA	4-methyl-umbellipheryl-β-D-galactoside/ β-galactosidase	NC	3.3–5.6; 2.6–6.5; nd	12	nd	12–4794
Roche Diagnostics	Cobas/Elecsys	ECLIA	Ruthenium derivatives	C	1.3–5.6[c]; 2.1–8.7[c]; nd	10	nd	10–4000
Siemens Healthineers	ADVIA Centaur[a]	CLIA	Acridinium esters	C	2.9–5.5; 1.8–2.0; 3.5–5.8	10	30[e]	10–500
Siemens Healthineers	Immulite XPi	CLIA	Adamantyl dioxetane phosphate/ Alkaline phosphatase	NC	3.2–4.9; 4.6–5.8; nd	2.2	nd	20.0–30,000

(continued)

Table 5.5 (continued)

Manufacturer	Platform	Method	tracer/enzyme	Assay	Imprecision (%): *intra-; inter-; total*	LOD[d] (kIU/L)	LOQ[d] (kIU/L)	Assay range (kIU/L)
Snibe	MAGLUMI	CLIA	ABEI	NC	2.8–9.1; 5.2–9.8; nd	10	nd	10–2800
Tosoh Bioscience	AIA	FEIA	4MUP/Alkaline phosphatase	NC	4.3–5.1; nd; 5.5–6.0	0.12	nd	0.12–200,000
Tosoh Bioscience	AIA CL2400	CLEIA	DIFURAT®/Alkaline phosphatase	NC	5.1–5.5; 5.8–6.6; nd	0.005	nd	0.005–2,500,000

Legend: *4MUP* 4-methyl-umbelliferyl phosphate, *ABEI* N-(aminobutil)-N-(ethyl)-isoluminol, *AMPPD* alkaline phosphatase-spiroadamantyl-methoxy-phosphoryloxy-phenyl-dioxetane, *C* competitive immunoassay, *CLIA* chemiluminescence immunoassay, *CLEIA* chemiluminescence enzyme immunoassay, *DIFURAT®* 3-(5-tert-Butyl-4,4-dimethyl-2,6,7-trioxabicyclo[3.2.0]hept-1-yl) phenylphosphate disodium salt, *ECLIA* electrochemiluminescence immunoassay, *ELFA* enzyme-linked fluorescence assay, *FEIA* fluorescence enzyme immunoassay, *FIA* fluoroimmunoassay, *NC* noncompetitive immunoassay, *nd* not declared, *TRACE* time-resolved amplified cryptate emission

[a]All methods are standardized with the reference preparation MRC 65/93 and use international units (kIU/L) except for Centaur and Kryptor which refer to a secondary standard and use arbitrary units (kAU/L)

[b]Precision defined by the NCCLS Protocol EP5-A

[c]Precision defined by the modified NCCLS Protocol EP5-A2

[d]LOD and LOQ defined by the CLSI protocol EP17-A

[e]Functional sensitivity defined as TgAb concentration with total CV ≤20%, determined for a period of 2 days using one lot of reagents and testing, by four instruments, multiple samples from normal patients

were about 5–8% percentages at TgAb concentrations of less than 4–6 kIU/L but rose quickly to approximately 30% just above this level and continued to rise asymptotically to 70–85% at 50–100 kIU/L with minimal further increment in prevalence thereafter [69, 103]. However, low concentrations of TgAb may be sometimes associated with strong interference, and, conversely, patients with high concentrations of TgAb show no evidence of interference with the Tg measurement. On the basis of these considerations, the term positivity (that means TgAb concentration higher than the URL) is used inappropriately, and many specimens with interfering TgAb were proved to be misclassified as TgAb negative using m-URL. In fact, since the relationship between TgAb concentration and their interference in Tg IMAs is not clear, with even low concentrations of TgAb being able to cause false-negative results, TgAb URL must be interpreted with caution, never forgetting that it is usually calculated to diagnose AITD and not to exclude the presence of potentially interfering TgAb. Most likely, false-negative misclassifications could be reduced or even eliminated by using the LOD/FS of the method employed. Thus, it is advisable to use high-sensitivity quantitative TgAb assays, in order to allow detection of the majority of possible TgAb interferences [44]. Therefore, according to these findings, two different cut-offs for TgAb could be defined, one for the diagnosis of AITD and one for the effects of TgAb on Tg measurement [104]. However, it is noteworthy that the LOD/FS cut-off is associate with about 20% false-positive cases. In addition, LOD/FS cut-off also has an inherent 15–20% between-run imprecision, leading to false fluctuations in TgAb status [4, 105]. Finally, the relevant difference between URLs supported concerns regarding inter-method bias; in fact, despite the attempt of harmonization, TgAb methods were too qualitatively and quantitatively variable to come up with any definitive and common cut-off or to establish conversion factors that would allow a change in method without disrupting serial TgAb monitoring [89]. The lack of satisfactory agreement between methods has an important practical implication: on the one hand, the clinicians/patients had to use always the same method to measure TgAb in the follow-up of DTC, and on the other hand, laboratories had to notify in time any change in TgAb methods to facilitate re-baselining. Moreover, as the qualitative characteristics of the TgAb secreted by individual patients remain constant over time during long-term monitoring, independent of changes in TgAb concentration, it is advisable to store a patient's sample in order to establish, if possible, the ratio between the old and the new method, allowing re-baselining and so the monitoring of TgAb trend [44, 91, 104, 106].

5.5.1.3 Comparison of Tg Measurement by Radioimmunoassay Versus Immunometric Assay

Competitive Tg assays (usually radioimmunoassay, RIA), using polyclonal antibodies, were reported to be less susceptible to TgAb interference than Tg IMA methods, and discordance between Tg measured by RIA versus IMA was adopted as an additional methodological benchmark for detecting TgAb interference [4, 107, 108]. It is unlikely, though, that this observation can be generalized to all Tg RIAs, as some authors have found the opposite [109]. Although TgAb interference with IMA methodology is always unidirectional (i.e. underestimation), the influence of TgAb on RIA measurements is variable and assay dependent. Early studies reported that TgAb caused overestimation of Tg measured by RIA, presumably when endogenous TgAb sequestered [^{125}I-Tg] tracer. In contrast, more recent studies have suggested that TgAb causes underestimation of Tg measured by RIA, presumably when the second antibody reagent precipitates endogenous TgAb-[125I-Tg] complexes [1]. Moreover, most RIA methods are less sensitive in detecting Tg than IMA ones. Hence, any benefits gained in robustness against TgAb interference might be negated by the inability to detect the low Tg levels that characterize cure [110, 111]. In summary, any sample with a positive TgAb result, measured by a sensitive test, should be considered unreliable for measuring serum Tg concentrations in patients with DTC. Several analytes have been investigated as alternatives to

Tg measurement to assess relapse/metastases in TgAb-positive DTC patients such as oncofetal fibronectin, Tg messenger RNA (mRNA), thyrotropin receptor mRNA, thyroid peroxidase mRNA and circulating mutated BRAF. However, in most studies it proved impossible to achieve clinically useful sensitivity and specificity levels for these analytes [8, 112].

5.5.1.4 Tg measurement by Tandem Mass Spectrometry: Liquid Chromatography (MS/MS-LC)

Tandem mass spectrometry-liquid chromatography (MS/MS-LC) recently emerged as a promising method to overcome interferences in Tg measurement [113, 114]. The MS/MS-LC workflow for protein measurements involves a digestion of the sample with trypsin. Trypsin cleaves protein in a predictable fashion into peptides, which are measured and identified by protein database matching. In fact trypsin digests all proteins in a sample, including Tg and any TgAb or other antibodies, by cleaving them at predictable sites. One can then specifically look for tryptic peptides that are proteotypic for Tg (based on predicted cleavage), without any interference by TgAb [115] (Fig. 5.2).

Current MS/MS-LC Tg assays have shown comparability to immunoassays in samples that are TgAb negative. In TgAb-positive samples, which have detectable Tg by Tg immunoassay, all published MS/MS-LC Tg assays still correlated with the immunoassay but demonstrated a slope of ~1.5, consistent with systematic under recovery of Tg (~50–60%) in the immunoassays. Finally, in samples that are TgAb positive but have an undetectable Tg by sensitive immunoassay, the Tg MS assays can detect Tg in 20–25%

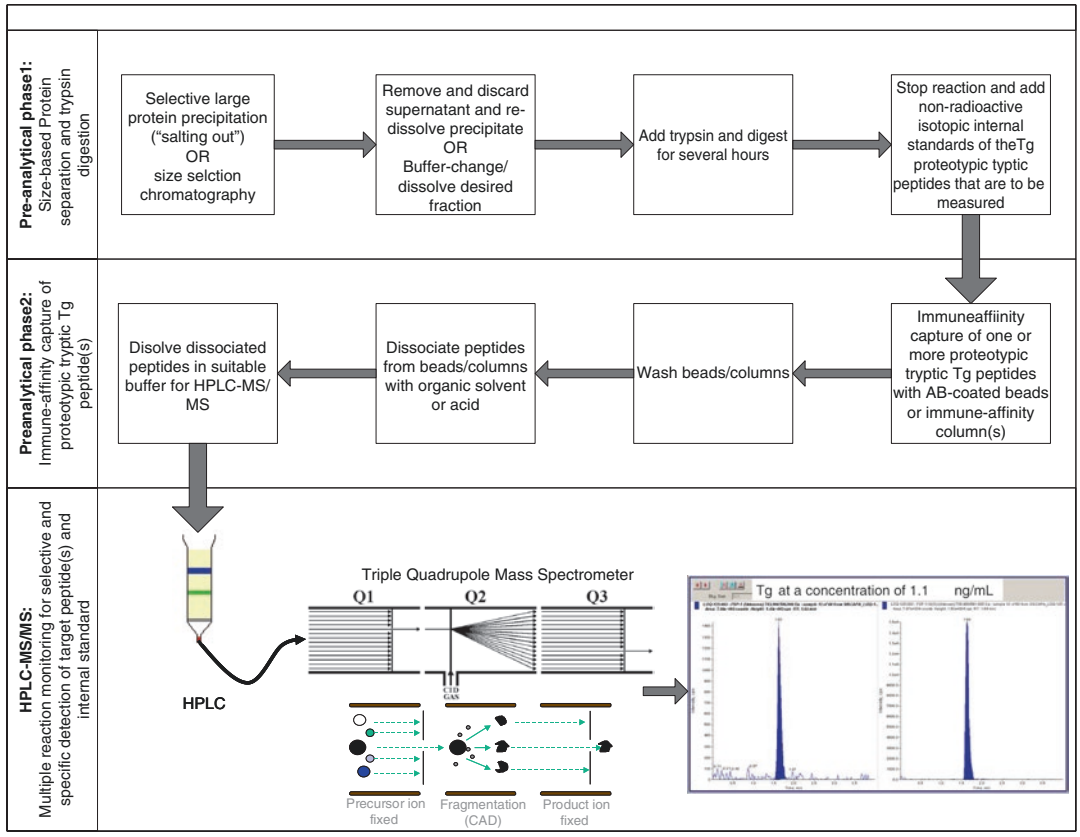

Fig. 5.2 Schematic depiction of the workflow for current (high-pressure) liquid chromatography tandem mass spectrometry (LC-MS/MS; HPLC-MS/MS) measurement of Tg (from [32], permission obtained)

of cases [116]. In practice, however, many problems affect Tg measurement by MS/MS-LC. First, one faces formidable "signal to noise" problems in identifying the quite low concentration of Tg peptides in a trillion-fold higher background abundance of all the other peptides from all the other proteins, and sample enrichment is required (i.e. immune affinity purification of the desired Tg target peptides from all the other peptides). Second, MS/MS-LC-based Tg assays are manual, complex, with a the long turnaround time (largely due to the several hours of tryptic digest). Third, the LOQ of current MS/MS-LC Tg assays is 0.5–1 μg/L, higher than that of high-sensitive Tg immunoassay (FS/LOQ ~0.1 μg/L) resulting in a suboptimal clinical sensitivity. Azmat and colleagues [117] recently evaluated the frequency of detectable Tg-MS/MS-LC with the functional sensitivity (FS) of 0.5 μg/L in patients with structural disease and compared performance of Tg MS/MS-LC versus Tg IMA, using either Immulite® assay with a FS of 0.9 μg/L or Beckman® assay with a FS of 0.1 μg/L in detecting structural disease in patients with positive TgAb. In patients with structural disease and positive TgAb, Tg MS/MS-LC was undetectable in 43.7% of patients. In the 26 patients with positive TgAb where Immulite assay was used, the sensitivity and specificity for detecting structural disease were at 44.4% and 94.1% for Tg MS/MS-LC assay and at 33.3% and 88.2% for Immulite assay. In the 74 patients with positive TgAb where Beckman assay was used, the sensitivity and specificity for detection of structural disease were 62.6% and 93.7% for the Tg MS/MS-LC and 72.7% and 71.4% for the Beckman assay, respectively. Overall, Tg MS/MS-LC was frequently undetectable and was less sensitive for detecting disease than a Tg immunoassay with functional sensitivity at 0.1 μg/L questioning the clinical usefulness of reflexing Tg measurement to MS/MS-LC in TgAb-positive patients [118]. Furthermore, in TgAb-positive patients with negative recovery measurement, RIA/IMA comparison and MS/MS-LC, falsely low Tg levels may still occur due to a faster biological clearance of TgAb-bound Tg [119].

5.5.2 Interfering Heterophilic Antibodies

Heterophilic antibodies (HAb) can bind animal antigens and form a bridge between capture and detection antibody leading to a falsely elevated (or, rarely, falsely decreased) Tg measurement in immunometric assays. These interferences are usually eliminated by the manufacturers by adding blocking agents to the assay, but a small percentage of patients (~1–3%) still show HAb interference on Tg measurement [120]. HAb interference may be detected either by recovery measurement or measurement of Tg in serially diluted sera (providing Tg concentrations are sufficiently high). An additional method, which is more specifically geared towards HAb interference, is to pretreat a serum aliquot with proprietary blocking agents and then compare the Tg result with an aliquot that was not pretreated [121]. In addition, as HAb interference is generally assay specific, the use of an alternate assay may both identify a false-positive sample and provide the correct test value. From the practical point of view, routine screening for the presence of HAb is not recommended; however, one or more of these tests should be performed in patients with discordant clinical findings, such as high serum Tg but negative imaging workup, positive imaging but undetectable Tg in the absence of TgAb and/or an unusual clinical course of Tg concentrations [122, 123].

5.5.3 Hook Effect

Thyroglobulin, as other tumour marker tests employing two-site noncompetitive IMAs, is prone to the so-called high-dose hook effect: this phenomenon is reported for the first time by Miles [124]. The excessively high concentrations of the analyte simultaneously saturate both capture and detecting antibodies. This prevents the formation of detectable antibody leading to the formation of stable capture antibody/analyte/detecting antibody complexes with a plot resembling a "fish hook" (Figs. 5.3 and 5.4) [3]. It affects mainly solid-phase assays where the capture antibody concentrations may be limiting.

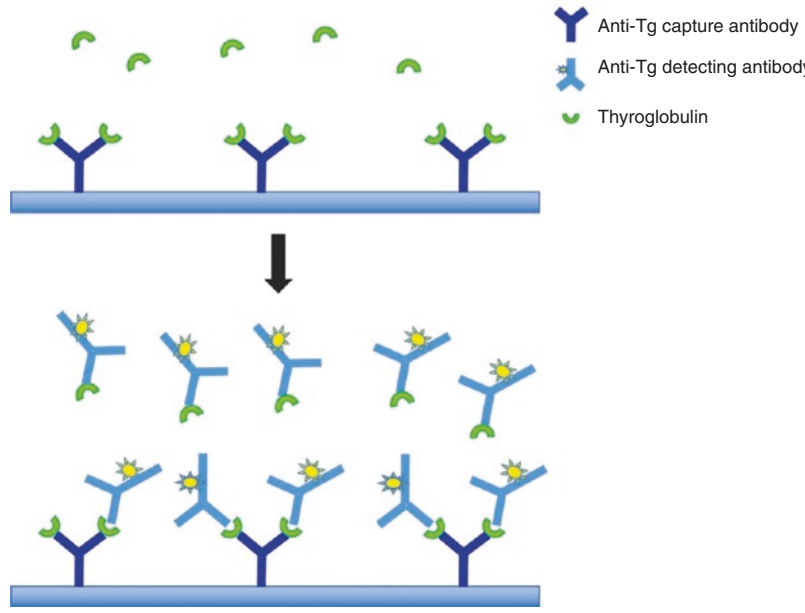

Fig. 5.3 High-dose hook effect: scheme of the principle

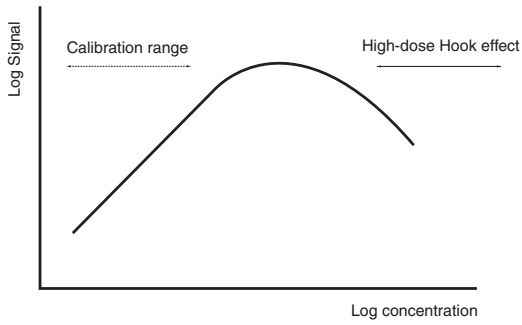

Fig. 5.4 Graphic representation of the high-dose hook effect

In this case a falsely low serum Tg measurement may have important clinical consequences. This phenomenon is demonstrated for alphafetoprotein, prolactin, chorionic gonadotropin, PSA, NSE, calcitonin and other tumour markers [125, 126]. In the case of Tg, published data show that the recent two-step sandwich IMAs are very resistant to high-dose hook effect, probably occurring only at very high concentrations of Tg (up to 200,000 mg/L) that is an unusual situation (about 0.1% of routine samples) [3, 127]. However, all laboratories providing tumour marker assays (i.e. Tg) should be alert to the possibility of hook effect ensuring a constant vigilance for this phenomenon: to avoid reporting falsely low results, some laboratories perform the Tg measurement on both neat and diluted serum (for cost reasons possible in few laboratories) or make a pool of patient specimens comparing it to the expected average of the batch of samples [128].

5.6 Role of Thyroglobulin and Thyroglobulin Autoantibodies in Managing Patients with Differentiated Thyroid Cancer

As previously mentioned preoperative Tg measurement is considered to have limited diagnostic value, although a number of studies report that an elevated preoperative serum thyroglobulin is a risk factor for nodular malignancy [23, 129, 130] and serum Tg measurement is sometime employed to strengthen or exclude a suspicion of DTC in patients with widespread metastases of unknown origin. Also, the relationship between the serum Tg levels and tumour burden may give indication of the efficiency of the tumour cells to secrete Tg and thereby determine the significance of postoperative serum Tg changes [3, 4, 44, 131].

5.6.1 Patients Treated by Total Thyroidectomy and Radioiodine Ablation

Current international clinical guidelines agree that Tg is a pivotal sensitive method for monitoring patients with DTC for the presence of residual or recurrent disease during follow-up after total thyroidectomy and, ideally, adjuvant ^{131}I remnant ablation. For a long time DTC follow-up was based on stimulated serum Tg in all patients who had had remnant ablation and negative cervical US and undetectable TSH-suppressed Tg within the first year after ablation. A "negative" TSH-stimulated Tg measurement and no other evidence of recurrent disease (i.e. negative clinical examination, neck US or additional imaging procedures, when indicated) predicted a very low risk of recurrence. The prognosis is excellent and life expectancy normal if response to the treatment is achieved within 6–12 months after treatment, both for low- and high-risk patients [132]. This approach (i.e. *dynamic risk stratification system*) allows tailoring follow-up intensity on an individual basis according to the response achieved after primary treatment [133] (Table 5.6).

Traditionally, "biochemical cure" was defined by a Tg levels <1–2 μg/L following TSH stimulation. More recently, however, novel highly sensitive Tg assays (with a functional sensitivity ≤0.1–0.2 μg/L) have been developed and became commercially available [9]. As rhTSH typically stimulates basal Tg approximately tenfold, the negative predictive value of a rhTSH-stimulated Tg value below the fixed cut-off of 1–2 μg/L is comparable to a basal high-sensitive Tg value below 0.10–0.20 μg/L, as consistently confirmed by literature [44]. Such concept was introduced in recent ATA 2015 guidelines [8] where interpretation criteria for basal and stimulated Tg were provided, as summarized in Table 5.7.

These criteria are simple and practical and allow clinician to modulate the intensity of further follow-up and to avoid inappropriate diagnostic procedures with relevant impacts on patients' comfort and overall costs. However, a relevant point of discussion is that most guidelines do not sufficiently address differences between different Tg assays in terms of the lower reporting limit (i.e. functional sensitivity or LOQ), analytical and clinical performance and appropriate cut-off limits. In fact, despite calibration against an international reference standard

Table 5.6 Response assessment after total thyroidectomy and radioiodine ablation according to ATA 2015 criteria [8]

Response	Definition
Excellent response	No clinical, biochemical or structural evidence of disease
Incomplete biochemical response	Abnormal Tg or rising anti-Tg antibody levels in the absence of localizable disease
Incomplete structural response	Persistent or newly identified loco-regional or distant metastases
Indeterminate response	Nonspecific biochemical or structural findings that cannot be confidently classified as either benign or malignant

Table 5.7 Response assessment after total thyroidectomy and radioiodine ablation: imaging and biochemical criteria according to ATA 2015 criteria [8]

Response	Imaging	Thyroglobulin [μg/L]
Excellent response	Negative	Basal Tg <0.2 μg/L *OR* Stimulated <1 μg/L
Incomplete biochemical response	Negative	Basal >1 μg/L *OR* Stimulated >10 μg/L
Incomplete structural response	Positive	Any value
Indeterminate response	Indeterminate findings	Basal 0.2–1 μg/L Stimulated 1–10 μg/L

(BCR®457), multiple assays analysing the same samples report different values due to heterogeneity in both Tg structure and assay reactivity. Additionally, different protocols are used by manufacturers to define analytical characteristics of different assays, and an assay with a declared higher functional sensitivity value may have a clinical performance equal to or better than one with a lower declared functional sensitivity. Accordingly, clinical thyroidologists and laboratory specialists are strongly advised to carefully evaluate the analytical and clinical performance of any newly introduced (highly sensitive) Tg assay, including a comparison between basal and stimulated values in the same assay, and to confirm cut-off and decision limits in their own DTC patient populations [44]. As a practical recommendation, it should be noted that Tg results cannot be reliably interpreted from samples collected immediately after surgery (i.e. post-surgical Tg half-life: 2–4 days) or up to 3 months after radioiodine therapy [62, 134]. Therefore, waiting 6–8 weeks after surgery and 3 months after radioiodine therapy is recommended [4, 44]. Finally, it is important to note that most patients who were enrolled in available studies on high-sensitive Tg were affected by low-risk and, even if less frequently, intermediate-risk DTC. Data on patients with high-risk tumours, however, are sparse and less robust. As a consequence, further studies in a broader spectrum of high-risk DTC patients are needed before applying the same approach in these cases, and for the moment, using a combination of ultrasound, stimulated Tg and diagnostic whole body scan is suggested [135].

5.6.2 Is There a Role for Tg Measurement in Patients Treated with Surgery Alone?

5.6.2.1 Lobectomy

According to current clinical guidelines, it is sufficient to treat low-risk patients with a thyroid microcarcinoma by resection of the affected thyroid lobe only, without complete thyroidectomy or ^{131}I ablation. In this situation, measuring Tg using either highly sensitive or conventional assays is essentially useless as Tg levels will not depend on the presence or absence of tumour foci but rather on the remaining thyroid lobe volume, current iodine status and TSH concentration. In such patients, the options for DTC follow-up are to perform cervical US and, if recurrence or metastasis are suspected, to secure the diagnosis through a fine needle biopsy [44]. More sophisticated Tg reference intervals, mathematically normalized to TSH level and residual thyroid tissue, tailored to individual patients should be useful in these cases [1]; for the moment, however, no reliable interpretation criteria are available.

5.6.2.2 Thyroidectomy

In patients with tumours <10–20 mm, no lymph node and/or distant metastases, a (near-)total thyroidectomy without radioiodine ablation is now considered a reasonable treatment [8]. These non-ablated patients may have a considerable thyroid remnant, and a TSH stimulation during follow-up is not useful as Tg will be detectable due to remaining healthy thyroid tissue and will obscure any possible tumour-related Tg level rise. In addition, the absolute Tg concentration will be significantly less useful in this scenario. However, a decrease of Tg concentrations over time was reported, and the number of patients with undetectable Tg significantly increased after 5 years of follow-up [136]. A retrospective evaluation of 86 patients with low-risk DTC treated by total thyroidectomy only (i.e. without radioiodine ablation) using a highly sensitive Tg assay (i.e. functional sensitivity, 0.1 μg/L) was reported [137]. Of the 76 patients without TgAb, the first Tg measurement (on T4), obtained at a mean time of 9 months after surgery, was ≤0.1 μg/L in 62% of cases, ≤0.3 μg/L in 82%, ≤1 μg/L in 91% and ≤2 μg/L in 96% of cases. After a median follow-up of 2.5 years, one patient had persistent disease, an unstimulated Tg concentration of 11 μg/L and an abnormal neck US, while two patients had Tg levels >2 μg/L with normal neck US. Within the first 2 years after total thyroidectomy, the unstimulated Tg level was <0.3 μg/L in 86% and ≤2 μg/L in 96% of the cases, respectively. However, the authors emphasize that the

results were strictly dependent on the completeness of surgery by a dedicated surgeon in a referral centre. More recently, Tg cut-off levels were proposed by Momesso and colleagues for DTC patients treated without radioiodine ablation [138]. However, relevant methodological problems hampered such study and precluded any reliable conclusion for the clinical practice. In facts, they employed five different Tg immunoassays over time with different functional sensitivities and arbitrarily selected Tg cut-off [139]. Then, although a stable low Tg concentration, combined with normal neck US, may be helpful in assessing whether there is concern for progressive disease in these patients, reliable interpretation criteria of Tg testing are still lacking, as underlined in the last version of ATA guidelines [8]. It is true that the trend in basal Tg should reflect changes in thyroid tissue mass and low Tg concentrations arising from small post-surgical thyroid remnants are expected after thyroidectomy, typically in the 0.1–0.5 μg/L range when TSH is suppressed [59]. However, (1) the volume of thyroid remnants is "surgeon dependent" with a high variability [140], and (2) TSH-suppression is no longer recommended in most low-risk and intermediate-risk DTC patients and TSH levels ranged between 0.1–0.5 and 2 mUI/L in such cases. Of course, Tg reference values may be significantly different in patients with small remnants and suppressed TSH after surgery and those with large remnants and non-suppressed TSH levels, respectively. Again, Tg values should be normalized to the volume of remnant tissue and TSH levels to obtain a reliable clinical information. Additionally, TSH levels may also impact the longitudinal evaluation of Tg [i.e. trend and doubling time] that recently showed prognostic utility [141]; in facts, aspecific TSH fluctuations can induce relevant Tg changes that, in turn, may falsely alert or reassure both patients and physicians. All considered, it seems very difficult, if not impossible, to provide general interpretation criteria for serum Tg in non-ablated DTC patients. Then, for the moment, Tg interpretation in these patients requires stable TSH levels, consistency of Tg assay employed across the follow-up and cautious clinical interpretation.

5.6.3 Managing Patients with Positive Thyroglobulin Antibodies

Serum levels of TgAb are not correlated with the tumour load of the patient but rather indicate the activity of the immune system. Furthermore, the mere presence of TgAb in serum has thus far not conclusively been shown to correlate with a worse or better overall prognosis. Chiovato et al. [142] demonstrated that the concentration of TgAb after thyroid ablation for thyroid carcinoma of 182 patients with thyroid autoimmune disease before treatment had a mean disappearance time of 3 years, indicating that the actual TgAb concentration is not very useful during that period for outcome prediction. However, TgAb can be used as "surrogate tumour marker" as disease-free patients with high TgAb concentrations typically display a progressive TgAb decline over time, even if some patients may not achieve full TgAb negativity, possibly because of the long-lived memory of plasma cells. In this context the trend is more important than the absolute level: a consistent reduction in the serum TgAb concentration thus seems to indicate that the patient is likely to be free of disease, while a consistent rise or *de novo* appearance of serum TgAb raises suspicion of recurrence, and an unchanged serum TgAb concentration must be regarded as indeterminate [109, 143, 144]. Unfortunately, robust quantitative criteria for the interpretation of a specific "rising" or "falling" of serum TgAb concentration are still lacking [74], and few caveats should be also mentioned (1) a very abrupt and extreme rise in serum Tg as in rapid development of metastatic thyroid carcinoma may compromise correct quantification of TgAb measurements by immune complex formation with falsely low TgAb concentrations; (2) a rapid, but transient, increase in measured TgAb concentration may occur shortly after thyroidectomy in patients with prior positive TgAb; and (3) a similar but slower reaction is seen after radioactive iodine treatment [145–147]. As a guide for TgAb trend interpretation, different authors have suggested that disease-free patients typically display a > 50% drop in TgAb in the

first post-operative year [148–150]. However, this figure is very likely to depend on the initial pretreatment concentration, and more studies and evidence are thus required to be able to use this as early risk assessment in clinical practice, in order to avoid uncertainty and additional expensive imaging procedures.

Finally, it should be emphasized that the recommendation to use serial TgAb concentrations as a surrogate tumour marker necessitates continuity of the method in the laboratory as changing methods disrupts TgAb monitoring. In this context it is worth noting that despite numeric differences between methods, the ratio between any two different TgAb methods appears constant for a given patient but different for different patients, reflecting TgAb heterogeneity. Establishing the ratio between an old and proposed new method on a specimen from the patient can be used to *re-baseline* the new method, which is an important approach in order to avoid misinterpretation of the long-term outcomes. However, this is rarely done in clinical practice [91]. Currently the data on TgAb use for thyroid cancer management is based on an initial treatment strategy of total ablation, since insufficient ablation will hamper both Tg and TgAb as tumour markers. For TgAb the important issue is continued presence of autoantigens as long as remnant thyroid cells are still present. In practice, while TgAb together with the other thyroid autoantibodies generally decrease after removal of the thyroid tissue by total ablation [142], their disappearance is not expected in other cases since autoimmunity will be continuously stimulated by the presence of thyroid autoantigens as well as by intrathyroidal lymphocytes. Importantly, no clinical data are currently available on the management of TgAb-positive patients treated by either lobectomy alone or thyroidectomy without radioiodine ablation.

Conclusions

The post-surgical follow-up of DTC is aimed at early identification of the small proportion of patients who have residual disease or will develop recurrence. In the absence of TgAb and heterophilic antibodies, Tg measurements are nowadays the reference standard for clinical management of patients previously treated for DTC. Even though the introduction of high-sensitive Tg assays is not without challenges, there is an increasing body of evidence that an undetectable highly sensitive Tg during LT4 treatment is sufficient with a high negative predictive value to forgo TSH stimulation. In the presence of TgAb, it is possible to follow the dynamic trend of TgAb themselves as surrogate markers. Robust data are urgently needed to define the clinical role, the interpretation criteria and the limitations of these markers in the increasing number of patients treated with lobectomy alone or total thyroidectomy not followed by radioiodine ablation. For the time being, the issue remains largely unaddressed, and no clear-cut recommendations for the clinical practice can be delivered before well-designed, large and multicentric studies addressing these issues have been published.

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