D. Thyroid Autoantibodies (TPOAb, TgAb and TRAb)

Autoimmune thyroid disease (AITD) causes cellular damage and alters thyroid gland function by humoral and cell-mediated mechanisms. Cellular damage occurs when sensitized T-lymphocytes and/or autoantibodies bind to thyroid cell membranes causing cell lysis and inflammatory reactions. Alterations in thyroid gland function result from the action of stimulating or blocking autoantibodies on cell membrane receptors. Three principal thyroid autoantigens are involved in AITD. These are thyroperoxidase (TPO), thyroglobulin (Tg) and the TSH receptor. Other autoantigens, such as the Sodium Iodide Symporter (NIS) have also been described, but as yet no diagnostic role in thyroid autoimmunity has been established (248). TSH receptor autoantibodies (TRAb) are heterogeneous and may either mimic the action of TSH and cause hyperthyroidism as observed in Graves' disease or alternatively, antagonize the action of TSH and cause hypothyroidism. The latter occurs most notably in the neonate as a result of a mother with antibodies due to AITD. TPO antibodies (TPOAb) have been involved in the tissue destructive processes associated with the hypothyroidism observed in Hashimoto's and atrophic thyroiditis. The appearance of TPOAb usually precedes the development of thyroid dysfunction. Some studies suggest that TPOAb may be cytotoxic to the thyroid (249,250). The pathologic role of TgAb remains unclear. In iodide sufficient areas, TgAb is primarily determined as an adjunct test to serum Tg measurement, because the presence of TgAb can interfere with the methods that quantitate Tg [Section-3 E6]. In iodide deficient areas, serum TgAb measurements may be useful for detecting autoimmune thyroid disease in patients with a nodular goiter and for monitoring iodide therapy for endemic goiter.

Laboratory tests that determine the cell-mediated aspects of the autoimmune process are not currently available. However, tests of the humoral response, i.e. thyroid autoantibodies, can be assessed in most clinical laboratories. Unfortunately, the diagnostic and prognostic use of thyroid autoantibody measurements is hampered by technical problems as discussed below. Although autoantibody tests have inherent clinical utility in a number of clinical situations, these tests should be selectively employed.

1. Clinical Significance of Thyroid Autoantibodies

TPOAb and/or TgAb are frequently present in the sera of patients with AITD (251). However, occasionally patients with AITD have negative thyroid autoantibody test results. TRAb are present in most patients with a history of or who currently have Graves' disease. During pregnancy, the presence of TRAb is a risk factor for fetal or neonatal dysfunction as a result of the transplacental passage of maternal TRAb (252,253). The prevalence of thyroid autoantibodies is increased when patients have non-thyroid autoimmune diseases such as type 1 diabetes and pernicious anemia (254). Aging is also associated with the appearance of thyroid autoantibodies (255). The clinical significance of low levels of thyroid autoantibodies in euthyroid subjects is still unknown (256). However, longitudinal studies suggest that TPOAb may be a risk factor for future thyroid dysfunction, including post-partum thyroiditis (PPT) as well as the development of autoimmune complications from treatment by a number of therapeutic agents (50,257,258). These include amiodarone therapy for heart disease, interferon-alpha therapy for chronic hepatitis C and lithium therapy for psychiatric disorders (75,259-262). The use of thyroid autoantibody measurements for monitoring the treatment for AITD is generally not recommended (263). This is not surprising since treatment of AITD addresses the consequence (thyroid dysfunction) and not the cause (autoimmunity) of the disease. However, changes in autoantibody concentrations often reflect a change in disease activity.

2. Nomenclature of Thyroid Antibody Tests

The nomenclature used for thyroid autoantibodies has proliferated, particularly in the case of TSH receptor antibodies (LATS, TSI, TBII, TSH-R and TRAb). The terms used in this monograph, TgAb, TPOAb and TRAb are those recommended internationally. These terms correspond to the molecular entities (immunoglobulins) which react with the specified autoantigens recognized by the laboratory test. Method differences may bias the measurement of these molecular entities, e.g.: methods may detect only IgG or IgG plus IgM; TPOAb or Ab directed to TPO and other membrane autoantigens; TSH inhibiting and/or TSH stimulating TRAb.

3. Specificity of Thyroid Antibody Tests

The use of thyroid autoantibody measurements has been hampered by specificity problems. Studies show that results vary widely depending on the method used. This is due to differences in both the sensitivity and specificity of the methods and the absence of adequate standardization. In the past few years, studies at the molecular level have shown that autoantibodies react with their target autoantigens, by binding to " conformational " domains or epitopes. The term "conformational" refers to the requirement for a specific three- dimensional structure for each of the epitopes recognized by the autoantibodies. Accordingly, assay results critically depend on the molecular structure of the antigen used in the test. Small changes in the structure of a given epitope may result in a decrease or a loss in autoantigen recognition by the antibodies targeted to this epitope. Recently, dual specificity TGPO antibodies, that recognize both Tg and TPO, have been demonstrated in the blood of patients with AITD *(264)*.

Guideline 29. Thyroid Antibody Method Sensitivity & Specificity Differences

Recognize and understand that the results of thyroid antibody tests are method-dependent.
Thyroid antibody methods recognize different epitopes in the heterogeneous antibody populations present in serum.
Thyroid antibody assay differences reflect different receptor preparations (receptor assays) or cells (bioassays) used in the assay.
Assay differences can result from contamination of the antigen reagent with other autoantigens.
Assay differences can result from the inherent assay design (i.e. competitive versus non-competitive immunoassay) as well as the signal used.
• Assay differences can result from the use of different secondary standards.

It has been known for years that autoantibodies are directed against few epitopes as compared to heterologous antibodies. Current methods differ widely in epitope recognition. Specificity differences can result from misrecognition of an epitope that leads to a bias regarding the autoantibody population tested. This results in vastly different reference intervals, even when methods are standardized to the same international reference preparation. Whatever the targeted autoantigen, thyroid autoantibodies are clearly not unique molecular entities but, rather, mixtures of immunoglobulins that only have in common their ability to interact with Tg, TPO or the TSH receptor.

Differences in the sensitivity of autoantibody tests may arise from the design of the assay (e.g. competitive RIA versus two-site IMA) as well as the physical method used for the signal (e.g. radioisotope versus chemiluminiscence). Differences in specificity may occur as a result of contamination of the autoantigen preparation by other autoantigens (e.g. thyroid microsomes versus purified TPO). Further, misrecognition of an epitope may lead to an underestimation of the total amount of circulating autoantibody present, resulting in decreased sensitivity.

Guideline 30. Functional Sensitivity of Thyroid Antibody Tests

Functional sensitivity assessment of thyroid autoantibody tests should:

Be determined with human serum pools containing a low autoantibody concentration
Be determined using the same protocol as described for TSH (Guideline 20) but with the between-run precision assessment made over a 6 to 12 month time-period to represent the appropriate clinical assessment interval.

Functional sensitivity should be determined with human serum pools containing a low autoantibody concentration. The protocol for functional sensitivity should be the same protocol as described for TSH (Guideline 20). The between-run precision for TgAb tests used for monitoring TgAb-positive DTC patients

should be assessed across a longer time-period (6 to 12 months) consistent with the interval used for serial monitoring in clinical practice.

4. Standardization of Thyroid Antibody Tests

Standardization of thyroid autoantibody tests is currently suboptimal. International Reference Preparations, MRC 65/93 for TgAb, MRC 66/387 for TPOAb are available from the National Council for Biological Standards and Control in London, UK (www.mrc.ac.uk). These preparations were made from a pool of serum from patients with autoimmune thyroid disease and were prepared and lyophilized 35 years ago!

Guideline 31. For Manufacturers Standardizing Thyroid Antibody Assays

Assays should be standardized against MRC International Reference Preparations:-
    MRC 65/93 for TgAb, MRC 66/387 for TPOAb and MRC 90/672 for TRAb
New International Reference Preparations should be prepared for TgAb and TPOAb.
Secondary standards should be fully characterized to avoid bias between different methods.
Reference preparations or recombinant antigen preparations should be used when available.

It is well known that lyophilized antibodies are prone to degradation over time. Degradation of the antibodies may have introduced a bias in the binding activity of these reference preparations towards more stable antibodies of unknown clinical relevance. Due to the scarcity of these preparations, they are only used as primary standards for calibrating assay methods. Commercial kits contain secondary standards that differ for each method. Currently, assay calibrations vary with the experimental conditions as well as the antigen preparation used by the manufacturer. This may introduce another bias in detecting the heterogeneous antibodies present in patient specimens. In the case of TRAb, the reference preparation MRC 90/672 is more recent (1990) but currently used by few manufacturers.

5. TPOAb Measurements

Thyroid Peroxidase (TPO) is a 110 kD membrane bound hemo-glycoprotein with a large extracellular domain, and a short transmembrane and intracellular domain. TPO is involved in thyroid hormone synthesis at the apical pole of the follicular cell. Several isoforms related to differential splicing of TPO RNA have been described. TPO molecules may also differ with respect to their three-dimensional structure, extent of glycosylation and heme binding. Most of the TPO molecules do not reach the apical membrane and are degraded intracellularly.

Guideline 32. Preferred TPOAb Methodology

Sensitive, specific TPOAb immunoassays, using suitable preparations of highly purified native or recombinant human TPO as the antigen, should replace the older insensitive, semi-quantitative anti-microsomal antibody (AMA) agglutination tests.
(Consensus Level 90%)

The clinical significance of a low TPOAb concentration requires more study.

TPO autoantibodies were initially described as anti-microsomal autoantibodies (AMA) since they were found to react with crude preparations of thyroid cell membranes. The microsomal antigen was later identified as TPO *(265)*. Older AMA immunofluorescence assays as well as passive tanned red cell agglutination tests are still currently in use in addition to the newer, more sensitive competitive and non-competitive TPOAb immunoassays. These new immunoassay methods are currently replacing the older AMA agglutination tests because they are quantitative, more sensitive and can easily be automated. However, there is wide variability in the sensitivity and specificity of these new TPOAb methods. Some of this variability stems from differences in the TPO preparations used in the various assay kits. When extracted from human thyroid tissue, TPO may be used as a crude membrane preparation or may be purified by different methods. The assay specificity may also

differ because of contamination by other thyroid antigens – notably Tg and/or variations in the three-dimensional structure of TPO. The use of recombinant human TPO (rhTPO) eliminates the risk of contamination but does not solve the problem of the differences in TPO structure that depend upon the technique used to isolate TPO. Most current TPOAb assays are quantitated in international units using the reference preparation MRC 66/387. Unfortunately, the use of this primary standard does not alleviate between-method variations as is evident from the broad variability in sensitivity limits claimed by the different kit manufacturers (range <0.3 to <20 kIU/L) and the differences in normal reference intervals.

(a) TPOAb Prevalence & Reference Intervals

The estimate of TPOAb prevalence depends on the sensitivity and specificity of the method employed. The recent NHANES III United States survey of ~17,000 subjects without apparent thyroid disease, reported detectable TPOAb levels in 12 % of subjects using a competitive immunoassay method *(18)*. Whether low levels of TPOAb detected in healthy individuals and/or patients with non-thyroid autoimmune diseases reflect normal physiology, the prodrome of AITD, or an assay specificity problem, remains unclear.

Normal reference values for TPOAb assays are highly variable and often arbitrarily established, so that a large majority of patients with AITD test positive, and most subjects without clinical evidence of AITD test negative. The lower normal limit appears to relate to technical factors. Specifically, assays citing a low detection limit (<10 kIU/L) typically report undetectable TPOAb levels in meticulously selected normal subjects. Such methods suggest that the presence of TPOAb is a pathologic finding. In contrast, TPOAb assays reporting higher detection limits (>10kIU/L) typically cite a TPOAb "normal reference range". Since such methods appear to have no enhanced sensitivity for detecting AITD, these "normal range" values may represent non-specific assay "noise" and may not be pathologically meaningful.

The recent 20-year follow-up study of the Whickham cohort reported that detectable TPOAb titers (measured as AMA) was not only a risk factor for hypothyroidism but that a detectable AMA preceded the development of an elevated TSH (Figure 5) *(35)*. This suggests that a detectable TPOAb is a risk factor for AITD (Guideline 34). However, individuals with low TPOAb levels would have had undetectable AMA by the older methods used in this study *(35)*. The clinical significance of low TPOAb levels that are not detectable by AMA agglutination methods remains to be established through longitudinal studies. Thus, whether individuals with low levels of TPOAb and/or TgAb should be considered normal remains in question until long-term follow-up studies on such individuals show that they do not have an increased risk for developing thyroid dysfunction.

Guideline 33. Reference Intervals for Thyroid Antibody Tests

Reference intervals for thyroid antibody tests should be established from 120 "Normal" subjects free from any history of thyroid disease: Subject selection should minimize the inclusion of persons with a predisposition for autoimmune thyroid disease. Normal subjects should be:

Male
Young (< 30 years of age)
Have serum TSH levels between 0.5 and 2.0 mIU/L
No goiter
No personal or family history of thyroid disease
No non-thyroid autoimmune diseases (e.g. lupus or diabetes)

The criteria employed for selecting subjects for the normal cohort used to establish an autoantibody normal reference range, is critical. Such a cohort should be comprised of young, biochemically euthyroid (TSH 0.5 to 2.0 mIU/L) male subjects with no goiter and no family history of AITD. This rigorous selection process would be least likely to include subjects with a predisposition to AITD.

(b) Clinical Uses of TPOAb Measurements

TPOAb is the most sensitive test for detecting autoimmune thyroid disease *(266)*. As shown schematically in Figure 5, TPOAb is typically the first abnormality to appear in the course of developing hypothyroidism secondary to Hashimotos' thyroiditis. In fact, when TPOAb is measured by a sensitive immunoassay, >95% of subjects with Hashimotos thyroiditis have detectable levels of TPOAb. Such methods also detect TPOAb in most (~85%) patients with Graves' disease *(254)*. Patients with TPOAb detected in early pregnancy are at risk for developing post-partum thyroiditis *(50)*. Patients with Down's syndrome have an increased risk of thyroid dysfunction due to autoimmune thyroid disease and annual screening with TSH and TPOAb is important *(267,268)*.
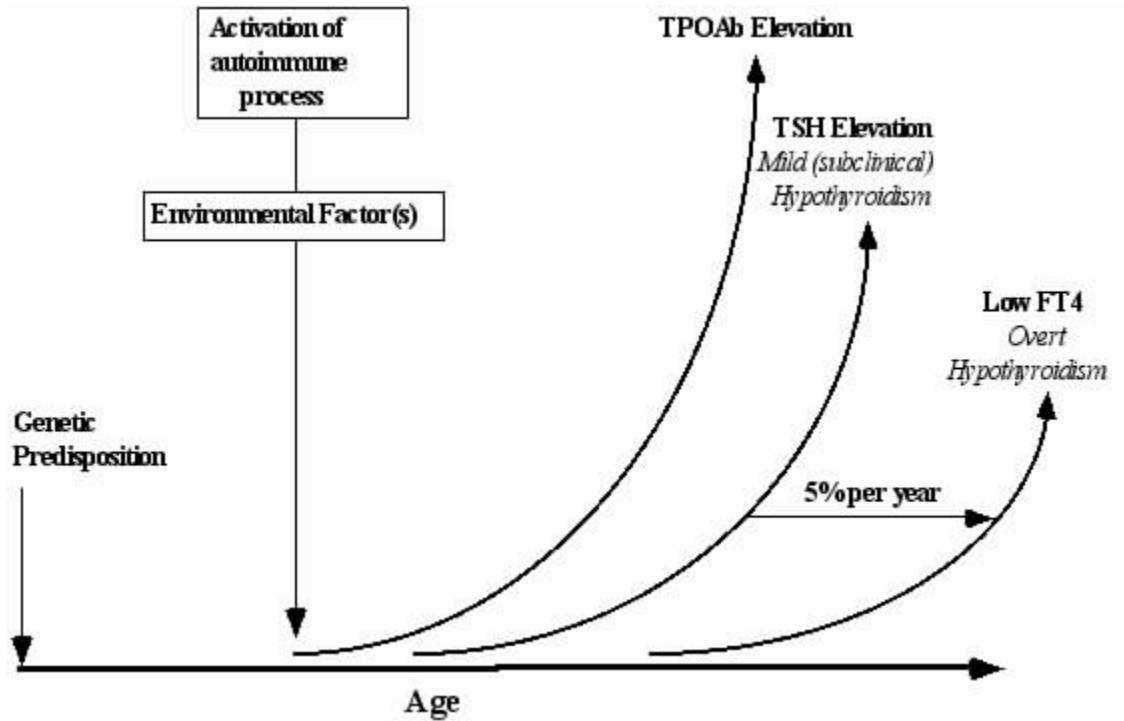

Fig 5. TPOAb Changes with Developing Autoimmune Thyroid Disease

Recent reports have suggested that the IQ of children born to mothers with increased TSH and/or detectable TPOAb during pregnancy may be compromised *(63-65)*. This has prompted recommendations that all pregnant women should have TSH and TPOAb levels measured in the first trimester of their pregnancy [Section-2 A3 and Guideline 4]. Further, TPOAb measurements may have a role in infertility, since high TPOAb levels are associated with a high risk of miscarriage and failure to conceive with in-vitro fertilization *(269)*.

Guideline 34. Recommended Uses for TPOAb Measurement

    Diagnosis of Autoimmune Thyroid Disease
    Risk factor for Autoimmune Thyroid Disease
    Risk factor for hypothyroidism during Interferon alpha, Interleukin-2 or Lithium therapy
    Risk factor for thyroid dysfunction during amiodarone therapy (see Guideline 5)
    Risk factor for hypothyroidism in Down's Syndrome patients
    Risk factor for thyroid dysfunction during pregnancy and for post-partum thyroiditis
    Risk factor for miscarriage and in-vitro fertilization failure

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The presence of TPOAb is well established as a risk factor for thyroid dysfunction when patients are being treated with lithium, amiodarone, interleukin-2 or interferon-alpha *(75,259,260,261,270)*. During interferon-alpha treatment, a preexisting thyroid autoimmune disorder or positive TPOAb titer are predisposing factors for the development of thyroid disease during therapy *(262)*. There appears however, to be no increased frequency of thyroid dysfunction during interferon-beta therapy *(271)*. The presence of TPOAb before therapy shows a sensitivity of 20%, a specificity of 95% and a predictive value of 66.6% for the development of thyroid dysfunction *(272)*.

6. Thyroglobulin Autoantibody (TgAb) Measurements

Thyroglobulin (Tg), the prothyroid globulin, is a high molecular weight (660 kDa) soluble glycoprotein made up of two identical subunits. Tg is present with a high degree of heterogeneity due to differences in post-translational modifications (glycosylation, iodination, sulfation etc). During the process of thyroid hormone synthesis and release, Tg is polymerized and degraded. Consequently, the immunologic structure of Tg is extremely complex. The characteristics of Tg preparations may vary widely depending on the starting human thyroid tissue and the purification process used. This is the first clue to explain why TgAb assays, as well as Tg assays [Section-3 E2] are so difficult to standardize.

(a) TgAb Methodology

As with TPOAb methods, the design of TgAb assays has evolved from immunofluorescence of thyroid tissue sections, to passive tanned red cell agglutination methods and to the more current, competitive and non-competitive immunoassays. This technical evolution has improved both the sensitivity and specificity of serum TgAb measurements. However, because the older and newer methods are still being used concurrently in clinical laboratories, the sensitivity and specificity of available methods can vary widely depending on the laboratory. Assays are calibrated with purified or crude preparations of TgAb by pooling patient sera or blood donor material. These various secondary standards are often, but not always, calibrated against the primary standard (MRC 65/93). However, standardization with MRC 65/93 does not ensure that different methods are quantitatively or qualitatively similar. Other reasons for method differences relate to the heterogeneity of the TgAb itself. The heterogeneity of TgAb is restricted in patients with AITD compared with other thyroid disorders such as differentiated thyroid carcinomas (DTC) in which the heterogeneity of TgAb appears less restricted *(273)*. This reflects differences in the expression of the different autoantibodies that may be normally expressed at very low levels in healthy individuals *(274)*. The inter-method variability of serum TgAb values may also reflect qualitative differences in TgAb affinity and epitope specificity in different serum samples from patients with different underlying thyroid and immunological conditions. Another reason for inter-method differences is that assay designs are prone to interference by high levels of circulating antigen (Tg), as is commonly the case with Graves' disease and metastatic DTC *(275)*.

Guideline 35. For Manufacturers Developing TgAb Methods

The epitope specificity of TgAb methods should be broad not restricted, since TgAb epitope specificity may be wider for TgAb-positive patients with DTC compared to patients with autoimmune thyroid disease.

(b) TgAb Prevalence & Reference Intervals

As with TPO antibodies, the prevalence and normal cut-off values for thyroglobulin antibodies depends on the sensitivity and specificity of the assay method *(276)*. The NHANES III survey reported a TgAb prevalence of ~10% for the general population, measured by competitive immunoassay *(18)*. The TgAb prevalence appears to be two-fold higher than normal for patients diagnosed with DTC (~20 %) *(276)*. As with TPOAb, the clinical significance of low TgAb levels, that would be undetectable by the older agglutination methods, remains unclear. It has been suggested that low levels may represent " natural " antibody in normal individuals or a " scavenger " antibody response to antigen release following thyroid surgery or radioactive iodide therapy. Alternatively, low levels might represent underlying silent AITD *(256)*. Different TgAb methods report

different normal threshold values, as discussed for TPOAb [Section-3 D5(a)]. Specifically, some TgAb methods report that normal subjects should have values below the assay detection level, other methods report a "normal range". When TgAb measurements are used as an adjunct test to serum Tg measurements, the significance of low TgAb levels relates less to the pathophysiology of its presence but more to the potential for low TgAb levels to interfere with the serum Tg method.

Guideline 36. TgAb Measurement in Non-Neoplastic Conditions

In iodide <u>sufficient</u> areas, it is not usually necessary or cost-effective to order <u>both</u> TPOAb and TgAb, because TPOAb-negative patients with detectable TgAb rarely display thyroid dysfunction.
In iodide <u>deficient</u> areas, serum TgAb measurements may be useful for detecting autoimmune thyroid disease when patients have a nodular goiter.
Monitoring iodide therapy for endemic goiter.

(c) Sensitivity and Precision of TgAb Measurement

Sensitive quantitative TgAb measurements are a critical adjunct test for serum Tg measurement. Qualitative agglutination tests are not sufficiently sensitive to detect the low TgAb concentrations that can interfere with serum Tg measurements (276). As with TPOAb assays [Section-3 D5(a)], the absolute values reported by different TgAb immunoassays are highly variable which precludes the use of different manufacturers tests for serial monitoring of DTC patients. There appear to be two classes of TgAb immunoassays. One class is characterized by low detection limits (<10 kIU/L) and an undetectable normal reference limit. Such methods suggest that the presence of T TgAb is a pathologic finding. The other class of assay reports higher detection limits (>10kIU/L) and cites a TgAb "normal reference range". The likelihood is that these detectable "normal range" values merely represent non-specific assay "noise" caused by assay insensitivity or problems with specificity since these low "normal range" values do not show evidence of interfering with serum Tg measurements [Section-3 E6].

Guideline 37. TgAb Measurement in Differentiated Thyroid Carcinomas (DTC)

The TgAb concentration should be measured in <u>ALL</u> patient sera prior to Tg analysis because low levels of TgAb can interfere with serum Tg measurements causing either falsely low or undetectable or high values depending on the Tg method used.

TgAb should be measured in every serum specimen sent to the laboratory for Tg testing.
Serial TgAb measurements should be made on all TgAb-positive DTC patients using the same manufacturer's method because serial TgAb values have prognostic significance for monitoring response to DTC treatment.
TgAb methods should be immunoassay not agglutination, because low levels of TgAb can interfere with serum Tg measurements made by most methods, and serial measurements must be quantitative not qualitative.
Serum Tg recovery tests do not reliably detect the presence of TgAb and should be discouraged as a method for detecting TgAb (Guideline 46).
Before changing the TgAb method, the laboratory should inform physician users and evaluate the relationship between the old and proposed new method values. Patients should be re-baselined if the difference between the methods is >10% CV.

(d) Clinical Uses of TgAb Measurement

There is some debate over the clinical utility of serum TgAb measurement for assessing the presence of thyroid autoimmunity. The United States NHANES III study reported that 3 % of subjects with no risk factors for thyroid disease had detectable TgAb without associated presence of TPOAb (18). Since this cohort had no associated TSH elevation, TgAb measurements do not appear to be a useful diagnostic test for AITD in areas of iodide sufficiency (256,279). In iodide deficient areas however, TgAb is believed to be useful for detecting

AITD, especially for patients with a nodular goiter. TgAb measurements are also useful for monitoring iodide therapy for endemic goiter, since iodinated Tg molecules are more immunogenic.

Serum TgAb testing is primarily used as an adjunct test when serum Tg measurements are requested. The clinical utility of TgAb measurements in sera from DTC patients is two-fold. First, sensitive and specific TgAb screening of sera in these cancer patients is necessary, because even low antibody concentrations can interfere with the Tg measurements made by most Tg methods [see Section-3 E6] *(275,276)*. Second, serial TgAb measurements themselves may serve as a surrgogate tumor marker test for TgAb-positive patients in whom Tg testing may be unreliable *(276)*. Specifically, TgAb-positive patients who are rendered disease-free typically become TgAb-negative within 1-4 years *(276,277,278)*. In contrast, patients who have persistent disease after treatment retain detectable TgAb concentrations. In fact, a rise in the TgAb level is often the first indication of recurrence in such patients *(276)*.

7. TSH Receptor Autoantibodies (TRAb)

The TSH receptor is a member of the superfamily of receptors with seven transmembrane domains linked to G proteins. The 60kb TSH receptor gene located on the long arm of chromosome *14q31* has been cloned and sequenced (272). Exons 1-9 code for the extracellular domain of the receptor (397 amino acids) and exon 10 codes for the transmembrane region (206 amino acids). Activation of G proteins by the hormone receptor complex results in stimulation of cAMP production by adenylate cyclase and inositol phosphate turnover by phospholipases *(280)*. Site-directed mutagenesis has shown that the 3-dimensional receptor structure is important for the interaction with TSH and/or TRAbs. There are three broad types of TRAb measured by either bioassay or receptor assay (Table 6). Receptor, or TSH Binding Inhibitory Immunoglobulin (TBII) assays do not measure biologic activity directly but assess whether the specimen contains immunoglobulins that can block the binding of TSH to an in vitro receptor preparation. TSH stimulating antibodies (TSAb) appear to bind the N-terminal portion of the extracellular domain and mimic the actions of TSH by inducing post-receptor signal transduction and cell stimulation. In contrast, the C-terminal region is more important for TSH receptor blocking antibodies (abbreviated TBAb or TSBAb) which block stimulation by either TSAb or TSH, causing hypothyroidism *(281)*. Thyroid growth-stimulating immunoglobulins (TGI) are less well characterized in this regard.

It has now been shown that the lack of correlation between TRAb levels and the clinical status of patients is largely because of circulating TRAb's that are heterogeneous. The fact that TRAb heterogeneity can coexist within an individual patient and change over time is one reason why it has been difficult to develop diagnostically accurate TRAb tests *(282,283)*. Indeed, the clinical presentation of Graves' patients who exhibit both TSAb and TBAb/TSBAb will likely depend on the relative concentration and affinity of the predominant antibody. A shift from stimulating to blocking TRAb may explain the spontaneous remission of Graves' disease during pregnancy as well as radioiodide induction of transient hypothyroidism *(281,284)*. It is important to note that bioassays that use cell preparations to measure the biologic effects of TRAb (stimulation, inhibition of TSH activity or growth) can detect functional changes in TRAb heterogeneity. In contrast, the receptor, or TSH Binding Inhibitory Immunoglobulin (TBII) type of assays, which are used by many clinical laboratories, merely measure the ability of a serum or IgG preparation to block the binding of a TSH preparation and do not measure the biological response (Table 6). This fundamental difference in assay design explains why bioassays and receptor assays usually display a weak correlation (r = 0.31-0.65) *(283,285)*.

(a) TRAb Methodology

The first report that there was a thyroid stimulator that differed from TSH with respect to its longer half-life (Long Acting Thyroid Stimulator or LATS) was published in 1956 using an in vivo bioassay *(286)*. LATS was later identified as an immunoglobulin. Like TSH, TRAbs stimulate both cAMP and the inositol phosphate pathways of the thyroid follicular cell, and thus both stimulate and block both thyroid hormone synthesis and the growth of the gland *(283)*.

The types of methods developed for TRAb measurements are classified relative to their functional activity, as shown in Table 6. Studies in mice and FRTL-5 cell lines as well as humans, show that a high concentration of

human chorionic gonadotropin (hCG) is also a weak TRAb agonist and can stimulate cAMP, iodide transport, and cell growth *(56)*. The marked hCG elevations secondary to choriocarcinoma can in rare cases cause a false positive TRAb result. However, the increase in hCG typically seen with normal pregnancy or in patients treated for a hydatiform mole are usually not high enough to elicit a false positive result.

(b) Bioassays (TSAb, TBAb/TSBAb and TGI)

Most current bioassays are based on TSH receptor activation of second messenger (cAMP) production from a cell preparation (FRTL-5/ CHO TSH-R) exposed to a serum specimen or IgG preparation *(287-289)*. The recent cloning of the TSH receptor has benefited bioassays by facilitating the development of TSH receptor transfected cell lines *(290,291)*. Although these bioassays are available in several commercial laboratories in the United States and Asia, they are less available in Europe because of regulations that affect the use of genetically altered organisms. Unfortunately, the correlation between TRAb assay results and clinical presentation is still poor. For example, the diagnostic sensitivity for Graves' disease using TRAb bioassays ranges from 62.5 to 81% *(283)*. New approaches employing chimeric assays may be able to target the loci of TRAb epitopes and TSH binding sites and thus provide a better correlation between assay response and clinical outcome *(281,284,292-294)*.

Table 6. TSH Receptor Antibody (TRAb) Methods

Antibody	Function	Detection Method
TSAb	Stimulates cAMP production, iodide uptake, thyroglobulin	cell bioassay (FRTL-5/ CHO TSH-R) % stimulation of TSH-induced cAMP synthesis compared to normal pooled serum
TBAb/ TSBAb	Inhibits TSH-induced cAMP production, iodide uptake, thyroglobulin synthesis	cell bioassay (same as above) % inhibition of TSH-induced cAMP compared to normal pooled serum
TGI	Stimulates thyroid cell growth	FRTL-5-cells, ^{3}H thymidine uptake/ mitotic arrest assay
TBII	Inhibits ^{125}I TSH binding to receptor	Receptor assay soluble porcine TSH-R or recombinant human TSH-R

TSAb: Thyroid stimulating antibodies
TBAb/TSBAb: TSH receptor blocking antibodies
TGI: Thyroid growth stimulating antibodies
TSH-R: TSH receptor
TBII: Thyroid binding inhibiting immunoglobulins

(c) Receptor (TBII) Assays

Thyroid binding inhibiting immunoglobulin (TBII) assays are commercially available and are used by many clinical laboratories. These methods quantify the inhibition of the binding of ^{125}I-labeled TSH to either solubilized porcine receptors, or more recently, recombinant human TSH receptors *(295-297)*. This type of method does not distinguish between stimulating and blocking TRAbs. TBII activity is typically quantified against a TRAb-positive serum calibrated against a reference calibrator serum. The most frequently used calibrator serum has been the MRC reference serum, LATS-B. A WHO standard (MRC 90/672) has recently become available. The inherent heterogeneity of TRAb in patient serum and the source of receptors used (porcine versus recombinant human) are likely causes for the wide variability observed between TBII methods, despite the use of the same standard *(283,298)*. Although TBII methods based on recombinant human TSH receptor are now available and may have a higher diagnostic sensitivity for Graves' disease, they do not appear

to offer improved specificity or sensitivity for predicting response to anti-thyroid drug (ATD) therapy
(297,299).

Guideline 38. TSH Receptor Antibody (TRAb) Tests

Clinical laboratory TRAb assays are either:

> Receptor or TSH binding inhibition tests (TBII) do not measure stimulatory activity directly but detect factors in the serum specimen that block the binding of a labeled TSH preparation to an in-vitro TSH receptor preparation. These tests are the more commonly used TRAb assays in clinical laboratories.
> TSH receptor bioassays (TSAb) use cells (FRTL-5 cells, or more recently CHO transfected with human TSH receptor) to detect thyroid stimulating immunoglobulins (TSAb) that either stimulate cAMP or iodide uptake. These tests are not routinely available in all countries.
> In general, there is a poor correlation between TSAb and TBII results (60-75%). TSAb assays claim to be positive in 80-100% and TBII assays positive in 70 to 90% of untreated Graves' hyperthyroid patients. Neither test has high specificity or sensitivity for predicting remission from Graves' hyperthyroidism.
> Normal hCG as well as abnormal hCG production in choriocarcinoma are known to interact with the TSH receptor which could lead to false positive results. This might be observed in rare cases of choriocarcinoma but not in normal pregnancy or treated hydatiform mole in which the level of hCG is not high enough to cause a false positive result.

(d) TRAb Reference Intervals

Despite the adoption of a new international reference preparation MRC 90/672, TRAb values are still method-dependent and reference intervals vary depending on the selection of the "normal" population used to determine the cut-off level for a positive result. This cut-off is generally defined as two standard deviations from the mean of normal subjects.

8. Clinical Uses of TRAb Measurement

The clinical use of TRAb measurements for the diagnosis and follow-up of AITD remains a matter of controversy and differs geographically. The differential diagnosis of hyperthyroidism can be resolved in most patients without resorting to TRAb testing. Nevertheless, the presence of TRAb may distinguish Graves' disease from factitious thyrotoxicosis and other manifestations of hyperthyroidism such as subacute or post-partum thyroiditis and toxic nodular goiter.

TRAb measurements have also been proposed as a means for predicting the course of Graves' disease. A declining TRAb level is often seen in hyperthyroid patients in clinical remission after treatment with antithyroid drugs (ATD). After ATD withdrawal, very high levels of TRAb correlate quite well with prompt relapse, but this situation involves very few patients. Conversely, a significant number of patients with undetectable or low TRAb levels will relapse. A meta-analysis of the relationship between TRAb levels and the risk of relapse has shown that 25% of patients are misclassified by TRAb assays *(263)*. This suggests that after ATD therapy, a follow-up of the patients is necessary whatever the TRAb level at the time of ATD withdrawal and that TRAb measurement is not cost effective for this purpose *(263)*.

There is general agreement that TRAb measurements can be used to predict fetal and/or neonatal thyroid dysfunction in pregnant women with a previous history of AITD *(8,252)*. High levels of TRAb in the mother during the third trimester of pregnancy suggest a risk of thyroid dysfunction in the offspring *(8,282)*. Two to 10% of pregnant women with very elevated TRAb deliver newborns with hyperthyroidism *(8)*. The risk for neonatal hyperthyroidism is negligible following successful treatment of hyperthyroidism with antithyroid drugs, but can develop after radioiodide treatment if TRAb levels remain elevated *(8)*. Euthyroid pregnant women (+/- L-T4 treatment) who have had prior radioiodide therapy for Graves' disease should have TRAb levels measured both in early pregnancy, when an elevated value is a significant risk factor for fetal hyperthyroidism, and during the third trimester, to evaluate for the risk of neonatal hyperthyroidism *(8)*. Pregnant women who take antithyroid drugs (ATD) for Graves' disease should have TRAb measured in the

third trimester. High TRAb levels in such patients should prompt a thorough clinical and biochemical evaluation of the neonate for hyperthyroidism, both at birth (cord blood) and at 4 – 7 days, after the effects of the transplacental passage of ATD have disappeared *(300)*. It is worth noting that the TBII receptor assays are often used for this purpose since they detect both stimulating (TSAb) and in rare cases, blocking antibodies (TBAb/TSBAb) which cause transient hypothyroidism in 1:180,000 of newborns *(301)*. It is also advisable to test for both stimulating and blocking antibodies because the expression of thyroid dysfunction may be different in the mother and the infant *(253)*.

Guideline 39. Clinical Uses of TRAb Measurement

To investigate the etiology of hyperthyroidism when the diagnosis is not clinically obvious.
A declining TRAb concentration during long-term antithyroid drug therapy is suggestive of remission. However TRAb measurements can be misleading in 25% of such patients.
TRAb measurements are useful to diagnose Graves' disease patients and for relating TRAb values to a treatment algorithm.
To evaluate patients suspected of "euthyroid Graves' opthalmopathy". Undetectable TRAb however, does not exclude the condition.
Although TSAb assays have theoretical advantages, some believe that TBII tests which detect both stimulating (TSAb) and the rare cases of blocking (TBAb/TSBAb) antibodies are equally useful.
For pregnant women with a past or present history of Graves' disease. Note: Pregnant women who are euthyroid after receiving prior antithyroid drug treatment for Graves' disease have a negligible risk for fetal or neonatal hyperthyroidism.
Euthyroid pregnant women (± L-T4 treatment) who have had prior radioiodide treatment for Graves' disease should have TRAb measured both early in pregnancy when a high value is a risk factor for fetal hyperthyroidism (2-10%), and during the third trimester to evaluate the risk of neonatal hyperthyroidism.
Pregnant women who take antithyroid drugs (ATD) for Graves' disease to maintain a euthyroid state during pregnancy should have TRAb measured in the third trimester. A high TBII value should prompt a clinical and biochemical evaluation of the neonate for hyperthyroidism, both at birth (cord blood) and at 4 – 7 days after the effects of transplacental passage of ATD have been lost.
The assessment of the risk of fetal and neonatal thyroid dysfunction necessitates the detection of either blocking or stimulating TRAb when mothers have no intact thyroid following past therapy for Graves' hyperthyroidism.
To identify neonates with transient hypothyroidism due to the presence of TSH receptor blocking antibodies.

Guideline 40. Improvements Needed in Thyroid Antibody Tests

Current thyroid autoantibody assays should be submitted to a comparative study of their analytical and clinical performances.
A comparison study of the antigen preparations currently in use would facilitate the identification of the method(s) best suited for clinical thyroid autoantibody testing.
The characteristics of the antigen preparations used in the test should be stated for all thyroid autoantibody assays.
Reference preparations of antigens should be made available.

The role of TRAb in thyroid-associated opthalmopathy (TAO) is uncertain *(302)*. TAO appears to be exacerbated by radioiodide therapy *(303)*. Furthermore, TRAb and other thyroid antibody levels increase significantly after radioiodide therapy *(304-306)*. This suggests that TRAb measurements prior to radioiodide therapy may be useful to predict the risk of TAO but as yet there are no prospective studies to document this observation.

9. Future Directions

It is important that a well-structured comparative study of the commercially available thyroid autoantibody assays be performed. This would provide irrefutable evidence that differences exist in the performance of current assay methods (296). It would also help to convince clinical laboratory scientists to avoid using assays that have poor clinical performance and encourage manufacturers to improve their products or drop them from the market.

Guideline 41. For Manufacturers Developing Thyroid Antibody Tests

Absolute or "gold standard" methods remain a target for the future.
The kit package insert should document the methods used to produce the antigen reagents, the assay design and all experimental conditions affecting the antigen-antibody interactions.
The specificity of the secondary standards should be selected relative to the interactions between the autoantibodies in patient sera and their specific antigen.
TPOAb and TgAb IMAs should be checked for hook effects using ~20 specimens with antibody concentrations >1,000 kIU/L and ~20 specimens with values above 10,000 kIU/L.
TgAb methods should be checked for high antigen (Tg) effects by spiking a range of sera containing low TgAb concentration to Tg levels >10,000 µg/L (ng/ml) and >100,000 µg/L (ng/ml).