

The National Academy of Clinical Biochemistry

Presents

LABORATORY MEDICINE PRACTICE GUIDELINES

LABORATORY SUPPORT FOR THE DIAGNOSIS OF THYROID DISEASE





LABORATORY MEDICINE PRACTICE GUIDELINES

Laboratory Support for the Diagnosis and Monitoring of Thyroid Disease

Table of Contents

Section 1. Foreword and Introduction

Section 2. Pre-analytic factors

Section 3. Thyroid Tests for the Laboratorian and Physician

A. Total Thyroxine (TT4) and Total Triiodothyronine (TT3) methods

B. Free Thyroxine (FT4) and Free Triiodothyronine (FT3) tests

C. Thyrotropin/ Thyroid Stimulating Hormone (TSH) measurement

D. Thyroid Autoantibodies:

- Thyroid Peroxidase Antibodies (TPOAb)
- Thyroglobulin Antibodies (TgAb)
- Thyrotrophin Receptor Antibodies (TRAb)

E. Thyroglobulin (Tg) Measurement

F. Calcitonin (CT) and ret Proto-oncogene

G. Urinary Iodide Measurement

H. Thyroid Fine Needle Aspiration (FNA) and Cytology

I. Screening for Congenital Hypothyroidism

Section 4. The Importance of the Laboratory - Physician Interface

Appendices and Glossary

References

Editors:

Laurence M. Demers, Ph.D., F.A.C.B.

Carole A. Spencer Ph.D., F.A.C.B.

Guidelines Committee:

The preparation of this revised monograph was achieved with the expert input of the editors, members of the guidelines committee, experts who submitted manuscripts for each section and many expert reviewers, who are listed in Appendix A. The material in this monograph represents the opinions of the editors and does not represent the official position of the National Academy of Clinical Biochemistry or any of the co-sponsoring organizations. The National Academy of Clinical Biochemistry is the official academy of the American Association of Clinical Chemistry.

Single copies for personal use may be printed from authorized Internet sources such as the NACB's Home Page (www.nacb.org), provided it is printed in its entirety, including this notice. Printing of selected portions of the document is also permitted for personal use provided the user also prints and attaches the title page and cover pages to the selected reprint or otherwise clearly identifies the reprint as having been produced by the NACB. Otherwise, this document may not be reproduced in whole or in part, stored in a retrieval system, translated into another language, or transmitted in any form without express written permission of the National Academy of Clinical Biochemistry (NACB, 2101 L Street, N.W., Washington, DC 20037-1526). Permission will ordinarily be granted provided the logo of the NACB and the following notice appear prominently at the front of the document: *Reproduced (translated) with permission of the National Academy of Clinical Biochemistry, Washington, DC*

Single or multiple copies may also be purchased from the NACB at the address above or by ordering through the Home Page (<http://www.nacb.org/>).

©2002 by the National Academy of Clinical Biochemistry.

We gratefully acknowledge the following individuals who contributed the original manuscripts upon which this monograph is based:

Zubair Baloch, M.D., Ph.D.,

University of Philadelphia Medical Center, Philadelphia, PA, USA

Pierre Carayon, M.D., D.Sc

U555 INSERM and Department of Biochemistry & Molecular Biology,
University of the Medeterranea Medical School, Marseille, France

Bernard Conte-Devolx, M.D. Ph.D

U555 INSERM and Department of Endocrinology,
University of the Medeterranea Medical School, Marseille, France

Ulla Feldt Rasmussen, M.D.

Department of Medicine, National University Hospital, Copenhagen, Denmark

Jean-François Henry M.D.

U555 INSERM and Department of Endocrine Surgery,
University of the Medeterranea Medical School, Marseille, France

Virginia LiVolsi, M.D.

University of Philadelphia Medical Center, Philadelphia, PA, USA

Patricia Niccoli-Sire, M.D.

U555 INSERM and Departments of Endocrinology and Surgery
University of the Medeterranea Medical School, Marseille, France

Rhys John, Ph.D., F.R.C.Path,

University Hospital of Wales, Cardiff, Wales, UK

Jean Ruf, M.D.

U555 INSERM and Department of Biochemistry & Molecular Biology,
University of the Medeterranea Medical School, Marseille, France

Peter PA Smyth, Ph.D.

University College Dublin, Dublin, Ireland

Carole A. Spencer, Ph.D., F.A.C.B.

University of Southern California, Los Angeles, California, USA

Jim R. Stockigt, M.D., F.R.A.C.P., F.R.C.P.A.,

Ewen Downie Metabolic Unit, Alfred Hospital, Melbourne, Victoria, Australia

Section 1. Foreword and Introduction

Physicians need quality laboratory testing support for the accurate diagnosis and cost-effective management of thyroid disorders. On occasion, when the clinical suspicion is strong, as in clinically overt hyperthyroidism in a young adult or with the presence of a rapidly growing thyroid mass laboratory thyroid hormone testing simply confirms the clinical suspicion. However in the majority of patients, thyroid disease symptoms are subtle in presentation so that only biochemical testing or cytopathologic evaluation can detect the disorder. However overt or obscure a patient's thyroid problem may be, an open collaboration between the physicians and clinical laboratory scientists is essential for optimal, cost-effective management of the patient with thyroid disease.

Thyroid dysfunction, especially thyroid insufficiency caused by a deficiency in iodide, is a worldwide problem. Iodide deficiency is not always uniform across a nation. Studies in both Europe and the United States suggest

that iodide deficiency should be considered more as a "pocket disorder", meaning that it can be more prevalent in some areas of a country compared with others (1-3). The creation of this updated monograph was a collaborative effort involving many thyroid experts from a number of professional organizations concerned with thyroid disease: American Association of Clinical Endocrinologists (AACE), Asia & Oceania Thyroid Association (AOTA), American Thyroid Association (ATA), British Thyroid Association (BTA), European Thyroid Association (ETA) and the Latin American Thyroid Society (LATS). These organizations are the authoritative bodies that spearhead thyroid research and have published standards of care for treating thyroid disease in each region of the world. Because geographic and economic factors impact the clinical use of thyroid tests to some extent, this monograph will focus on the technical aspects of thyroid testing and the performance criteria needed for optimal clinical utility of thyroid tests in an increasingly cost-sensitive global environment. Individual clinicians and laboratories around the world favour different thyroid hormone testing strategies. (4). This monograph cannot accommodate all these variations in thought and opinion but we hope that readers of this monograph will appreciate our efforts to consolidate some of these differences into a recommended strategy. We believe that most of the commonly performed tests and diagnostic procedures used to diagnose and treat thyroid disorders are included in this text. The monograph is designed to give both clinical laboratory scientists and practicing physicians an overview regarding the current strengths and limitations of those thyroid tests most commonly used in clinical practice. Consensus recommendations are made throughout the monograph. The consensus level is > 95%, unless otherwise indicated. We continue to welcome constructive comments that would improve the monograph for a future revision.

A. Additional Resources

Current clinical guidelines are published in the following references (4-11). In addition, the textbooks "Thyroid" and "The Thyroid and Its Diseases" (www.thyroidmanager.org) are useful references (12,13). A list of symptoms suggesting the presence of thyroid disease together with the ICD-9 codes recommended to Medicare by the American Thyroid Association is available on the ATA website (www.thyroid.org). Clinical practice guidelines may vary, depending on the region of the country. More information can be obtained from each of the thyroid organizations: Asia & Oceania Thyroid Association (AOTA = www.dnm.kuhp.kyoto-u.ac.jp/AOTA); American Thyroid Association (ATA = www.thyroid.org); European Thyroid Association (ETA = www.eurothyroid.com) and Latin American Thyroid Society (LATS = www.lats.org).

B. Historical Perspective

Over the past forty years, improvements in the sensitivity and specificity of biochemical thyroid tests, as well as the development of fine needle aspiration biopsy (FNA) and improved cytological techniques, have dramatically impacted clinical strategies for detecting and treating thyroid disorders. In the 1950s, only one serum-based thyroid test was available - an indirect estimate of the total (free + protein-bound) thyroxine (T4) concentration, using the protein bound iodide (PBI) technique. Today, urine iodide concentrations are measured directly by dry or wet-ash techniques and are used to estimate dietary iodide intake. The development of competitive immunoassays in the early 1970s and more recently, non-competitive immunometric assay (IMA) methods have progressively improved the specificity and sensitivity of thyroid hormone testing. Currently, serum-based tests are available for measuring the concentration of both the total (TT4 and TT3) and free (FT4 and FT3) thyroid hormones in the circulation (14,15). In addition, measurements of the thyroid hormone binding plasma proteins, Thyroxine Binding globulin (TBG), Transthyretin (TTR)/Prealbumin (TBPA) and Albumin are available (16). Improvements in the sensitivity of assays to measure the pituitary thyroid stimulating hormone, thyrotropin (TSH) now allow TSH to be used for detecting both hyper- and hypothyroidism. Furthermore, measurement of the thyroid gland precursor protein, Thyroglobulin (Tg) as well as the measurement of Calcitonin (CT) in serum have become important tumor markers for managing patients with differentiated and medullary thyroid carcinomas, respectively. The recognition that autoimmunity is a major cause of thyroid dysfunction has led to the development of more sensitive and specific tests for autoantibodies to thyroid peroxidase (TPOAb), thyroglobulin (TgAb) and the TSH receptor (TRAb). Current thyroid tests are usually performed on serum by either manual or automated methods that employ specific antibodies (17). Methodology continues to evolve as performance standards are established and new technology and instrumentation are developed.



Section 2. Pre-Analytic Factors

Fortunately, most pre-analytic variables have little effect on serum TSH measurements - the most common thyroid test used initially to assess thyroid status in ambulatory patients. Pre-analytic variables and interfering substances present in specimens may influence the binding of thyroid hormones to plasma proteins and thus decrease the diagnostic accuracy of total and free thyroid hormone measurements, more frequently than serum TSH (see Table 1). As discussed in [Section-2 B2 and Section-3 B3(c)viii] both FT4 and TSH values may be diagnostically misleading in the hospitalized setting of severe nonthyroidal illness (NTI). Indeed, euthyroid patients frequently have abnormal serum TSH and/or total and free thyroid hormone concentrations as a result of NTI, or secondary to medications that might interfere with hormone secretion or synthesis. When there is a strong suspicion that one of these variables might affect test results, consulting advice from the expert physician or clinical biochemist is frequently needed.

Table 1. Causes of FT4/TSH Discordance in the Absence of Serious Associated Illness

Mis-leading Test	Result		Likely Causes	Action
	TSH	FT4		
FT4	↑	N	1. Untreated - mild hypothyroidism 2. Treated - inadequate L-T4 dose or non-compliance	1. Measure TPO Ab. Confirm TSH after 6 weeks 2. Increase L-T4 dose/counsel compliance
	↓ or N	N or ↓	1. Mild (subclinical) hyperthyroidism 2. Overtreatment with T3-containing prep.	1. ? Autonomous functioning goiter. 2. Measure FT3 to rule out T3-toxicosis.
	N	↑	1. Common during L-T4 treatment. 2. Abnormal Binding proteins (i.e. FDH) 3. Antibody interferences (T4 antibody, HAMA or rheumatoid factor)	1. Expect higher FT4 in L-T4 Rx. hypo. 2 & 3. Check FT4 by alternate FT4 method ideally one using physical separation i.e. equilibrium dialysis or ultrafiltration
	N	↓	1. Binding-protein competitor drugs [see Section-3 B3(c)vi] 2. Pregnancy	1. Check FT4 by method using minimal dilution 2. Check FT4 by albumin -insensitive method. Use method- and trimester-specific reference ranges
TSH	↑	N	1. Dysequilibrium (first 6-8 weeks of L-T4 Rx. for 1° hypothyroidism) 2. HAMA & other interferences	1. Recheck TSH before adjusting L-T4 dose. High TSH persists for months after Rx. severe hypo. 2. Check TSH (new specimen) by alternate method
	↓	N	1. Dysequilibrium (first 2-3 months post Rx. for hyperthyroidism) 2. Medications, i.e. glucocorticoids, dopamine	1. Use FT4 and FT3 during early Rx. of hyper to monitor thyroid status. TSH may take months to normalize after starting Rx. for severe hyperthyroidism
	N or ↑	↑	1. TSH-secreting pituitary adenoma	1. Check TSH (new specimen) by alternate method 2. TRH-stim or thyroid hormone suppression test 3. TSH alpha subunit 4. Pituitary imaging.
	N	↓	1. Central hypothyroidism	1. Reduced bioactivity of immunoreactive TSH 2. ? other signs of pituitary deficiency 3. ? blunted (< 2 fold) TRH response

In addition to basic physiologic variability, individual patient variables such as genetic abnormalities in thyroid binding proteins or severe nonthyroidal illness (NTI) may impact the sensitivity and specificity of a thyroid test. Also, iatrogenic factors such as thyroid and nonthyroidal medications such as glucocorticoids or beta-blockers; and specimen variables, including autoantibodies to thyroid hormones and Tg as well as heterophilic antibodies (HAMA) can affect the diagnostic accuracy resulting in test result misinterpretation. Table 2 lists the pre-analytic factors to consider when interpreting thyroid tests.

A. Physiologic Variables

For practical purposes, variables such as age, gender, race, season, phase of menstrual cycle, cigarette smoking, exercise, fasting or phlebotomy-induced stasis have minor effects on the reference intervals for thyroid tests in ambulatory adults (18). Since the differences in these physiological variables are less than the method-to-method differences encountered in clinical practice they are considered inconsequential.

Table 2.

A. Physiologic Variables	• TSH/free T4 relationship • Age • Pregnancy • Biologic variation
B. Pathologic Variables	• Thyroid gland dysfunction • Hepatic or renal dysfunction • Medications • Systemic illnesses
C. Specimen-related Variables	• Interfering factors

Guideline 1. General Guidelines for Laboratories & Physicians
☐ Laboratories should store (at 4-8°C) all serum specimens used for thyroid testing for at least one week after the results have been reported to allow physicians time to order additional tests when necessary. ☐ Specimens from differentiated thyroid cancer patients sent for serum Thyroglobulin (Tg) measurement should be archived (at -20°C) for a minimum of six months.

1. The Serum TSH/ FT4 Relationship

An understanding of the normal relationship between serum levels of free T4 (FT4) and TSH is essential when interpreting thyroid tests. Needless to say, an intact hypothalamic-pituitary axis is a prerequisite if TSH measurements are to be used to determine primary thyroid dysfunction (19). A number of clinical conditions and pharmaceutical agents disrupt the FT4/TSH relationship. As shown in Table 1, it is more common to encounter misleading FT4 tests than misleading serum TSH measurements.

When hypothalamic-pituitary function is normal, a log/linear inverse relationship between serum TSH and free T4 concentrations is produced by negative feedback inhibition of pituitary TSH secretion by thyroid hormones. Thus, thyroid function can be determined either directly, by measuring the primary thyroid gland product, T4 (preferably as free T4) or indirectly, by assessing the TSH level, which inversely reflects the thyroid hormone concentration sensed by the pituitary. It follows that high TSH and low FT4 is characteristic of hypothyroidism and low TSH and high FT4 is characteristic of hyperthyroidism. In fact, now that the sensitivity and specificity of TSH assays have improved, it is recognised that the indirect approach (serum TSH measurement) offers better sensitivity for detecting thyroid dysfunction than does FT4 testing (10).

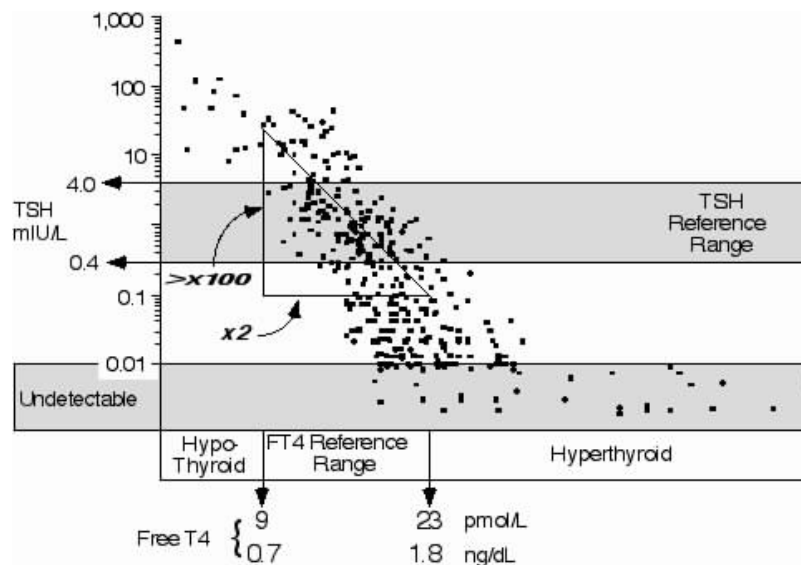


Fig 1. The relationship between serum TSH and FT4 concentrations in individuals with stable thyroid status and normal hypothalamic-pituitary function. Adapted from reference (20).

There are two reasons for using a TSH-centered strategy for ambulatory patients: 1) As shown in Figure 1, serum TSH and FT4 concentrations exhibit an inverse log/linear relationship such that small alterations in FT4 will produce a much larger response in serum TSH (20).

2) The narrow individual variations in thyroid hormone test values together with twin studies suggest that each individual has a genetically determined FT4 set-point (21, 22). Any mild FT4 excess or deficiency will be sensed by the pituitary, relative to that individual's FT4 set-point, and cause an amplified, inverse response in TSH secretion. It follows that in the early stages of developing thyroid dysfunction, a serum TSH abnormality will precede the development of an abnormal FT4 because TSH responds exponentially to subtle FT4 changes that are within the population reference limits. This is because population reference limits are broad, reflecting the different FT4 set-points of the individual members of the cohort of normal subjects studied.

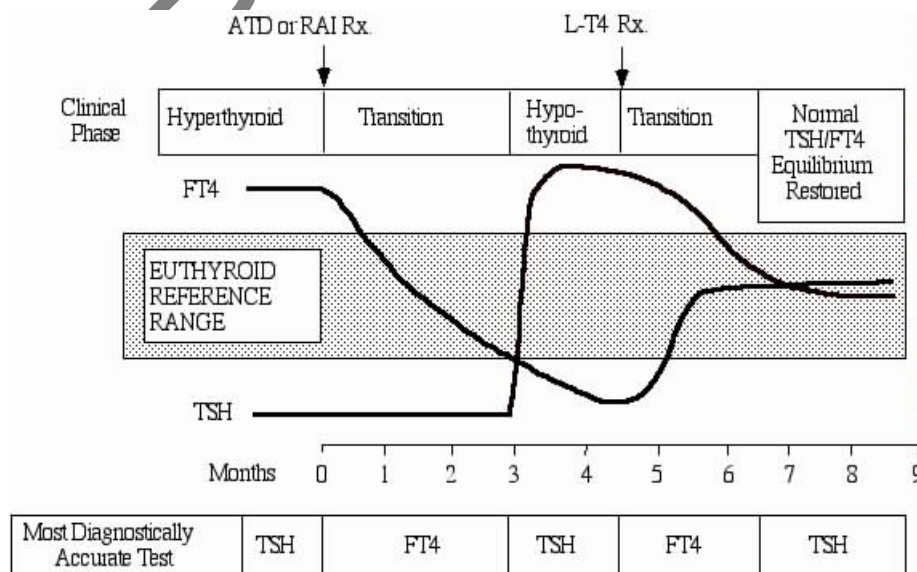


Fig 2. The lag in pituitary TSH reset during transition periods of unstable thyroid status following treatment for hyper- or hypothyroidism.

Guideline 2. Thyroid Testing for Ambulatory Patients

- *Patients with stable thyroid status:* When thyroid status is stable and hypothalamic-pituitary function is intact, serum TSH measurement is more sensitive than free T4 (FT4) for detecting mild (subclinical) thyroid hormone excess or deficiency. The superior diagnostic sensitivity of serum TSH reflects the log/linear relationship between TSH and FT4 and the exquisite sensitivity of the pituitary to sense free T4 abnormalities relative to the individual's genetic free T4 set-point.
- *Patients with unstable thyroid status:* Serum FT4 measurement is a more reliable indicator of thyroid status than TSH when thyroid status is unstable, such as during the first 2-3 months of treatment for hypo- or hyperthyroidism. Patients with chronic, severe hypothyroidism may develop pituitary thyrotroph hyperplasia that can mimic a pituitary adenoma, but resolves after several months of L-T4 replacement therapy. In hypothyroid patients suspected of intermittent or non-compliance with L-T4 replacement therapy, both TSH and FT4 should be used for monitoring. Non-compliant patients may exhibit discordant serum TSH and FT4 values (high TSH/high FT4) because of persistent disequilibrium between FT4 and TSH.

Currently, measurement of the serum TSH concentration is the most reliable indicator of thyroid status at the tissue level. Studies of mild (subclinical) thyroid hormone excess or deficiency (abnormal TSH/normal range FT4 and FT3) find abnormalities in markers of thyroid hormone action in a variety of tissues (heart, brain, bone, liver and kidney). These abnormalities typically reverse when treatment to normalize serum TSH is initiated (23-26).

It is important to recognize the clinical situations where serum TSH or FT4 levels may be diagnostically misleading (see Table 1). These include abnormalities in hypothalamic or pituitary function, including TSH-producing pituitary tumors (27-29). Also, as shown in Figure 2, serum TSH values are diagnostically misleading during transition periods of unstable thyroid status, such as occurs in the early phase of treating hyper- or hypothyroidism or changing the dose of L-T4. Specifically, it takes 6-12 weeks for pituitary TSH secretion to re-equilibrate to the new thyroid hormone status (30). These periods of unstable thyroid status may also occur following an episode of thyroiditis, including post-partum thyroiditis when discordant TSH and FT4 values may also be encountered.

Drugs that influence pituitary TSH secretion (i.e. dopamine and glucocorticoids) or thyroid hormone binding to plasma proteins, may also cause discordant TSH values [Section-3 B3(c)vi].

2. Effects of Chronological Age on Thyroid Test Reference Ranges

(a) Adults

Despite studies showing minor differences between older and younger subjects, adult age-adjusted reference ranges for thyroid hormones and TSH are unnecessary (18,31-33). With respect to euthyroid elderly individuals, the TSH mean value increases each decade as does the prevalence for both low and high serum TSH concentrations compared with younger individuals (18,34,35). Despite the wider serum TSH variability seen in older individuals, there appears to be no justification for using a widened or age-adjusted reference range (31,32). This conservative approach is justified by reports that mildly suppressed or elevated serum TSH is associated with increased cardiovascular morbidity and mortality (36,37).

(b) Neonates, Infants and Children

In children, the hypothalamic/pituitary/thyroid axis undergoes progressive maturation and modulation. Specifically, there is a continuous decrease in the TSH/FT4 ratio from the time of mid-gestation until after the completion of puberty (38-43). As a result, higher TSH concentrations are typically seen in children (44). This maturation process dictates the use of age-specific reference limits. However, there are significant differences between FT4 and TSH measurements made by different methods [see Sections 3B and 3 C]. Since most manufacturers have not independently established age-specific reference intervals, these limits can be calculated

for different assays by adjusting the upper and lower limits of the adult range by the ratio between child and adult values, such as indicated in Table 3.

Lower serum total and FT3 levels (measured by most methods) are seen with pregnancy, during the neonatal period, in the elderly and during caloric deprivation (15). Furthermore, higher total and free FT3 concentrations are typically seen in euthyroid children. This suggests that the upper T3 limit for young patients (less than 20 years of age) should be established as a gradient: between 6.7 pmol/L (0.44 ng/dL) for adults, up to 8.3 pmol/L (0.54 ng/dL) for children under three years of age (45).

Table 3*. Relative TSH and FT4 Reference Ranges during Gestation and Childhood

Age	TSH Child/ Adult Ratio	TSH Ranges mIU/L	FT4 Child/ Adult Ratio	FT4 Ranges pmol/L (ng/dL)
Midgestation Fetus	2.41	0.7-11	0.2	2-4 (0.15-0.34)
LBW cord serum	4.49	1.3-20	0.8	8-17 (0.64-1.4)
Term infants	4.28	1.3-19	1	10-22 (0.8-1.9)
3 days	3.66	1.1-17	2.3	22-49 (1.8-4.1)
10 weeks	2.13	0.6-10	1	9-21 (0.8-1.7)
14 months	1.4	0.4-7.0	0.8	8-17 (0.6-1.4)
5 years	1.2	0.4-6.0	0.9	9-20 (0.8-1.7)
14 years	0.97	0.4-5.0	0.8	8-17 (0.6-1.4)
Adult	1	0.4-4.0	1	9-22 (0.8-1.8)

* Data taken from reference (42). FT4 measured by direct equilibrium dialysis.

Guideline 3. Thyroid Testing of Infants and Children

The hypothalamic-pituitary-thyroid axis matures throughout infancy until the end of puberty.

- ☐ Both TSH and FT4 concentrations are higher in children, especially in the first week of life and throughout the first year. Failure to recognize this could lead to missing and/or under-treating cases of congenital hypothyroidism.
- ☐ Age-related normal reference limits should be used for all tests (see Table 3).

3. Pregnancy

During pregnancy, estrogen production increases progressively elevating the mean TBG concentration. TBG levels plateau at 2 to 3 times the pre-pregnancy level by 20 weeks of gestation (46,47). This rise in TBG results in a shift in the TT4 and TT3 reference range to approximately 1.5 times the non-pregnant level by 16 weeks of gestation (48-50). These changes are associated with a fall in serum TSH during the first trimester, such that subnormal serum TSH may be seen in approximately 20 % of normal pregnancies (46,47,51). This decrease in TSH is attributed to the thyroid stimulating activity of human chorionic gonadotropin (hCG) that has structural homology with pituitary TSH (52,53). The peak rise in hCG and the nadir in serum TSH occur together at about 10-12 weeks of gestation. In approximately 10 % of such cases (i.e. 2 % of all pregnancies) the increase in free T4 reaches supranormal values and, when prolonged, may lead to a syndrome entitled "gestational transient thyrotoxicosis" (GTT) that is characterized by more or less pronounced symptoms and signs of thyrotoxicosis (52-54). This condition is frequently associated with hyperemesis in the first trimester of pregnancy (55,56).

The fall in TSH during the first trimester of pregnancy is associated with a modest increase in FT4 (46,47,51). Thereafter, in the second and third trimesters there is now consensus that serum FT4 and FT3 levels decrease to approximately 20 to 40 percent below the normal mean, a decrease in free hormone that is further amplified when the iodide nutrition status of the mother is restricted or deficient (46,47,51). In some patients, FT4 may fall below the lower reference limit for non-pregnant patients (51,57-60). The frequency of subnormal FT4 concentrations in this setting is method-dependent (57,59,60). Patients receiving L-T4 replacement therapy who become pregnant may require an increased dose to maintain normal serum TSH levels (61,62). The thyroid status of these patients should be checked with TSH + FT4 during each trimester. The L-T4 dose should be adjusted to maintain normal TSH and FT4 concentrations. Serum Tg concentrations typically rise during normal pregnancy (46). Patients with differentiated thyroid carcinomas (DTC) with thyroid tissue still present typically show a two-fold rise in serum Tg with a return to baseline by 6 to 8 weeks postpartum.

Guideline 4. Thyroid Testing of Pregnant Patients

Mounting evidence suggests that hypothyroidism during early pregnancy has a detrimental effect on fetal outcome (fetal wastage and lower infant IQ).

- ☐ Pre-pregnancy or first trimester screening for thyroid dysfunction using serum TSH and TPOAb measurements is important both for detecting mild thyroid insufficiency (TSH > 4.0 mIU/L) and for assessing risk for post-partum thyroiditis (elevated TPOAb).
- ☐ Initiation of levothyroxine (L-T4) therapy should be considered if the serum TSH level is >4.0mIU/L in the first trimester of pregnancy.
- ☐ A high serum TPOAb concentration during the first trimester is a risk factor for post-partum thyroiditis.
- ☐ Serum TSH should be used to assess thyroid status during each trimester when pregnant patients are taking L-T4 therapy, with more frequent measurement if L-T4 dosage is changed.
- ☐ Trimester-specific reference intervals should be used when reporting thyroid test values for pregnant patients.
- ☐ TT4 and TT3 measurements may be useful during pregnancy if reliable FT4 measurements are not available, as long as the reference ranges are increased by 1.5-fold relative to non-pregnant ranges.
- ☐ FT3 and FT4 reference ranges in pregnancy are method-dependent and should be established independently for each method.
- ☐ Measurement of serum thyroglobulin (Tg) in DTC patients during pregnancy should be avoided. Serum Tg rises during normal pregnancy and returns to baseline levels post-partum. This rise is also seen in pregnant DTC patients with remnant normal thyroid or tumor tissue present and is not necessarily a cause for alarm.

Decreased availability of maternal thyroid hormone may be a critical factor impairing the neurologic development of the fetus in the early stages of gestation, before the fetal thyroid gland becomes active. Several recent studies report both increased fetal loss as well as IQ deficits in infants born to mothers with either undiagnosed hypothyroidism, low range FT4 or TPOAb positivity (63-65). However, one study suggests that early identification and treatment of mild (subclinical) hypothyroidism may prevent the long-term effects of low thyroid hormone levels on the psychomotor and auditory systems of the neonate (66).

B. Pathologic Variables

1. Medications

Medications can cause both in vivo and in vitro effects on thyroid tests. This may lead to misinterpretation of laboratory results and inappropriate diagnoses, unnecessary further testing and escalating health care costs (67,68).

(a) In Vivo Effects

In general, the serum TSH level is affected less by medications than thyroid hormone concentrations (Table 1). For example, Estrogen-induced TBG elevations raise serum TT4 levels but do not affect the serum TSH concentration, because pituitary TSH secretion is controlled by the FT4 independent of binding-protein effects.

Glucocorticoids in large doses can lower the serum T3 level and inhibit TSH secretion (69,70). Dopamine also inhibits TSH secretion and may even mask the raised TSH level of primary hypothyroidism in sick hospitalized patients (71). Propranolol is sometimes used to treat manifestations of thyrotoxicosis and has an inhibitory effect on T4 to T3 conversion. Propranolol given to individuals without thyroid disease can cause an elevation in TSH as a result of the impaired T4 to T3 conversion (72).

Iodide, contained in solutions used to sterilize the skin and radioopaque dyes and contrast media used in coronary angiography and CT-scans, can cause both hyper and hypothyroidism in susceptible individuals (73). In addition, the iodide-containing anti-arrhythmic drug Amiodarone used to treat heart patients has complex effects on thyroid gland function that can induce either hypothyroidism or hyperthyroidism in susceptible patients with positive TPOAb (74-78).

Guideline 5. Patients taking Amiodarone Medication

Amiodarone therapy can induce the development of hypo- or hyperthyroidism in 14-18% of patients with apparently normal thyroid glands or with preexisting abnormalities.

- *Pretreatment* –thorough physical thyroid examination together with baseline TSH and TPOAb. FT4 and FT3 tests are only necessary if TSH is abnormal. Positive TPOAb is a risk factor for the development of thyroid dysfunction during treatment.
 - *First 6 months.* Abnormal tests may occur in the first six months after initiating therapy. TSH may be discordant with thyroid hormone levels (high TSH/highT4/low T3). TSH usually normalizes with long-term therapy if patients remain euthyroid.
 - *Long-term follow-up.* Monitor thyroid status every 6 months with TSH. Serum TSH is the most reliable indicator of thyroid status during therapy.
 - *Hypothyroidism.* Preexisting Hashimoto's thyroiditis and/or TPOAb-positivity is a risk factor for developing hypothyroidism at any time during therapy.
 - *Hyperthyroidism.* Low serum TSH suggests hyperthyroidism. T3 (total and free) usually remains low during therapy but may be normal. A high T3 is suspicious for hyperthyroidism.
- Two types of amiodarone-induced hyperthyroidism may develop during therapy, although mixed forms are frequently seen (20%). Distinction between two types often difficult. Decreased flow on color flow doppler and elevated interleukin-6 suggests Type II. Direct therapy at both Type I and II if etiology is uncertain.
- *Type I* = Iodine-induced. Recommended treatment = simultaneous administration of thionamides and potassium perchlorate (if available). Some recommend iopanoic acid before thyroidectomy. Most groups recommend that amiodarone be stopped. Seen more often in areas of low iodine intake. However, in iodine-sufficient areas, radioiodine uptakes may be low precluding radioiodine as a therapeutic option. In iodide-deficient regions, uptakes may be normal or elevated.
 - *Type a:* Nodular goiter. More common in iodine-deficient areas, i.e. Europe.
 - *Type Ib:* Graves' disease. More common in iodine-sufficient areas, i.e. United States.
 - *Type II* = amiodarone-induced destructive thyroiditis – a self-limiting condition. Recommended treatment = glucocorticoids and/or beta-blockers if cardiac status allows. When hyperthyroidism is severe, surgery with pre-treatment with iopanoic may be considered. Radioiodine uptake is typically low or suppressed. Type II is more commonly seen in iodine-sufficient areas.

- *Type I AIH* appears to be induced in abnormal thyroid glands by the excess of iodide contained in the drug. A combination of thionamide drugs and potassium perchlorate has often been used to treat such cases.
- *Type II AIH* appears to result from a destructive thyroiditis that is often treated with prednisone and thionamide drugs. Some studies report elevated IL-6 levels in Type II (79). Serum T3 (free and total) is typically low during therapy. A paradoxically normal or high T3 is useful to support the diagnosis of Amiodarone-induced hyperthyroidism.

Lithium can cause hypo- or hyperthyroidism in as many as 10% of lithium-treated patients, especially those with a positive TPOAb titer (81-83). Some therapeutic and diagnostic agents (i.e. Phenytoin, Carbamazepine or

Furosemide/Frusemide) may competitively inhibit thyroid hormone binding to serum proteins in the specimen, and acutely increase FT4 resulting in a reduction in serum TT4 values through a feedback mechanism [see Section- 3 B3(c)vi].

(b) In Vitro Effects

Intravenous Heparin administration, through in-vitro stimulation of lipoprotein lipase can liberate free fatty acids (FFA), which inhibit T4 binding to serum proteins and falsely elevates FT4 [Section-3 B3(c)vii] (84). In certain pathologic conditions such as uremia, abnormal serum constituents such as indole acetic acid may accumulate and interfere with thyroid hormone binding (85). Methods employing fluorescent signals may be sensitive to the presence of fluorophore-related therapeutic or diagnostic agents in the specimen (86).

2. Nonthyroidal Illness (NTI)

Patients who are seriously ill often have abnormalities in their thyroid tests but usually do not have thyroid dysfunction (87,88). These abnormalities are seen with both acute and chronic critical illnesses and thought to arise from a maladjusted central inhibition of hypothalamic releasing hormones, including TRH (89,90). The terms "nonthyroidal illness" or NTI, as well as "euthyroid sick" and "low-T4 syndrome" are often used to describe this subset of patients (91). As shown in Figure 3, the spectrum of changes in thyroid tests relates both to the severity and stage of illness, as well as to technical factors that affect the methods and in some cases the medications given to these patients.

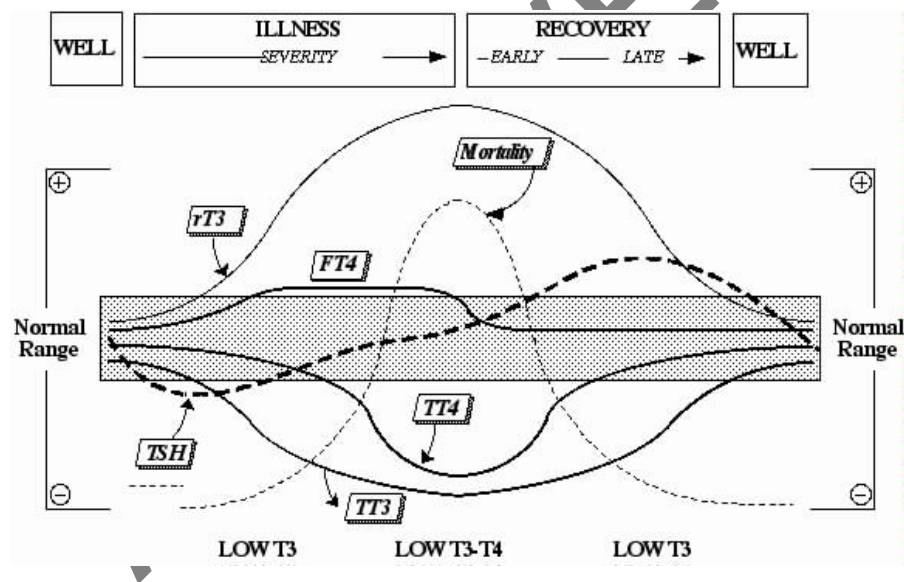


Fig 3. Changes in thyroid tests during the course of NTI.

Most hospitalized patients have low serum TT3 and FT3 concentrations, as measured by most methods (14,97). As the severity of the illness increases, serum TT4 typically falls because of a disruption of binding protein affinities, possibly caused by T4-binding inhibitors in the circulation (91,98,99). It should be noted that subnormal TT4 values only develop when the severity of illness is critical (usually sepsis). Such patients are usually in an ICU setting. If a low TT4 is not associated with an elevated serum TSH (>20mIU/L) and the patient is not profoundly sick, a diagnosis of central hypothyroidism secondary to pituitary or hypothalamic deficiency should be considered.

Guideline 6. For Testing of Hospitalized Patients with Non Thyroidal Illness (NTI)

- ☐ Acute or chronic NTI has complex effects on thyroid function tests. Whenever possible, diagnostic testing should be deferred until the illness has resolved, except when the patient's history or clinical features suggest the presence of thyroid dysfunction.

- ❑ Physicians should recognize that some thyroid tests are inherently non-interpretable in severely sick patients, or patients receiving multiple medications.
- ❑ TSH in the absence of dopamine or glucocorticoid administration, is the more reliable test for NTI patients.
- ❑ Estimates of free or total T4 in NTI should be interpreted with caution, in conjunction with a serum TSH measurement. Both T4 + TSH measurements are the most reliable way for distinguishing true primary thyroid dysfunction (concordant T4/TSH abnormalities) from transient abnormalities resulting from NTI per se (discordant T4/TSH abnormalities).
- ❑ An abnormal FT4 test in the setting of serious somatic disease is unreliable, since the FT4 methods used by clinical laboratories lack diagnostic specificity for evaluating sick patients.
- ❑ An abnormal FT4 result in a hospitalized patient should be confirmed by a reflex TT4 measurement. If both TT4 and FT4 are abnormal (in the same direction) a thyroid condition may be present. When TT4 and FT4 are discordant, the FT4 abnormality is unlikely due to thyroid dysfunction and more likely a result of the illness, medications or an artifact of the test.
- ❑ TT4 abnormalities should be assessed relative to the severity of illness, since the low TT4 state of NTI is typically only seen in severely sick patients with a high mortality rate. A low TT4 concentration in a patient not in intensive care is suspicious for hypothyroidism.
- ❑ A raised total or free T3 is a useful indicator of hyperthyroidism in a hospitalized patient, but a normal or low T3 does not rule it out.
- ❑ Reverse T3 testing is rarely helpful in the hospital setting, because paradoxically normal or low values can result from impaired renal function and low binding protein concentrations. Furthermore, the test is not readily available in most laboratories.

FT4 and FT3 estimate values are method dependent and may be either spuriously high or low, depending on the methodologic principles underlying the test. For example, FT4 tests are unreliable if the method is sensitive to the release of FFA generated in vitro following IV heparin infusion [see Section-3 B3(c)vii] or is sensitive to dilutional artifacts (84,94,97,98,100,101). FT4 methods such as equilibrium dialysis and ultrafiltration that physically separate free from protein-bound hormone usually generate normal or elevated values for critically ill patients [see Section-2 B2 and Section-3 B3(c)viii] (94,102). These elevated values often represent I.V. heparin effects (101).

Serum TSH concentrations remain within normal limits in the majority of NTI patients, provided that no dopamine or glucocorticoid therapy is administered (87,93). However, in acute NTI there may be a mild, transient fall in serum TSH into the 0.02-0.3 mIU/L range, followed by a rebound to mildly elevated values during recovery (103). In the hospitalized setting, it is critical to use a TSH assay with a functional sensitivity <0.02 mIU/L. Without this level of sensitivity, sick hyperthyroid patients with profoundly low serum TSH values (<0.02 mIU/L) cannot be reliably discriminated from patients with merely a transient mild TSH suppression caused by NTI (0.02-0.3 mIU/L). Minor elevations in TSH are less diagnostic for hypothyroidism in the hospitalized setting. Sick hypothyroid patients typically exhibit the combination of a low T4 and elevated TSH (>20 mIU/L) (92).

It is clear that the diagnosis and treatment of thyroid dysfunction in the presence of a severe NTI is not simple, and is best done with the help of an endocrine specialist. Empiric treatment of the low TT4 state of NTI has not improved outcome and is still considered experimental (104-106). Serum TT4 measurements may be more diagnostically helpful than using current FT4 immunoassay tests with variable diagnostic accuracy for assessing sick patients, provided that TT4 values are interpreted in relation to the severity of illness. For example, the low T4 state of NTI is primarily seen in severely sick patients, usually in an intensive care setting (71). Low TT4 values for hospitalized patients who are not severely sick should prompt an evaluation for hypothyroidism. Although the diagnostic specificity of TSH is reduced in the presence of somatic illnesses, a detectable serum TSH value in the 0.02-20 mIU/L range, when measured by an assay with a functional sensitivity ≤ 0.02 mIU/L, usually rules out significant thyroid dysfunction, provided that hypothalamic-pituitary function is intact and the patient is not receiving medications that affect pituitary TSH secretion. However, in general it is best to avoid routine thyroid testing in hospitalized patients if at all possible.

C. Specimen Variables

1. Stability

A few studies have examined the effects of storing blood samples on serum concentrations of total and free thyroid hormones, TSH and Tg (107). In general, these studies suggest that thyroid hormones are relatively stable whether stored at room temperature, refrigerated or frozen. Some studies have reported that T4 in serum is stable for months when stored at -4°C or years when frozen at -10°C (108,109). TSH and TT4 in dried whole blood spots used to screen for neonatal hypothyroidism are also stable for months when stored with a desiccant. TSH in serum has been reported to be slightly more stable than T4 (110). It is important to note however, as discussed above, that non-frozen specimens from patients receiving heparin are prone to in-vitro generation of FFA that can spuriously elevate FT4 when measured by some methods (84).

2. Serum Constituents

Hemolysis, lipemia, and hyperbilirubinemia do not produce significant interference in immunoassays, in general. However, free fatty acids can displace T4 from serum binding proteins, which may partly explain the low TT4 values often seen in NTI (100).

3. Heterophilic Antibodies (HAMA)

Heterophilic antibodies may be encountered in patient sera. HAMA fall into two classes (111). They are either relatively weak multispecific, polyreactive antibodies that are frequently IgM rheumatoid factor or may be broadly reactive antibodies induced by infections or exposure to therapies containing monoclonal antibodies (112-114). These are sometimes called human anti-mouse antibodies (HAMA). Alternatively, they can be specific human anti-animal immunoglobulins (HAAA) that are produced against well-defined specific antigens following exposure to a therapeutic agent containing animal antigens (i.e. murine antibody) or by coincidental immunization through workplace exposure (i.e. animal handlers) (115). Either HAMA or HAAA affect IMA methodology more than competitive immunoassays by forming a bridge between the capture and signal antibodies, thereby creating a false signal, resulting in an inappropriately high value (116,117). The inappropriate result may not necessarily be abnormal, but merely inappropriately normal. Manufacturers are currently employing various approaches to deal with the HAMA issue with varying degrees of success, including the use of chimeric antibody combinations and blocking agents to neutralize the effects of HAMA on their methods (118).

4. Sample Collection and Processing

Most manufacturers recommend serum as the preferred specimen, rather than EDTA- or heparinized-plasma. For optimal results and maximum serum yield, it is recommended that whole blood samples be allowed to clot for at least 30 minutes before centrifugation and separation. Serum can be stored at 4-8°C for up to one week. Storage at -20°C is recommended if the assay is to be delayed for more than one week. Collection of serum in barrier gel tubes does not affect the results of most TSH and thyroid hormone tests.

Guideline 7. Investigation of Discordant Thyroid Test Results

Discordant thyroid test results can result from technical interference or rare clinical conditions:

- ☐ **Technical Interference:** Technical interference can sometimes be detected by measuring the specimen with a different manufacturer's method, since the magnitude of most interferences is method-dependent. Alternatively, non-linearity in dilutions of the specimen may indicate a technical interference with TT4, TT3 or TSH measurements. Note: a 100-fold dilution of a "normal" serum theoretically causes insignificant reduction (<2%) in the FT4 concentration. It is not recommended to dilute the FT4 and FT3 tests used by clinical laboratories because such tests are binding protein dependent and do not give linear dilution responses.
- ☐ **Rare Clinical Conditions:** Unexpectedly abnormal or discordant thyroid test values may be seen with some rare, but clinically significant conditions such as central hypothyroidism, TSH-secreting pituitary tumors, thyroid hormone resistance, or the presence of heterophilic antibodies (HAMA) or thyroid hormone (T4 and/or T3) autoantibodies.

5. Thyroid Test Performance Standards

(a) Biologic Variation

The serum levels of the thyroid hormones as well as their precursor protein, thyroglobulin (Tg) are quite stable within individuals over a 1 to 4 year period (Table 4) (22,119). All thyroid analytes display a greater inter-individual variability compared to intra-individual variability (Table 4) (33,119,120). The stability of intra-individual serum T4 concentrations reflects the long half-life (7 days) of thyroxine and the genetically imposed free T4 setpoint (21). The stability of intra-individual T3 concentrations reflects autoregulation of the rate of T4 to T3 conversion (121). Inter-individual variability is especially high for serum Tg concentrations, because individuals have differences in thyroid mass, TSH status and may have conditions associated with thyroid injury (i.e. thyroiditis) - all conditions that influence serum Tg concentrations (122). Serum TSH levels also display high variability, both within and between individuals (22). This primarily reflects the short half-life of TSH (~60 minutes) together with its circadian and diurnal variations, the levels peak during the night and reach a nadir sometime between 1000 to 1600 hrs (123,124). The amplitude of the diurnal TSH variability across a 24-hour period is approximately 2-fold (123,124). However, since this magnitude of change lies within the normal TSH reference interval for the population as a whole (~0.4 to 4.0 mIU/L) it does not compromise the utility of a single TSH value for diagnosing thyroid dysfunction. Furthermore, TSH is typically measured during the day in the outpatient setting when the magnitude of TSH variability is the least.

The performance of a laboratory test can be evaluated both biologically and analytically. Table 4 shows the biological variation of various thyroid serum analytes, expressed as inter-individual variability and intra-individual variability, across different periods of time (22,33,119,120,125). Analytical performance is typically assessed in the laboratory by parameters such as:

- ☐ Within and between-run precision assessed at different analyte concentrations
- ☐ Minimum detection limit (analytical sensitivity) (126,127)
- ☐ Functional Sensitivity (defined by % CV, relative to the analyte-specific biologic and methodologic variation)
- ☐ Linearity of measurements made across the reportable range of values
- ☐ Recovery of analyte added to the standard matrix
- ☐ Normal reference interval (mean $\pm$ 2 standard deviation of values) for a cohort of subjects without disease
- ☐ Correlation with a reference method

Although analytical performance parameters are the foundation of most laboratory quality control and quality assurance programs, it is widely accepted that analytical performance goals should be established on biological principles (within- and between-individual variation) as well as clinical need (33). It has been proposed that total analytical error should ideally be less than half the within-subject biological coefficient of variation (%CV) (33,125,128-130).

Table 4. Intra-individual and Inter-individual Variability in Serum Thyroid Tests

Serum Analyte	Time span	%CV*	%CV**
TT4 /FT4	1 week	3.5	10.8
	6 weeks	5.3	13.0
	1year	9.2	17.1
TT3 /FT3	1 week	8.7	18.0
	6 weeks	5.6	14.8
	1year	12.0	16.8
Thyrotropin (TSH)	1 week	19.3	19.7
	6 weeks	20.6	53.3
	1 year	22.4	37.8

Thyroglobulin (Tg)	1 week	4.4	12.6
	6 weeks	8.7	66.6
	4 months	14.0	35.0
	*intra-individual **inter-individual		

Data taken from references (22,33,119,120,125).

For diagnostic testing, thyroid test results are reported together with a "normal" reference interval that reflects inter-individual variability. This reference range provides for a benchmark for case finding. Reference ranges, however, cannot be used to determine whether differences exist between two consecutive test results made during the monitoring of a patient's treatment constitute a clinically significant change, or merely reflect the technical (between-run imprecision) and biologic (intra-individual variance) variability of the measurement (131). The "normal" reference interval is usually irrelevant during the post-operative clinical management when using tumor markers such as Tg (132). Clearly, method bias and precision goals need not be as stringent when a measurement is used for diagnostic testing compared with using serial measurements for patient monitoring. While the "normal" reference interval stated on the typical laboratory report helps the physician to make a primary diagnosis, it does not give relevant information to help the physician assess the significance of changes resulting from treatment.

Table 5. Bias and Precision Targets for Thyroid Tests

Test	TT4	FT4	TT3	FT3	TSH	Tg
Normal	nmol/L(µg/dL)	pmol/L(ng/dL)	nmol/L(ng/dL)	pmol/L(ng/L)	mIU/L	µg/L(ng/mL)
Reference Range	58-160/4.5-12.6	9-23/0.7-1.8	1.2-2.7/80-180	3.5-7.7/23-50	0.4-4.0	3.0-40.0
Within person %CV	6.0	9.5	5.6	7.9	19.7	8.7
Between person %CV	12.1	12.1	14.8	22.5	27.2	66.6
W	3.5	3.8	4.0	6.0	14.3	16.8
X	1.3	2.4	1.4	2.0	5.2	2.2
Y	7.0	7.7	7.9	11.9	28.6	33.6
Z	2.7	4.8	2.8	4.0	10.3	4.4

W= Suggested percentage goal for maximum bias in diagnostic testing

X= Suggested percentage goal for maximum bias in monitoring an individual

Y= Suggested percentage goal for maximum imprecision in diagnostic testing

Z= Suggested percentage goal for maximum imprecision for monitoring an individual

Table 5 shows bias and precision goals for the principal thyroid tests for both diagnostic testing and monitoring. The values shown are calculated from studies of within- and inter-individual precision estimates from various studies and are based on well-established concepts (22,33,119,120,130,133,134).

Table 5 and Guideline 8 show the magnitude of change in consecutive results (approximating mean normal analyte concentrations) that constitute a clinically significant difference in each test (22,120). These benchmarks should help the physician gauge the clinical significance of changes seen during serial monitoring the treatment of patients with thyroid disorders.

Guideline 8. Guidelines for Interpreting Thyroid Test Results

- ☐ For diagnostic testing (case-finding) thyroid test results are typically reported together with a "normal" reference interval that reflects between-person variability.
- ☐ The "normal" reference interval does not indicate the magnitude of difference between test results that constitutes a clinically significant change.

Analytical variability together with between-person and within-person estimates of biological variability suggests that the magnitude of difference in thyroid test values that would be clinically significant when monitoring a patient's response to therapy are:

TT4 =	28 (2.2) nmol/L (µg/dL)
FT4 =	6 (0.5) pmol/L (ng/dL)
TT3 =	0.55 (35) nmol/L (ng/dL)
FT3 =	1.5 (0.1) pmol/L (ng/dL)
TSH =	0.75 mIU/L
Tg =	1.5 µg/L (ng/mL)

ARCHIVED



Section 3: Thyroid Tests for the Clinical Biochemist and Physician

A. Total Thyroxine (TT4) and Total Triiodothyronine (TT3) Methods

Thyroxine (T4) is the principal hormone secreted by the thyroid gland. All the T4 in the circulation is derived from thyroidal secretion. In contrast, only about 20% of circulating triiodothyronine (T3) is of thyroidal origin. Most of the T3 in blood is produced enzymatically in nonthyroidal tissues by 5'-monodeiodination of T4 (121). In fact, T4 appears to function as a pro-hormone for the production of the more biologically active form of thyroid hormone, T3. In the circulation, most (~99.98%) T4 is bound to specific plasma proteins, thyroxine-binding globulin (TBG) (60-75%), TTR/TBPA (prealbumin/transthyretin) (15-30%) and albumin (~10%) (12,16). Approximately 99.7% of T3 in the circulation is bound to plasma proteins, specifically TBG. This represents a 10-fold weaker protein-binding than seen for T4 (12). Protein-bound thyroid hormones do not enter cells and are thus considered to be biologically inert and function as storage reservoirs for circulating thyroid hormone. In contrast, the minute free hormone fractions readily enter cells by specific membrane transport mechanisms to exert their biological effects. In the pituitary, the negative feedback of thyroid hormone on TSH secretion is mediated primarily by T3 that is produced at the site from the free T4 entering the thyrotroph cells.

Technically, it has been easier to develop methods to measure the total (free + protein-bound) thyroid hormone concentrations, as compared with tests that estimate the minute free hormone concentrations. This is because total hormone concentrations (TT4 and TT3) are measured at nanomolar levels whereas free hormone concentrations (FT4 and FT3) are measured in the picomole range and to be valid, must be free from interference by the much higher total hormone concentration.

1. Methods for measuring Total Thyroid Hormones

Serum total T4 and total T3 methods (TT4 and TT3) have evolved through a variety of technologies over the past four decades. The PBI tests of the 1950s that estimated the TT4 concentration as "protein-bound iodide" were replaced in the 1960s first by competitive protein binding methods and later in the 1970s by radioimmunoassay (RIA) methods. Currently, serum TT4 and TT3 concentrations are measured by competitive immunoassay methods that are now mostly non-isotopic and use enzymes, fluorescence or chemiluminescence molecules as signals (135). Total hormone assays necessitate the inclusion of an inhibitor (displacing or blocking agent) such as 8-anilino-1-naphthalene-sulphonic acid (ANS), or salicylate to release the hormone from binding proteins (136). The displacement of hormone binding from serum proteins by such agents, together with the large sample dilution employed in modern assays, facilitates the binding of hormone to the antibody reagent. The ten-fold lower TT3 concentration, as compared with TT4 in blood, presents both a technical sensitivity and precision challenge despite the use of a higher specimen volume (137). Although reliable high-range TT3 measurement is critical for diagnosing hyperthyroidism, reliable normal-range measurement is also important for adjusting antithyroid drug dosage and detecting hyperthyroidism in sick hospitalized patients, in whom a paradoxically normal T3 value may indicate hyperthyroidism.

Despite the availability of highly purified preparations of crystalline L-thyroxine and L-triiodothyronine (i.e. from the United States Pharmacopoeia (16201 Twinbrook Parkway, Rockville, MD 20852) no TT4 or TT3 reference methods have yet been established (138,139). Further, the hygroscopic nature of the crystalline preparations can affect the accuracy of gravimetric weighing (140). Secondly, the diluents used to reconstitute L-T4 and L-T3 preparations for use as calibrators are either modified protein matrices or human serum pools that have been stripped of hormone by various means. In either case, the protein composition of the matrix used for the calibrators is not identical to patient sera. This can result in the protein binding inhibitor reagent (e.g. ANS) releasing different quantities of hormone from calibrator matrix proteins than from the TBG in patient specimens. This may impact the diagnostic accuracy of testing when the binding proteins are abnormal, such as in NTI.

Guideline 9. For Manufacturers Developing TT4 and TT3 Methods

Method biases should be reduced by:

- ☐ The development of L-T4 and L-T3 reference preparations and establishing international reference methods.
- ☐ Ensuring that instruments are not sensitive to differences between human serum and the calibrator matrix.
- ☐ Ensuring that during the test process, the amount of thyroid hormone released from serum binding proteins is the same as that released in the presence of the calibrator diluent.

2. Diagnostic Accuracy of Total Hormone Measurements

The diagnostic accuracy of total thyroid hormone measurements would equal that of free hormone if all patients had identical levels of binding proteins (TBG, TTR/TBPA and albumin) with similar affinities for thyroid hormones. Unfortunately, abnormal serum TT4 and TT3 concentrations are more commonly encountered as a result of binding protein abnormalities than result from true thyroid dysfunction. Patients with serum TBG abnormalities secondary to pregnancy or estrogen therapy, as well as genetic abnormalities in binding proteins, are frequently encountered in clinical practice (141). Abnormal TBG concentrations and/or affinity for thyroid hormone can distort the relationship between total and free hormone measurements (142). Additionally, some patient sera contain other abnormal binding proteins such as autoantibodies to thyroid hormones that render total hormone measurements diagnostically unreliable (143-145). These binding protein abnormalities compromise the use of TT4 and TT3 measurements as stand-alone thyroid tests. Instead, serum TT4 and TT3 measurements are typically made as part of a two-test panel that includes an assessment of binding protein status, made either directly by TBG immunoassay or by an “uptake” test [Section-3 B2(b)]. Specifically, a mathematical relationship between the total hormone concentration and the “uptake” result is used as a free hormone “index” (146). Free hormone indices (FT4I and FT3I) have been used as free hormone estimate tests for three decades but are rapidly being replaced by one-test free hormone estimate immunoassays [Section-3 B3].

3. Serum TT4 and TT3 Normal Reference Intervals

Serum TT4 values vary between methods to some extent. Typical reference ranges approximate 58 - 160 nmol/L (4.5-12.6 µg/dL). Likewise, serum TT3 values are method dependent, with reference ranges approximating 1.2 - 2.7 nmol/L (80 -180 ng/dL).

Guideline 10. Serum Total T4 (TT4) and Total T3 (TT3) Measurements

Abnormal serum TT4 and TT3 concentrations are more commonly encountered as a result of binding protein abnormalities and not thyroid dysfunction.

- ☐ Free T4 estimate tests (FT4) are preferred over TT4 measurement when TBG concentration is abnormal. However, FT4 tests but may be diagnostically inaccurate when the affinity of TBG is altered or abnormal T4-binding proteins are present.
- ☐ Total hormone assays (TT4 and TT3) should remain readily available to evaluate discordant free hormone tests.



B. Free Thyroxine (FT4) and Free Triiodothyronine (FT3) Estimate Tests

Thyroxine in blood is more tightly bound to serum proteins than is T₃, consequently the free T₄ (FT₄) bioavailable fraction is less than free T₃ (0.02% versus 0.2%, FT₄ versus FT₃, respectively). Unfortunately, the physical techniques used for separating the minute free hormone fractions from the predominant protein-bound moieties are technically demanding, inconvenient to use and relatively expensive for routine clinical laboratory use. Those methods that employ physical separation of free from bound hormone (i.e. equilibrium dialysis, ultrafiltration and gel filtration) are typically only available in reference laboratories. Routine clinical laboratories typically use a variety of free hormone tests that estimate the free hormone concentration in the presence of protein-bound hormone. These free hormone estimate tests employ either a two-test strategy to calculate a free hormone “index” [see Section-3 B2] or a variety of ligand assay approaches (14,145,147). In reality, despite manufacturers claims, most if not all FT₄ and FT₃ estimate tests are binding-protein dependent to some extent (148,149). This binding protein dependence negatively impacts the diagnostic accuracy of the free hormone methods that are subject to a variety of interference’s that can cause misinterpretation or inappropriate abnormal results (Table 1). Such interferences include sensitivity to abnormal binding proteins, in-vivo or in-vitro effects of various drugs [Section-3 B3(c)vi], high FFA levels and endogenous or exogenous inhibitors of hormone binding to proteins that are present in certain pathological conditions (60).

1. Nomenclature of Free T₄ (FT₄) and Free T₃ (FT₃) Estimate Methods

Considerable confusion encompasses the nomenclature of free thyroid hormone tests. Controversy continues regarding the technical validity of the measurements themselves and their clinical utility in conditions associated with binding protein abnormalities (145,147,148,150,151). Free hormone testing in clinical laboratories are made using either index methods that require two separate tests, test ligand assays or physical separation methods that isolate free from protein-bound hormone prior to direct measurement of hormone in the free fraction. Ligand assays are standardized either with solutions containing gravimetrically established concentrations of hormone, or use calibrators with values assigned by a physical separation method (i.e. equilibrium dialysis and/or ultrafiltration). Physical separation methods typically are manual, technically demanding and quite expensive for routine clinical use. Index and ligand assay tests are more commonly used in the clinical laboratory setting, where they are typically performed on automated immunoassay analyzer platforms (17).

Guideline 11. Free Hormone Test Nomenclature

- The free hormone methods used by most clinical laboratories (indexes and immunoassays) do not employ physical separation of bound from free hormone and do not measure free hormone concentrations directly! These tests are typically binding protein dependent to some extent and should more appropriately be called **“Free Hormone Estimate”** tests, abbreviated FT₄E and FT₃E.
- In general, Free Hormone Estimate tests overestimate the FT₄ level at high protein concentrations and underestimate FT₄ at low protein concentrations.

Unfortunately, a confusing plethora of terms have been used to distinguish the different free hormone methods and the literature is filled with inconsistencies in the nomenclature of these tests. Currently, there is no clear methodologic distinction between terms such as “T₇”, “effective thyroxine ratio”, “one-step”, “analog”, “two step”, “backtitration”, “sequential”, “immunoextraction” or “immunosequestration”, “ligand assay” because manufacturers have modified the original techniques or adapted them for automation (147). Following the launch of the original one-step “analog” tests in the 1970s, the term “analog” became mired in confusion (147). This first generation of hormone-analog tests were shown to be severely binding-protein dependent and have since been replaced by a new generation of labeled-antibody “analog” tests which are more resistant to the presence of abnormal binding proteins (147,152). Unfortunately, manufacturers rarely disclose all assay constituents or the number of steps involved in an automated procedure so that it is not possible to use the method’s nomenclature (two-step, analog etc) to predict its diagnostic accuracy for assessing patients with binding protein abnormalities (152).

2. Index Methods: FT4I and FT3I

Index methods are free hormone estimate tests that require two separate measurements (*146*). One test is a total hormone measurement (TT4 or TT3) the other is an assessment of thyroid binding protein concentration using either an immunoassay for TBG or a T4 or T3 “uptake” test called Thyroid Hormone Binding Ratio (THBR). Alternatively, indexes may be calculated from a TT4 measurement paired with an estimate of the free T4 fraction determined by isotopic dialysis. In this case, the quality and purity of the tracer employed critically impacts the accuracy of the index (*149,153,154*).

(a) Indexes using TBG Measurement

Calculation of a FT4I using TBG only improves diagnostic accuracy compared with TT4 when the TT4 abnormality results from an abnormal concentration of TBG. In addition, the TT4/TBG index approach is not fully TBG independent, nor does it correct for non TBG-related binding protein abnormalities or for TBG molecules which have abnormal affinity (*141,155-158*). Thus, despite the theoretical advantages of using a direct TBG measurement, TT4/TBG indexes are rarely used because TBG binding capacity can be altered independent of changes in the concentration of TBG protein, especially in patients with NTI (*99*). In addition, TBG binding reflects 60 – 75% of the binding capacity thus relying on TBG alone excludes hormone binding to transthyretin and albumin.

(b) Indexes using a Thyroid Hormone Binding Ratio (THBR) or “Uptake” Test

“Uptake” tests have been used to estimate protein binding of thyroid hormones since the 1950s. Two different types of “uptake tests” have been used. “Classical” uptake tests add a trace amount of radiolabeled T3 or T4 to the specimen and allow the labeled hormone to distribute across the thyroxine binding proteins in exactly the same way as endogenous hormone (*146,154*). Since only a trace amount of labeled T3 or T4 is used, the original equilibrium is barely disturbed. The distribution of the tracer is dependent upon the saturation of the binding proteins. Addition of a secondary binder or adsorbent (anion exchange resin, talc, polyurethane sponge, charcoal, or antibody-coated bead, etc.) results in a redistribution of the T3 or T4 tracer into a new equilibrium, that now includes the binder. The tracer counts sequestered by the adsorbent are dependent on the saturation of the binding proteins: the higher the saturation of the binding proteins, the greater the amount of tracer in the adsorbent. The uptake of added tracer into the adsorbent results in an indirect measure of TBG. When the TBG concentration is low, TBG binding sites are highly saturated with T4 so that a smaller amount of added T3 tracer binding will bind to TBG and more will be being taken up by the adsorbent. Conversely, when the TBG concentration is high, TBG saturation with T4 is low, more tracer binds unoccupied TBG binding sites and less becomes bound to the adsorbent. Unfortunately the relationship between THBR and TBG concentration is non-linear, such that index testing usually does not correct TT4 abnormalities resulting from grossly abnormal TBG concentrations (*158*).

It has been recommended that a normal serum sample standard be used to normalize the response of the assays and allow for the reporting of the result as a ratio to normal i.e. a “Thyroid Hormone Binding Ratio (THBR)” (*154*). “Classic” uptake assays used T3 tracer because the lower T3-TBG binding affinity relative to T4-TBG resulted in a higher isotopic uptake by adsorbent and thus shorter counting times. However, since the validity of using a T3-uptake test to correct a TT4 value is questionable, some current non-isotopic assays use a “T4-uptake”. Many manufacturers still use the “classical” approach to produce T3 uptake assays in which the mean normal percent uptake can vary from 25% to 40% (bound counts/total counts). Traditionally, the free thyroxine index, sometimes called a “T7” is derived from the product of a T3-uptake test and a TT4 measurement, often expressed as a % uptake (adsorbent bound counts divided by total counts).

Guideline 12. Thyroid Hormone Binding Ratio (THBR) or “Uptake” Tests

- “Uptake” tests should be called “Thyroid Hormone Binding Ratio” tests, abbreviated THBR and include an indication of which hormone is used, i.e. THBR (T4) or THBR (T3).
- A T4 signal is preferred over T3 for THBR measurements, to better reflect T4 binding protein abnormalities.
- THBR values should be reported as a ratio with normal serum, the latter having an assigned value of 1.00.
- THBR calculations should be based on the ratio between absorbent counts divided by the total minus absorbent counts, rather than the ratio between absorbent counts and total counts.
- The THBR result should be reported in addition to the total hormone and free hormone index value.
- THBR tests should not be used as an independent measurement of thyroid status, but should be interpreted in association with a TT4 and/or TT3 measurement and used to produce free hormone estimates (FT4 or FT3 indexes).

“Classic” T3-uptake or THBR tests are typically influenced by the endogenous T4 concentration of the specimen. This limitation can be circumvented by using a very large excess of a non-isotopically labeled T4 tracer with an affinity for thyroid binding proteins comparable to that of T4. Current THBR tests usually produce normal FT4I and FT3I values when TBG abnormalities are mild (i.e. pregnancy). However, some of these tests may produce inappropriately abnormal index values when patients have grossly abnormal binding proteins (congenital TBG high or low, familial dysalbuminemic hyperthyroxinemia (FDH), thyroid hormone autoantibodies and NTI) and in the presence of some medications that influence thyroid hormone protein binding [Section-3 B3(c)vi].

(c) Indexes using a Free Hormone Fraction Determination

The first free hormone tests developed in the 1960s were indexes, calculated from the product of the free hormone fraction from a dialysate multiplied by the TT4 measurement (made by PBI and later RIA) (159,160). The free fraction index approach was later extended to measure the rate of transfer of isotopically-labeled hormone across a membrane separating two chambers containing the same undiluted specimen. The free hormone indexes calculated with isotopic free fractions are not completely independent of TBG concentration and furthermore are influenced by radiochemical purity, the buffer matrix and the dilution factor employed (161,162).

3. Ligand Assays for FT4 and FT3 Estimation

These methods employ either a “two-step” or “one-step” approach. Specifically, two-step assays use a physical separation of free from protein-bound hormone before free hormone is measured by a sensitive immunoassay, or alternatively, an antibody is used to immunoextract a proportion of ligand out of the specimen before quantitation. In contrast, one-step ligand assays attempt to quantify free hormone in the presence of binding proteins. Two-step methods are less prone to non-specific artifacts. One-step methods may become invalid when the specimen and the standards differ in their affinity for the assay tracer (60,145,150).

(a) Ligand Assays employing Physical Separation

FT4 methods that physically isolate free from protein-bound hormone before employing a sensitive immunoassay to measure the free hormone concentration are standardized using solutions containing gravimetrically prepared standard preparations of T4. The physical isolation of free from protein-bound hormone is accomplished with either a semi-permeable membrane using a dialysis chamber, an ultrafiltration technique, or a Sephadex LH-20 resin adsorption column (161-165). An exceedingly sensitive T4 RIA method is needed to measure the picomole concentrations of FT4 in dialysates or free fraction isolates, as compared with total hormone measurements in the nanomole range. Although there are no officially acknowledged “gold standard” free hormone methods, it is generally considered that methods that employ physical separation are

least influenced by binding proteins, and by inference, provide free hormone values that best reflect the circulating free hormone level (94,166). However, dialysis methods employing a dilution step may underestimate FT4 when binding inhibitors are present in the specimen and adsorption of T4 to membrane materials may be an issue (94,166). In contrast, such methods may overestimate FT4 in sera from heparin-treated patients as a result of in-vitro generation of FFA [see Section-3 B3(c)vii] (84,97,98,100,101,167-170). This in-vitro heparin effect is the primary cause of spuriously high FT4 values in NTI patients (101). Physical separation methods are too labor intensive and expensive for routine use by clinical laboratories and are usually only available in reference laboratories. FT3 methods employing physical separation are only available in some specialized research laboratories (102).

(b) Ligand Assays without Physical Separation

Most of the free hormone immunoassays in current use employ a specific, high affinity hormone antibody to sequester a small amount of total hormone from the specimen. The antibody unoccupied antibody-binding sites that are usually inversely proportional to the free hormone concentration and are quantified using the hormone labeled with radioactivity, fluorescence- or chemiluminescence. The signal output is then converted to a free hormone concentration using calibrators with free hormone values assigned by a method employing physical separation. The actual proportion of total thyroid hormone sequestered varies with the method design, but greatly exceeds the actual free hormone concentration and should be <1-2% in order to minimize perturbation of the free-bound equilibrium. The active sequestering of hormone by the anti-thyroid hormone antibody reagent in the assay results in a continuous stripping of hormone from binding proteins and perturbation of the bound to free equilibrium. The key to the validity of these methods is twofold. First, it is necessary to use conditions that maintain the free to protein-bound hormone equilibrium, and to minimize dilution effects that weaken the influence of any endogenous inhibitors present in the specimen. Secondly, it is important to use serum calibrators containing known free hormone concentrations that behave in the assay in an identical manner to the patient specimens. Three general approaches have been used to develop comparable FT4 and FT3 immunoassay methods: (i) two-step labeled-hormone; (ii) one-step labeled-analog; and (iii) labeled antibody.

Guideline 13. For Manufacturers Developing Free Hormone Estimate Tests

- ☐ Methods that do not physically separate bound from free hormone should extract no more than 1-2% of the total hormone concentration off the binding proteins, so that the thermodynamic equilibrium is maintained as much as possible.
- ☐ Minimize dilution effects that weaken the influence of any endogenous inhibitors present in the specimen.
- ☐ Use serum calibrators containing known free hormone concentrations that behave in the assay in an identical manner to the patient specimens.
- ☐ Perform the test procedure at 37°C.

(i) Two-Step, Labeled-Hormone/Back-Titration Methods

Two-step methods were first developed for research purposes in the late 1970s and were subsequently adapted to produce commercial FT4 and FT3 methods. During a first incubation step, these methods used a high affinity ($>1 \times 10^{11}$ L/mol) anti-hormone antibody bound to a solid support (ultrafine Sephadex, antibody-coated tube or particles) to sequester a small proportion of total hormone from a diluted serum specimen. After a short incubation period, unbound assay constituents are washed away before the second step is performed in which sufficient labeled hormone is added to bind to all the unoccupied antibody-binding sites. After washing, the amount of labeled hormone bound to the solid-phase antibody is quantified relative to gravimetric standards or calibrators that have free hormone values assigned by a reference method. One-step labeled hormone-analog methods were introduced in the late 1970s. These new tests were less labor-intensive than two-step techniques. As a result, two-step methods lost popularity despite comparative studies showing that they were less affected by albumin concentration and binding protein abnormalities that negatively impact the diagnostic accuracy of the one-step analog tests (147,171-173).

(ii) One-Step, Labeled Hormone-Analog Methods

The physicochemical validity of the one-step labeled hormone-analog tests were dependent upon the development of a hormone analog with a molecular structure that was totally non-reactive with serum proteins, but could react with unoccupied hormone antibody sites. When these conditions are met, the hormone-analog, which is chemically coupled to a signal molecule such as an isotope or enzyme, can compete with free hormone for a limited number of antibody-binding sites in a classical competitive immunoassay format. Though conceptually attractive, this approach is technically difficult to achieve in practice, despite early claims of success. The hormone-analog methods were principally engineered to give normal FT4 values in high TBG states (i.e. pregnancy). However, they were found to have poor diagnostic accuracy in the presence of abnormal albumin concentrations, FDH, NTI, high FFA levels or with thyroid hormone autoantibodies. Considerable efforts were made during the 1980s to correct these problems by the addition of proprietary chemicals to block analog binding to albumin or by empirically adjusting calibrator values to correct for protein-dependent biases. However, after a decade of criticism, most hormone-analog methods have been abandoned because these problems could not be resolved (147).

(iii) Labeled Antibody Methods

Labeled antibody methods also measure free hormone as a function of the fractional occupancy of hormone-antibody binding sites. This competitive approach uses specific immunoabsorbents to assess the unoccupied antibody binding sites in the reaction mixture. A related approach has been the use of solid-phase unlabeled hormone/protein complexes (sometimes referred to as “analogs”) that do not react significantly with serum proteins, to quantify unoccupied binding sites on the anti-hormone antibody in the liquid-phase. The physiochemical basis of these labeled-antibody methods suggests that they may be as susceptible to the same errors as the older labeled-hormone analog methods. However, physicochemical differences arising from the binding of analog to the solid support confer kinetic differences that results in decreased analog affinity for endogenous binding proteins and a more reliable free hormone measurement. The labeled antibody approach is currently the favored free hormone testing approach on most automated platforms.

(c) Performance of FT4 and FT3 Tests in Different Clinical Situations

The only reason to select a free thyroid hormone method (FT4 or FT3) in preference to a total thyroid hormone test (TT4 or TT3) is to improve the diagnostic accuracy for detecting hypo- and hyperthyroidism in patients with thyroid hormone binding abnormalities that compromise the diagnostic accuracy of total hormone measurements (60). Unfortunately, the diagnostic accuracy of current free hormone methods cannot be predicted from either their method classification (one-step, two-step, labeled antibody etc) or by in-vitro tests of their technical validity, such as a specimen dilution test. The index tests (FT4I and FT3I) as well as current ligand assay methods, are all protein dependent to some extent, and may give unreliable values when binding proteins are significantly abnormal (148). Free hormone tests should be performed at 37°C since tests performed at ambient temperature falsely increase values when specimens have a very low TBG concentration (174,175).

The impetus for developing free hormone tests has been the high frequency of binding-protein abnormalities that cause discordance between total and free thyroid hormone concentrations. Unfortunately, no current FT4 method is universally valid in all clinical conditions. When the concentration of TBG is abnormal, most FT4 methods give results that are more diagnostically useful than TT4 measurement. However, pre-analytical or analytical assay artifacts arise in many situations associated with binding protein abnormalities: when the binding of the assay tracer to albumin is abnormal; in the presence of medications that displace T4 from TBG; during critical phases of NTI; and in pregnancy (see Table 1). The frequency of these FT4 assay artifacts suggests that TSH or the TSH/FT4 relationship is a more reliable thyroid parameter to use than an estimate of FT4 alone.

When it is suspected that a FT4 result is discrepant, FT4 should be checked using a different manufacturer's method (usually measured in a different laboratory). Additionally, or alternatively the FT4/ TT4 relationship can be checked for discordance since interference seldom affects both measurements to the same degree and in the same direction.

Guideline 14. Clinical Utility of Serum Free T3 Estimate Tests

Serum T3 measurement has little specificity or sensitivity for diagnosing hypothyroidism, since enhanced T4 to T3 conversion maintains normal T3 concentrations until hypothyroidism becomes severe. Patients with NTI or caloric deprivation typically have low total and free T3 values. Serum T3 measurements, interpreted together with FT4, and are useful to diagnose complex or unusual presentations of hyperthyroidism and certain rare conditions:

- ☐ A high serum T3 is often an early sign of recurrence of Graves' hyperthyroidism.
- ☐ The TT3/TT4 ratio can be used to investigate Graves' versus non-Graves' hyperthyroidism. Specifically, a high TT3/TT4 ratio (>20 ng/μg metric or >0.024 molar) suggests thyroidal stimulation characteristic of Graves' disease.
- ☐ Serum T3 measurement can be used to monitor the acute response to treatment for Graves' thyrotoxicosis.
- ☐ A high or paradoxically normal serum T3 may indicate hyperthyroidism in an NTI patient with suppressed TSH (< 0.01 mIU/L).
- ☐ A high or paradoxically normal serum T3 may indicate amiodarone-induced hyperthyroidism.
- ☐ Patients with goiter living in areas of iodide deficiency should have FT3 measured in addition to TSH to detect T3 thyrotoxicosis caused by focal or multifocal autonomy.
- ☐ A high serum T3 is frequently found with congenital goiter, due to defective organification of iodide (TPO defect) or defective synthesis of thyroglobulin.
- ☐ A high serum T3 usually precedes iodide-induced thyrotoxicosis when patients have multinodular long-standing goiter.
- ☐ A high serum T3 is often seen with TSH-secreting pituitary tumors.
- ☐ A high serum T3 is often seen in thyroid hormone resistance syndromes that usually present without clinical hyperthyroidism.
- ☐ Serum T3 measurement is useful for monitoring compliance with L-T3 suppression therapy prior to ¹³¹I scan for DTC.
- ☐ Serum T3 measurement is useful for distinguishing mild (subclinical) hyperthyroidism (low TSH/ normal FT4) from T3-toxicosis, sometimes caused by T3-containing health-foods.
- ☐ Serum T3 measurement is useful for investigating iodide deficiency (characterized by low T4/high T3).
- ☐ Serum T3 measurement can be useful during antithyroid drug therapy to identify persistent T3 excess, despite normal or low serum T4.
- ☐ Serum T3 measurement can be used to detect early recurrence of thyrotoxicosis after cessation of antithyroid drug therapy
- ☐ Serum T3 measurement can be used to establish the extent of T3 excess during suppressive L-T4 therapy or after an intentional T4 overdose.

(i) Pregnancy

The increase in serum TBG and the low albumin concentrations associated with pregnancy results in wide method-dependent variations in FT4 measurements [see Section-2 A3] (47,59). Albumin-dependent methods can produce low FT4 values in up to 50 percent of patients and are unsuitable for assessing thyroid status during pregnancy because of the marked negative bias attributable to the progressive decline in the serum albumin concentration by the third trimester (59). Conversely, methods such as tracer dialysis tend to show a positive bias in relation to standards, possibly due to tracer impurities (60). The use of method- and trimester-specific reference ranges might improve the diagnostic accuracy of free hormone testing in pregnancy. However, few if any manufacturers have developed such information for their methods.

(ii) Premature Infants

A low thyroxine level without an elevated TSH is commonly encountered in premature infants of less than 28 weeks gestation (39,176). There is some clinical evidence to suggest that L-T4 treatment may improve neurological outcome (176). However, as described above, method differences in FT4 methods are likely to compromise the reliability of detecting hypothyroxinemia of prematurity.

Guideline 15. Abnormal Thyroid Hormone Binding Proteins Effects on FT4 Tests

Binding protein abnormalities cause pre-analytical or analytical FT4 assay artifacts. Thyroid function should be assessed from the TSH-TT4 relationship when:

- ☐ The binding of assay tracer to albumin is abnormal (i.e. FDH).
- ☐ The patient is taking medications that displace T4 from TBG, i.e. Phenytoin, Carbamazepine or Furosemide (Frusemide).
- ☐ The patient has a critical or severe non-thyroidal illness.

(iii) Genetic Abnormalities in Binding Proteins

Heredity and acquired variations in albumin, and TBG with altered affinity for either T4 or T3 can cause abnormal total hormone concentrations in euthyroid subjects with normal free hormone concentrations (141). The albumin variant responsible for familial dysalbuminemic hyperthyroxinemia (FDH) has a markedly increased affinity for T4 and numerous T4-analog tracers, resulting in spuriously high serum free T4 estimates with these tracers (145,177). In FDH, serum TT4 and FT4I values, as well as some FT4 ligand assays, give supra-normal values, whereas serum TT3, FT3, TSH and FT4 measured by other methods, including equilibrium dialysis, are normal (177). Failure to recognize the presence of the FDH albumin variant that can occur with a prevalence of up to 1:1000 in some Latin-American populations can result in inappropriate thyroid test result interpretation leading to thyroid gland ablation (178).

(iv) Autoantibodies

Some patient sera contain autoantibodies to thyroid hormone that result in methodologic artifacts in total or free hormone measurements (143,145). Such antibody interferences are method-dependent. Tracer T4 or T3 bound to the endogenous antibody is falsely classified as bound by adsorption methods, or free by double antibody methods, leading to falsely low or falsely high serum TT4 or TT3 values, respectively (144,145). The T4 tracer analogs used in some FT4 tests may bind to these autoantibodies, leading to spuriously high serum FT4 results. There have even been reports of anti-solid phase antibodies interfering in labeled-antibody assays for free thyroid hormones (179).

(v) Thyrotoxicosis and Hypothyroidism

The relationship between free and total T4 and T3 in thyrotoxicosis is non-linear. In severe thyrotoxicosis, the elevations in TT4 and FT4 are disproportionate. This non-linearity reflects both a decrease in TBG levels and an overwhelming of the TBG binding capacity despite increased binding to TTR and albumin (180). Similarly, FT3 concentrations may be underestimated as a result of high T4-TBG binding. The converse situation exists in severe hypothyroidism, in which there is reduced occupancy of all binding proteins (180). In this situation, an excess of unoccupied binding sites may blunt the FT4 response to treatment. This suggests that an initial L-T4 loading dose is the most rapid approach for restoring a therapeutic FT4 level in a hypothyroid patient.

(vi) Drugs that Compete for Thyroid Hormone Binding

Some therapeutic and diagnostic agents such as Phenytoin, Carbamazepine or Furosemide/Frusemide may

competitively inhibit thyroid hormone binding to serum proteins in the specimen. The reduced binding-protein availability results in an acute increase in FT4 and in some instances increased hormone action as evidenced by a reduction in TSH (181). The increased FT4 measurements are influenced by the serum dilution used by the method and are also seen with dialysis methods (182,183). During the chronic administration of such competitor drugs, there is enhanced clearance of hormone. However, eventually the system re-establishes a "normal" equilibrium and FT4 levels normalize at the expense of a low TT4 concentration. The withdrawal of drug at this point would cause an initial fall in FT4 as more carrier protein becomes available, with re-normalization of FT4 as the equilibrium is re-established through an increased release of hormone from the thyroid gland. The time-scale and magnitude of these competitor effects differ with the half-life of the competitor agent.

A number of medications and factors compete with the binding of T4 and T3 to TBG causing an acute increase in the availability of FT4 or FT3. Many of these competing agents of thyroid hormone binding are frequently prescribed therapeutic agents that differ in their affinity for TBG relative to T4 (96,184). Furosemide, for example binds to TBG but with an affinity that is about three-fold less than T4 whereas aspirin binds seven-fold less than T4 (170,185). The competition in vivo observed with such agents relates to their affinity for TBG rather than their therapeutic levels, the free fraction or their affinity for non-TBG proteins, especially albumin (170,186).

Current FT4 assays that employ a dilution factor may fail to detect an elevation in FT4 secondary to the presence of binding-protein competitors. For example, a specimen containing both T4 (free fraction 1:4000) and a competitive inhibitor (free fraction 1:100) subjected to stepwise dilution will sustain the FT4 concentration up to a 1:100 dilution, secondary to progressive dissociation of T4 from binding proteins. In contrast, the free drug concentration would decrease markedly only after a 1:10 dilution. Thus the hormone-displacing effect of drugs competing for T4 binding will be underestimated in FT4 assays employing high specimen dilution. The use of symmetric equilibrium dialysis and ultrafiltration of undiluted serum can minimize this artifact (94,165,187,188).

(vii) Heparin Treatment Artifacts

It is well known that in the presence of a normal albumin concentration, non-esterified fatty acid (FFA) concentrations > 3mmol/L will increase FT4 by displacing the hormone from TBG (84,97,98,100,101,167-170). Serum from patients treated with heparin, including low-molecular weight heparin preparations, may exhibit spuriously high FT4 values secondary to in vitro heparin-induced lipase activity that increases FFA. This problem is seen even with heparin doses as low as 10 units and is exacerbated with storage of the specimen. Increased serum triglyceride levels, low serum albumin concentrations or prolonged assay incubation at 37°C can accentuate this problem.

(viii) Critical Nonthyroidal Illness

There is a large body of evidence collected over more than two decades, that report on the specificity of various FT4 methods in hospitalized patients with NTI [Section-2 B2]. This literature can be confusing, and is complicated by the heterogeneity of the patient populations studied and the method-dependence of the results. Manufacturers have progressively modified their methods over time, in an attempt to improve their specificity in this setting and other situations when binding proteins are abnormal. However, the exact composition of current methods remains proprietary and it is difficult for manufacturers to obtain pedigreed specimens from such patients to rigorously test their methods. In one recent FT4 method comparison study, a marked method-dependent difference was seen on the seventh day following bone marrow transplantation in euthyroid subjects receiving multiple drug therapies that included heparin and glucocorticoids (101). In this study, the TT4 concentrations were normal in most of the subjects (95%) and the serum TSH was < 0.1 mIU/L in approximately half of the subjects. This was consistent with the glucocorticoid therapy the patients were receiving. In contrast, both elevated and subnormal values were reported by different FT4 methods. It appeared that the supranormal FT4 estimates reported by some methods in 20 to 40% of patients, probably reflected the

I.V. heparin effect discussed above [Section-3 B3(c)vii]. In contrast, analog tracer methods that are subject to the influence of tracer binding to albumin, gave subnormal FT4 estimates in 20-30% of patients (101). Such FT4 measurement artifacts, giving rise to a discordance between FT4 and TSH results, increase the risk of an erroneous diagnosis of either thyrotoxicosis or secondary hypothyroidism and suggest that TT4 measurements may be more reliable in the setting of a critical illness.

(d) FT4 Method Validation

Unfortunately, most free hormone estimate methods receive inadequate evaluation prior to their introduction for clinical use. Manufacturers rarely extend the validation of their methods beyond the study of ambulatory hypo- and hyperthyroid patients, pregnant patients and a catchall category of “NTI/hospitalized patients”. However, there is currently no consensus as to the best criteria to use for evaluating these free T4 estimate methods. It is insufficient to merely demonstrate that a new method can distinguish between hypothyroid, normal and hyperthyroid values, and to show comparability with existing methods - any free hormone estimate method will satisfy these criteria without necessarily giving information about the true physiologic free hormone concentration.

Guideline 16. For Manufacturers: Assessment of FT4 Estimate Test Diagnostic Accuracy

- The diagnostic accuracy of the method should be tested using pedigreed specimens from ambulatory patients with the following binding protein disturbances:
 - TBG abnormalities (high estrogen & congenital TBG excess and deficiency)
 - Familial Dysalbuminemic Hyperthyroxinemia (FDH)
 - Increased Transthyretin (TTR) affinity
 - T4 and T3 Autoantibodies
 - Rheumatoid Factor
- Test the method for interference with normal serum specimens spiked with relevant concentrations of common inhibitors at concentrations that cause displacement of hormone from binding proteins in undiluted serum, effects which are lost after dilution i.e.:
 - Furosemide (Frusemide) 30 µM
 - Disalicylic acid 300 µM
 - Phenytoin 75 µM
 - Carbamazepine 8 µM
- List all known interferences with the magnitude and direction of resulting errors
- Document in-vitro heparin effects on NEFA generation during the assay incubation

New methods should either be tested with pedigreed clinical samples, especially those that may challenge the assay validity, or alternatively, by manipulating the constituents of a normal serum sample to test a particular criterion (148). Whichever approach is adopted, the key questions relate to the similarity between samples and standards, because all assays are generally comparable. Other approaches include testing the quantitative recovery of added L-T4, or determining the effects of serum dilution, since a 100-fold dilution of a “normal” serum theoretically causes an insignificant reduction (less than 2%) in the FT4 concentration (94,152) (58,189). These approaches however, just test the “protein dependence” of the method, i.e. the degree to which free T4 is dependent on the dissociation of free from bound hormone (148). These approaches will predictably give an unfavorable assessment of methods that involve a high degree of sample dilution compared to those methods that minimize sample dilution. There is no evidence however, to document whether these approaches truly reflect diagnostic accuracy of the method when used to evaluate difficult clinical specimens. Ultimately, as with any diagnostic method, the specificity of a free T4 method will only become evident after testing a full spectrum of specimens from individuals with and without thyroid dysfunction associated with binding protein abnormalities or medications known to affect thyroid hormone binding to plasma proteins. An unexpected interference may only be noted after methods have been in use for some time, as in the effects of rheumatoid factor that can produce spuriously high serum free T4 estimates (112). Non-specific fluorescence due to the presence in the blood of substances such as organic acids in patients with uremia can be another cause of non-specific interference (190).

The preferred approach is to pay particular attention to specimens that are likely to cause non-specific interference in the assay result (98). Ideally, in the ambulatory patient setting these would include samples that have: a) TBG abnormalities (pregnancy, oral contraceptive therapy, and congenital TBG excess and deficiency); b) Familial Dysalbuminemic Hyperthyroxinemia (FDH); c) T4 and T3 autoantibodies; d) interfering substances such as rheumatoid factor and and e) a wide spectrum of drug therapies. In the hospital setting, three classes of patients should be tested: a) patients without thyroid dysfunction but with low or high TT4 due to NTI; b) patients with documented hypothyroidism associated with severe NTI and, c) patients with documented hyperthyroidism associated with NTI. However, it is prohibitively difficult for manufacturers to obtain pedigreed specimens from such patients. Since no manufacturers have tested their methods adequately in critically sick patients, it is difficult for physicians to have confidence that abnormal FT4 results in such patients reflects true thyroid dysfunction rather than NTI. Thus in hospitalized patients with suspected thyroid dysfunction, a combination of serum TSH and TT4 measurements may provide more information than only a FT4 test, provided that the TT4 value is interpreted relative to the degree of severity of the illness. Specifically, the low TT4 state of NTI is usually restricted to severely sick patients in an intensive care setting. A low TT4 value in a patient not critically ill should prompt a consideration of pituitary dysfunction. In ambulatory patients, serum FT4 measurements are often more diagnostically accurate than a TT4 measurement. However, when an abnormal FT4 result does not fit the clinical picture, or there is an unexplained discordance in the TSH to FT4 relationship, it may be necessary to order a TT4 test as confirmation. Alternatively, the laboratory could either send the specimen to a different laboratory that uses a different manufacturer's FT4 method, or to a reference laboratory that can perform a FT4 measurement using a physical separation method, such as equilibrium dialysis or ultrafiltration.

(e) Interferences with Thyroid Tests

Ideally, a thyroid hormone test should display zero interference with any compound, drug or endogenous substance (i.e. bilirubin) in any specimen, at any concentration. Studies available from manufacturers vary widely in the number of compounds studied and in the concentrations tested. Usually the laboratory can only proactively detect interference from a "sanity check" of the relationship between the FT4 and TSH result. If only one test is measured, interference is usually first suspected by the physician who observes an inconsistency between the reported value and the clinical status of the patient. Classic laboratory checks of verifying the specimen identity and performing dilution, may not always detect interference. Interferences with either TT4 or FT4 measurements typically elicit inappropriately abnormal values in the face of a normal serum TSH level (Table 1). Interferences with competitive or non-competitive immunoassays fall into three classes: (i) cross-reactivity problems, (ii) endogenous analyte antibodies and (iii) drug interactions (191).

(i) Cross-reactivity

Cross-reactivity problems result from the inability of the antibody reagent to discriminate selectively between analyte and a structurally related molecule (192). Thyroid hormone assays are less susceptible to this type of interference than TSH, because iodothyronine antibody reagents are selected for specificity by screening with purified preparations. The availability of monoclonal and affinity-purified polyclonal antibodies has reduced the cross-reactivity of current T4 and T3 tests to less than 0.1% for all studied iodinated precursors and metabolites of L-T4. However, there have been reports of 3-3',5-triiodothyroacetic acid (TRIAc) interfering in FT3 assays and D-T4 interference in FT4 assays (14,135).

(ii) Endogenous Autoantibodies

Endogenous autoantibodies to both T4 and T3 have been frequently found in the serum of patients with autoimmune thyroid as well as non-thyroidal disorders. Despite their high prevalence, interference caused by such autoantibodies is relatively rare. Such interferences are characterized by either falsely low or falsely high values, depending on the type and composition of the assay used (193).

(iii) Drug Interferences

Drug Interferences can result from the in-vitro presence of therapeutic or diagnostic agents in the serum specimen in sufficient quantities to interfere with thyroid tests (67,68). Thyroid test methods employing fluorescent signals may be sensitive to the presence of fluorophor-related therapeutic or diagnostic agents in the specimen (190). In the case of I.V. heparin administration, the in vitro activation of lipoprotein lipases results in the generation of FFA in vitro that may falsely elevate FT4 values [see Section-3 B3(c)vii] (84,97,98,100,101,167-170).

(f) Serum FT4 and FT3 Normal Reference Intervals

Physical separation methods are used to assign values to the calibrators employed for most FT4 estimate tests. There is closer agreement between the reference intervals of the various ligand assays used by clinical laboratories than there is between the various methods that employ physical separation. Reference intervals for FT4 immunoassay methods approximate 9-23 pmol/L (0.7 –1.8 ng/dL). In contrast, the upper FT4 limit for methods such as equilibrium dialysis that employ physical separation extends above 30 pmol/L (2.5 ng/dL). Reference intervals for FT3 immunoassay methods approximate 3.5-7.7 pmol/L (0.2 – 0.5 ng/dL). FT3 methods that employ physical separation are currently only available as research assays (102).

(g) Standardization or Calibration

There are no internationally developed standard materials or methods for free hormone measurements. Although candidate reference methods have been suggested for TT4 measurements, it will be difficult to adapt such methods for free hormones (139). Each method and manufacturer approaches the problem of standardization from its own unique perspective.

FT4 estimate methods that require two independent assays (tracer equilibrium dialysis and ultrafiltration as well as index methods) use a total hormone measurement and a measurement of the free fraction of the hormone. Total hormone assays are standardized with gravimetrically prepared calibrators from high purity hormone materials, which are commercially available. The free fraction is determined as radioactive counts in a dialysate or ultrafiltrate. Alternatively, in the case of the index methods, the saturation or binding capacity of the binding protein(s) is measured using a thyroid hormone binding ratio (THBR) test, sometimes referred to as an “uptake” test. THBR tests are standardized against sera with normal binding proteins and assigned a value of 1.00 [Section-3 B2(b)].

The more complicated situation occurs with the ligand free hormone estimate assays. In general these tests are provided with standards that have known or assigned free hormone values determined by a reference method (usually equilibrium dialysis with RIA of the FT4 concentration of the dialysate). This is typically performed by the manufacturer for the purpose of establishing free hormone values for the human serum based calibrators containing the hormone and its binding protein(s) for inclusion in the kit. Alternatively, in the case of highly bound hormones, such as thyroxine, the Law of Mass Action can be used to calculate the free hormone concentration (194). The total hormone concentration, a measurement of the total binding capacity for the hormone in that serum sample, and the equilibrium constant provide the necessary information to calculate the free hormone concentration. This approach is valid for calibrators and controls manufactured in human serum that contains a normal TBG binding capacity. This allows the manufacturer to make calibrators and controls at fixed levels.

The use of calibrators, prepared as described above, also compensates for the over-extraction of hormone from their binding proteins. Specifically, in the case of thyroxine and triiodothyronine, the antibody in the kit may bind the free hormone and extract a significant amount (~1-2%) of the bound hormone. If assayed directly, the concentration of free hormone would be elevated due to the over extraction. However, the use of calibrators with known free hormone levels and in human serum permits the assignment of the specific signal levels from the assay readout system (whether, isotopic, enzymatic, fluorescence, or chemiluminescence) to specific known concentrations of free hormone in a proportional relationship. However, this will only be valid if the percent of hormone extracted from the calibrator is identical to that from the patient specimen. This is often not the case for specimens containing binding protein abnormalities (i.e. congenital high and low TBG, FDH, NTI etc).

4. Free Hormone Measurement – the Future

The era of immunoassay methods for the quantification of thyroid and steroid hormones in biological fluids began in the 1970's. That era is now drawing to a close. The era of advanced mass spectrometry for quantifying hormones in biological fluids is now emerging (138). There is no reason to doubt that mass spectrometry will provide better quantification because of greater analytical specificity and less analytical interference than immunoassays. So far such techniques have only being applied to TT4 determination (139). However, for total hormone assays, the requirement for complete hormone release from protein-hormone complexes will remain. For free hormone assays, the requirement for a physical separation of free hormone and protein bound hormone, prior to quantification will also remain. In order to accomplish the later, new separation technology will be needed, before any method can be regarded as a gold standard. The implicit dilution of small molecules is a limitation of equilibrium dialysis that needs to be overcome. Ultrafiltration shows promise, but current methods are either too leaky or too impractical for this task. Mass spectrometry measurements of hormones that form complexes with serum proteins will only be as good as the specimen preparation steps associated with the quantification. However, the ideal free hormone reference method would be a technique that employs ultrafiltration at 37°C, to avoid dilution effects and the direct measurement of free hormone in the ultrafiltrate by mass spectrometry.

Guideline 17. For Laboratories Performing FT4 and FT3 testing

- ☐ Physicians should have ready access to information on the effects of drugs and the diagnostic accuracy of the test used for assessing the thyroid status of patients with various binding protein abnormalities and severe illnesses.
- ☐ When requested by the physician, the laboratory should be prepared to confirm a questionable result by performing a total hormone measurement or by re-measuring FT4 by a reference method that physically separates free from bound hormone, such as direct equilibrium dialysis or ultrafiltration.
- ☐ Questionable results on specimens should be checked for interference by re-measurement made with a different manufacturer's method. (Send out to a different laboratory if necessary.)



C. Thyrotropin/Thyroid Stimulating Hormone (TSH)

For more than twenty-five years, TSH methods have been able to detect the TSH elevations that are characteristic of primary hypothyroidism. Modern-day TSH methods however, with their enhanced sensitivity are also capable of detecting the low TSH values typical of hyperthyroidism. These new methods are often based on non-isotopic immunometric assay (IMA) principles and are available on a variety of automated immunoassay analyzer platforms. Most of the current methods are capable of achieving a functional sensitivity of 0.02 mIU/L or less, which is a necessary detection limit for the full range of TSH values observed between hypo- and hyperthyroidism. With this level of sensitivity, it is possible to distinguish the profound TSH suppression typical of severe Graves' thyrotoxicosis ($\text{TSH} < 0.01 \text{ mIU/L}$) from the TSH suppression (0.01 – 0.1 mIU/L) observed with mild (subclinical) hyperthyroidism and in some patients with a non-thyroidal illness (NTI).

In the last decade the diagnostic strategy for using TSH measurements has changed as a result of the sensitivity improvements in these assays. It is now recognized that the TSH measurement is a more sensitive test than FT4 for detecting both hypo- and hyperthyroidism. As a result, some countries now promote a TSH-first strategy for diagnosing thyroid dysfunction in ambulatory patients (provided that the TSH method has a functional sensitivity $\leq 0.02 \text{ mIU/L}$). Other countries still favor the TSH + FT4 panel approach, because the TSH-first strategy can miss patients with central hypothyroidism [Section-3 C4(f)] or TSH-secreting pituitary tumors [Section-3 C4(g)] (19,195-197). An additional disadvantage of the TSH-centered strategy is that the TSH-FT4 relationship cannot be used as a “sanity check” for interferences or detection of unusual conditions characterized by discordance in the ratio of TSH/FT4 (Table 1).

1. Specificity

(a) TSH Heterogeneity

TSH is a heterogeneous molecule and different TSH isoforms circulate in the blood and are present in the pituitary extracts used for assay standardization (Medical Research Council (MRC) 80/558). In the future, recombinant human TSH (rhTSH) preparations might be used as primary standards for standardizing TSH immunoassays (198). Current TSH IMA methods use TSH monoclonal antibodies that virtually eliminate cross-reactivity with other glycoprotein hormones. These methods however, may detect different epitopes of abnormal TSH isoforms secreted by some euthyroid individuals, as well as some patients with abnormal pituitary conditions. For example, patients with central hypothyroidism caused by pituitary or hypothalamic dysfunction, secrete TSH isoforms with abnormal amounts of glycosylation and reduced biological activity. These isoforms are measured as paradoxically normal or even elevated serum TSH concentrations by most methods (195,197,199). Likewise, paradoxically normal serum TSH levels may be seen in patients with hyperthyroidism due to a TSH-secreting pituitary tumor, that appears to secrete TSH isoforms with enhanced biologic activity (196,200,201).

(b) Technical Problems

Technical problems, especially with the washing step, may result in falsely high TSH values (202). Additionally, any interfering substance in the specimen (eg heterophilic antibodies, HAMA) that produces a high background or a false bridge between the capture and signal antibodies will create a high signal on the solid support that will be read out as a falsely high result [see Section-2C3] (203,202).

(c) Methods for Detecting Interference with a TSH Result

The conventional laboratory approach to verifying an analyte concentration such as dilution may not always detect an interference problem. Since methods vary in their susceptibility to most interfering substances, the most practical way to test for interference is to measure the TSH concentration in the specimen using a different manufacturer's method, and to check for a significant discordance between the TSH values. When the

variability of TSH measurements made on the same specimen with different methods exceeds expectations (>50% difference), interference may be present. Biologic checks may also be useful to verify an unexpected result. Inappropriately low TSH values could be checked by a TRH-stimulation test, which is expected to elevate TSH more than 2-fold (≥ 4.0 mIU/L increment) in normal individuals (204). In cases where TSH appears inappropriately elevated, a thyroid hormone suppression test (1mg L-T4 or 200µg L-T3, po) would be expected to suppress serum TSH more than 90 % by 48 hours in normal individuals.

Guideline 18. Investigation of Discordant Serum TSH Values in Ambulatory Patients

A discordant TSH result in an ambulatory patient with stable thyroid status may be a technical error. Specificity loss can result from laboratory error, interfering substances (i.e. heterophilic antibodies), or the presence of an unusual TSH isoform (see Guideline 7 and Table 1). Physicians can request that their laboratory perform the following checks:

- ☐ Confirm specimen identity (i.e. have laboratory check for a switched specimen in the run).
- ☐ When TSH is unexpectedly high, ask the laboratory to re-measure the specimen diluted, preferably in thyrotoxic serum, to check for parallelism.
- ☐ Request that the laboratory analyze the specimen by a different manufacturer's method (send to a different laboratory if necessary). If the between-method variability for a sample is > 50%, an interfering substance may be present.
- ☐ Once a technical problem has been excluded, biologic checks may be useful:
 - Use a TRH stimulation test for investigating a discordant low TSH result, expect a 2-fold (≥ 4.0 mIU/L increment) response in TSH in normal individuals.
 - Use a thyroid hormone suppression test to verify a discordant high TSH level. Normal response to 1mg of L-T4 or 200µg L-T3 administered p.o. is a suppressed serum TSH of more than 90 % by 48 hours.

2. Sensitivity

Historically, the "quality" of a serum TSH method has been determined from a clinical benchmark - the assay's ability to discriminate euthyroid levels (~ 0.4 to 4.0 mIU/L) from the profoundly low (<0.01 mIU/L) TSH concentration typical of overt Graves' thyrotoxicosis. Most TSH methods now claim a detection limit of 0.02 mIU/L or less ("third generation" assays) (202).

Guideline 19. Definition of Functional Sensitivity

Functional Sensitivity should be used to determine the Lowest Detection Limit of the assay.

- TSH assay functional sensitivity is defined as a 20 % between-run coefficient of variation (CV) determined by the recommended protocol (see Guideline 20).

TSH assay functional sensitivity is defined by the 20 % between-run coefficient of variation (CV) determined by the recommended protocol (see Guideline 20).

Guideline 20. Protocol for TSH Functional Sensitivity & Between–Run Precision

Measure human serum pools covering the assay range in at least 10 different runs. The lowest pool value should be 10% above the detection limit and the highest pool value should be 90% of the upper assay limit.

- ☐ Carry-over should be assessed by analyzing the highest pool followed by the lowest pool.
- ☐ Use the same test mode as for patient specimens (i.e. singlicate or duplicate).
- ☐ The instrument operator should be blinded to the presence of test pools in the run.
- ☐ Runs should be spaced over a clinically representative interval (i.e. 6 to 8 weeks for TSH in an outpatient setting).
- ☐ Use at least two different lots of reagents and two different instrument calibrations during the testing period.
- ☐ When running the same assay on two similar instruments, blind duplicates should be run on each instrument periodically to verify correlation.

Manufacturers have largely abandoned the use of the “analytical sensitivity” parameter for determining the sensitivity of a TSH assay because it is calculated from the within-run precision of the zero calibrator which does not reflect the sensitivity of the test in clinical practice (126,127). Instead, a “functional sensitivity” parameter has been adopted (202). Functional sensitivity is calculated from the 20% between-run coefficient of variation (CV) for the method and is used to establish the lowest reportable limit for the test (202).

Functional sensitivity should be determined by strictly adhering to the recommended protocol (Guideline 20) that is designed to assess the minimum detection limit of the assay in clinical practice and ensure that the parameter realistically represents the lowest detection limit of the assay. The protocol is designed to take into account a variety of factors that can influence TSH method imprecision in clinical practice. These include:

- ☐ Matrix differences between patient serum and the standard diluent
- ☐ Erosion of precision over time
- ☐ Lot-to-lot variability in the reagents supplied by the manufacturer
- ☐ Differences between instrument calibration and technical operators
- ☐ Carry-over from high to low specimens (205)

The use of the functional sensitivity limit as the lowest detection limit is a conservative approach to ensure that any TSH result reported is not merely assay “noise”. Further, the 20% between-run CV approximates the maximum imprecision required for diagnostic testing (Table 5).

Guideline 21. For Laboratories Performing TSH Testing

Functional sensitivity is the most important performance criterion that should influence the selection of a TSH method. Practical factors such as instrumentation, incubation time, cost, and technical support though important, should be secondary considerations. Laboratories should use calibration intervals that optimize functional sensitivity, even if re-calibration needs to be more frequent than recommended by the manufacturer:

- ☐ Select a TSH method that has a functional sensitivity ≤ 0.02 mIU/L
- ☐ Establish functional sensitivity independent of the manufacturer by using Guideline 20
- ☐ There is no scientific justification to reflex from a less sensitive to a more sensitive test. (Insensitivity causes falsely high, not falsely low, values that are missed by reflex testing!)

3. TSH Reference Intervals

Despite some gender, age and ethnicity-related differences in TSH levels revealed by the recently published NHANES III US survey, it is not considered necessary to adjust the reference interval for these factors in clinical practice (18). Serum TSH levels exhibit a diurnal variation with the peak occurring during the night and the nadir, which approximates to 50% of the peak value, occurring between 1000 and 1600 hours (123,124).

This biologic variation does not influence the diagnostic interpretation of the test result since most clinical TSH measurements are performed on ambulatory patients between 0800 and 1800 hours and TSH reference intervals are more commonly established from specimens collected during this time period. Serum TSH reference intervals should be established using specimens from TPOAb-negative, ambulatory, euthyroid subjects who have no personal or family history of thyroid dysfunction and no visible goiter. The variation in the reference intervals for different methods reflects differences in antibody epitope recognition by the different kit reagents and the rigor applied to the selection of appropriate normal subjects.

Serum TSH concentrations determined in normal euthyroid subjects are skewed with a relatively long "tail" towards the higher values of the distribution. The values become more normally distributed when log-transformed. For reference range calculations, it is customary to log-transform the TSH results to calculate the 95% reference interval (typical population mean value ~1.5 mIU/L, range 0.4 to 4.0 mIU/L in iodide-sufficient populations) (202,206). However, given the high prevalence of mild (subclinical) hypothyroidism in the general population, it is likely that the current upper limit of the population reference range is skewed by the inclusion of persons with occult thyroid dysfunction (18).

Guideline 22. TSH Reference Intervals

TSH reference intervals should be established from the 95 % confidence limits of the log-transformed values of at least 120 rigorously screened normal euthyroid volunteers who have:

- ☐ No detectable thyroid autoantibodies, TPOAb or TgAb (measured by sensitive immunoassay)
- ☐ No personal or family history of thyroid dysfunction
- ☐ No visible or palpable goiter
- ☐ No medications (except estrogen).

(a) TSH Upper Reference Limits

Over the last two decades, the upper reference limit for TSH has steadily declined from ~10 to approximately ~4.0-4.5 mIU/L. This decrease reflects a number of factors including the improved sensitivity and specificity of current monoclonal antibody based immunometric assays, the recognition that normal TSH values are log-distributed and importantly, improvements in the sensitivity and specificity of the thyroid antibody tests that are used to pre-screen subjects. The recent follow-up study of the Whickham cohort has found that individuals with a serum TSH >2.0 mIU/L at their primary evaluation had an increased odds ratio of developing hypothyroidism over the next 20 years, especially if thyroid antibodies were elevated (35). An increased odds-ratio for hypothyroidism was even seen in antibody-negative subjects. It is likely that such subjects had low levels of thyroid antibodies that could not be detected by the insensitive microsomal antibody agglutination tests used in the initial study (207). Even the current sensitive TPOAb immunoassays may not identify all individuals with occult thyroid insufficiency. In the future, it is likely that the upper limit of the serum TSH euthyroid reference range will be reduced to 2.5 mIU/L because >95% of rigorously screened normal euthyroid volunteers have serum TSH values between 0.4 and 2.5 mIU/L.

(b) TSH Lower Reference Limits

Before the immunometric assay era, TSH methods were too insensitive to detect values in the lower end of the reference range (209). Current methods however, are capable of measuring TSH at the lower end and now cite lower limits between 0.2 and 0.4 mIU/L (202). As the sensitivity of the methods has improved, there has been an increased interest in defining the true lower limit of normal to better determine the presence of mild (subclinical) hyperthyroidism. Current studies suggest that TSH values in the 0.1 to 0.4 mIU/L range may represent thyroid hormone excess and in elderly patients might be associated with an increased risk of atrial fibrillation, and cardiovascular mortality (36,37). It is therefore important to carefully exclude subjects with a goiter and any illness or stress in the normal cohort selected for reference range study.

4. Clinical Use of Serum TSH Measurements

(a) Screening for Thyroid Dysfunction in Ambulatory Patients

Most professional societies recommend that TSH be used for case finding or screening for thyroid dysfunction in ambulatory patients, provided that the TSH assay used has a functional sensitivity at or below 0.02 mIU/L (4,10,210). The TSH assay sensitivity stipulation is critical for the reliable detection of subnormal values, since less sensitive TSH assays are prone to produce false negative (normal range) results on specimens with subnormal TSH concentrations (202). The log/linear relationship between TSH and FT4 dictates that serum TSH is the preferred test, since only TSH can detect mild (subclinical) degrees of thyroid hormone excess or deficiency (Figure 1) [Section-2 A1]. Mild (subclinical) thyroid dysfunction, characterized by an abnormal TSH associated with a normal range FT4 have reported prevalences in population surveys of ~10% and 2%, for subclinical hypo- and hyperthyroidism, respectively (10,18,25,211). Despite the clinical sensitivity of TSH, a TSH-centered strategy has inherently two primary limitations. First, it assumes that hypothalamic-pituitary function is intact and normal. Second, it assumes that the patients thyroid status is stable, i.e. the patient has had no recent therapy for hypo-or hyperthyroidism [Section-2 A1 and Figure 2] (19). If either of these criteria is not met, serum TSH results can be diagnostically misleading (Table 1).

When investigating the cause of an abnormal serum TSH in the presence of normal FT4 and FT3, it is important to recognize that TSH is a labile hormone and subject to nonthyroidal pituitary influences (glucocorticoids, somatostatin, dopamine etc) that can disrupt the TSH/FT4 relationship (69,70,71,212). It is important to confirm any TSH abnormality in a fresh specimen drawn after ~3 weeks before assigning a diagnosis of mild (subclinical) thyroid dysfunction as the cause of an isolated TSH abnormality. After confirming a high TSH abnormality, a TPOAb measurement is a useful test for establishing the presence of thyroid autoimmunity as the cause of mild (subclinical) hypothyroidism. The higher the TPOAb concentration, the more rapid the development of thyroid failure. After confirming a low TSH abnormality it can be difficult to unequivocally establish a diagnosis of mild (subclinical) hyperthyroidism, especially if the patient is elderly and not receiving L-T4 therapy (34). If a multinodular goiter is present, thyroid autonomy is the likely cause of mild (subclinical) hyperthyroidism (213).

There is no consensus regarding the optimal age to begin the screening process. The American Thyroid Association guidelines recommend screening at age 35 and every 5 years thereafter (10). Decision analysis appears to support the cost-effectiveness of this strategy, especially for women (215). The strategy for using TSH to screen for mild (subclinical) hypo- and hyperthyroidism will remain in dispute until there is more agreement on the clinical consequences and outcome of having a chronically abnormal TSH. Also there needs to be agreement as to the level of the TSH abnormality that should indicate the need for treatment (216,217).

There is mounting evidence to suggest that patients with a persistent TSH abnormality may be exposed to greater risk if left untreated. Specifically, a recent study reported a higher cardiovascular mortality rate when patients had a chronically low serum TSH (37). Further, there are an increasing number of reports that indicate that mild hypothyroidism in early pregnancy increases fetal wastage and impairs the IQ of the offspring (63-65)). Such studies support the efficacy of early thyroid function screening, especially in women during their childbearing years.

(b) Elderly Patients

Most studies support screening for thyroid dysfunction in the elderly (10,35,214). The prevalence of both a low and high TSH (associated with normal FT4) is increased in the elderly compared with younger patients. Hashimotos' thyroiditis, associated with a high TSH and detectable TPOAb, is encountered with increasing prevalence as we get older. (35). The incidence of a low TSH is also increased in the elderly (35). A low TSH may be transient but is a persistent finding in approximately 2 % of elderly individuals, with no other apparent evidence of thyroid dysfunction (36,214). This could be due to a change in the FT4/TSH set-point, a change in TSH bioactivity or mild thyroid hormone excess (218). A recent study by Parle et al showed a higher cardiovascular mortality rate in such patients (37). This suggests that the cause of a persistently low TSH level

should be actively investigated (37). Multinodular goiter should be ruled out as the cause especially in areas of iodide deficiency (213). Medication history should be thoroughly reviewed (including over-the-counter preparations, some of which contain T3). If a goiter is absent and the medication history negative, a serum TSH should be rechecked together with TPOAb measurements after 4 to 6 weeks. If the TSH is still low and TPOAb is positive, the possibility of autoimmune thyroid dysfunction should be considered. Treatment of low TSH should be made on a case-by-case basis.

(c) L-T4 Replacement Therapy

It is now well documented that hypothyroid patients have serum FT4 values in the upper third of the reference interval when the L-T4 replacement dose is titrated to bring the serum TSH into the therapeutic target range (0.5-2.0 mIU/L) (219,220).

Levothyroxine (L-T4) and not dessiccated thyroid, is the preferred long-term replacement medication for hypothyroidism.

A euthyroid state is usually achieved in adults with a L-T4 dose averaging 1.6 µg/kg body weight/day. Children require higher doses (up to 4.0 µg/kg bw/day) and older individuals require lower doses (1.0 µg/kg bw/day) (221,222). The initial dose and the optimal time needed to establish the full replacement dose should be individualized relative to age, weight and cardiac status. The requirements for an increase in thyroxine during pregnancy [Section-2 A3] and in post-menopausal women just starting hormone replacement therapy (223) may also be increased.

A serum TSH result between 0.5 and 2.0 mIU/L is generally considered the therapeutic target for a standard L-T4 replacement dose for primary hypothyroidism.

A serum FT4 concentration in the upper third of the reference interval is the therapeutic target for L-T4 replacement therapy when patients have central hypothyroidism due to pituitary and/or hypothalamic dysfunction.

A typical schedule for gradually titrating to a full replacement dose involves giving L-T4 in 25 µg increments each 6–8 weeks until the full replacement dose is achieved (serum TSH 0.5-2.0 mIU/L). As shown in figure 2, TSH is slow to re-equilibrate to a new thyroxine level. Patients with chronic, severe hypothyroidism may develop pituitary thyrotroph hyperplasia which can mimic a pituitary adenoma, but which resolves after several months of L-T4 replacement therapy (224). Patients taking Rifampin and anticonvulsants that influence the metabolism of L-T4 may also need an increase in their dose of L-T4 to maintain the TSH within the therapeutic target range.

Both free T4 and TSH should be used for monitoring hypothyroid patients suspected of intermittent or non-compliance with their L-T4 therapy. The paradoxical association of a high FT4 + high TSH is often an indication that compliance may be an issue. Specifically, acute ingestion of missed L-T4 doses before a clinic visit will raise the FT4 but fail to normalize the serum TSH because of the “lag effect” (Figure 2). In essence, the serum TSH is analogous to the hemoglobin A1c as a long-term free T4 sensor! At least 6 weeks is needed before retesting TSH following a change in the dose of L-T4 or brand of thyroid medication. Annual TSH testing of patients receiving a stable dose of L-T4 is recommended. The optimal time for TSH testing is not influenced by the time of day the L-T4 dose is ingested (133). However, the daily dose should be withheld when FT4 is used as the therapeutic endpoint, since serum FT4 is significantly increased (~13%) above baseline for 9 hours after ingesting the last dose (225).

Ideally L-T4 should be taken before eating; at the same time of day and at least 4 hours apart from any other medications or vitamins. Many medications can influence T4 absorption/metabolism (especially Cholestyramine, Ferrous Sulfate, Soy Protein, Sucralfate, antacids containing Aluminum Hydroxide, anticonvulsants or Rifampin) (4,226).

(d) L-T4 Suppression Therapy

L-T4 administration designed to suppress serum TSH levels to subnormal values is typically reserved for patients with well-differentiated thyroid carcinoma for which thyrotropin is considered a trophic factor (227). The efficacy of L-T4 suppression therapy has been determined from uncontrolled retrospective studies that have yielded conflicting results (228,229).

It is important to individualize the degree of TSH suppression by weighing patient factors such as age, clinical status including cardiac factors and DTC recurrence risk against the potentially deleterious effects of iatrogenic mild (subclinical) hyperthyroidism on the heart and bone (36). Many physicians use a serum TSH target of 0.05-0.1 mIU/L for low-risk patients and a TSH of <0.01 mIU/L for high-risk patients. Some physicians reduce the L-T4 dose to give low-normal TSH values when patients have undetectable serum thyroglobulin (Tg) levels and no recurrences 5-10 years after thyroidectomy. Suppression therapy for non-endemic goiters is generally considered ineffective (230). Furthermore, patients with nodular goiters often already have suppressed TSH concentrations as a result of thyroid gland autonomy (213).

Guideline 23. Levothyroxine (L-T4) Replacement Therapy for Primary Hypothyroidism

- ❑ L-T4, not desiccated thyroid, is the preferred medication for long-term replacement therapy for hypothyroidism.
- ❑ A euthyroid state is usually achieved with an average L-T4 dose of 1.6 µg/kg body weight/day. The initial dose and time to achieve full replacement should be individualized relative to age, weight and cardiac status. An initial L-T4 dose is normally 50-100 µg daily. Serum TSH measurement after six weeks will indicate the need for dose adjustment by 25-50 µg increments.
- ❑ Children require higher doses of L-T4, up to 4.0 µg/kg bw/day, due to rapid metabolism. Serum TSH and FT4 values should be assessed using age-specific and method-specific reference ranges (Table 3).
- ❑ A serum TSH level between 0.5 and 2.0 mIU/L is generally considered the optimal therapeutic target for the L-T4 replacement dose for primary hypothyroidism.
- ❑ TSH is slow to re-equilibrate to a new thyroxine status (Guideline 2). Six to 8 weeks is needed before retesting TSH after changing the L-T4 dose or brand of thyroid medication.
- ❑ Intermittent or non-compliance with levothyroxine (L-T4) replacement therapy will result in discordant serum TSH and FT4 values (high TSH/high FT4) because of a persistently unstable thyroid state (Guideline 2). Both TSH and FT4 should be used for monitoring such patients.
- ❑ Thyroxine requirements decline with age. Older individuals may require less than 1.0 µg/kg bw/day and may need to be titrated slowly. Some physicians prefer to gradually titrate such patients. An initial dose of 25 µg is recommended for patients with evidence of ischemic heart disease followed by dose increments of 25 µg every 3-4 weeks until the full replacement dose is achieved. Some believe that a higher target TSH (0.5-3.0 mIU/L) value may be appropriate for the elderly patient.
- ❑ In severe hypothyroidism an initial L-T4 loading dose is the most rapid means for restoring a therapeutic FT4 level because the excess of unoccupied binding sites may blunt the FT4 response to treatment.
- ❑ Thyroxine requirements increase during pregnancy. Thyroid status should be checked with TSH + FT4 during each trimester of pregnancy. The L-T4 dose should be increased (usually by 50 µg/day) to maintain a serum TSH between 0.5 and 2.0 mIU/L and a serum FT4 in the upper third of the normal reference interval.
- ❑ Post-menopausal women starting hormone replacement therapy may need an increase in their L-T4 dose to keep the serum TSH within the therapeutic target.
- ❑ TSH testing of patients receiving a stable L-T4 dose is recommended on an annual basis. The ideal time for TSH testing is not influenced by the time of day the L-T4 dose is ingested.
- ❑ Ideally L-T4 should be taken before eating, at the same time of day, and at least 4 hours apart from any other medications or vitamins. Bedtime dosing should be 2 hours after the last meal.
- ❑ Patients beginning chronic therapy with cholestyramine, ferrous sulfate, calcium carbonate, soy protein, sucralfate and antacids containing aluminum hydroxide that influence L-T4 absorption may require a larger L-T4 dose to maintain TSH within the therapeutic target range.
- ❑ Patients taking Rifampin and anticonvulsants that influence the metabolism of L-T4 may also need an increased L-T4 dose to maintain the TSH within the therapeutic target range.

Guideline 24. Levothyroxine (L-T4) Suppression Therapy

- ❑ Serum TSH is considered a growth factor for differentiated thyroid cancer (DTC). The typical L-T4 dose used to suppress serum TSH in DTC patients is 2.1 µg/kg body weight/day.
- ❑ The target serum TSH level for L-T4 suppression therapy for DTC should be individualized relative to the patient's age and clinical status including cardiac factors and DTC recurrence risk.
- ❑ Many physicians use a serum TSH target value of 0.05-0.1 mIU/L for low-risk patients and a TSH of <0.01 mIU/L for high-risk patients.
- ❑ Some physicians use a low-normal range therapeutic target for TSH when patients have undetectable serum Tg levels and have had no recurrence 5-10 years after thyroidectomy.
- ❑ If iodide intake is sufficient, L-T4 suppression therapy is rarely an effective treatment strategy for reducing the size of goiters.
- ❑ Over time, multi-nodular goiters typically develop autonomy that is characterized by a subnormal serum TSH level. Serum TSH should be checked before initiating L-T4 suppression therapy in such patients.

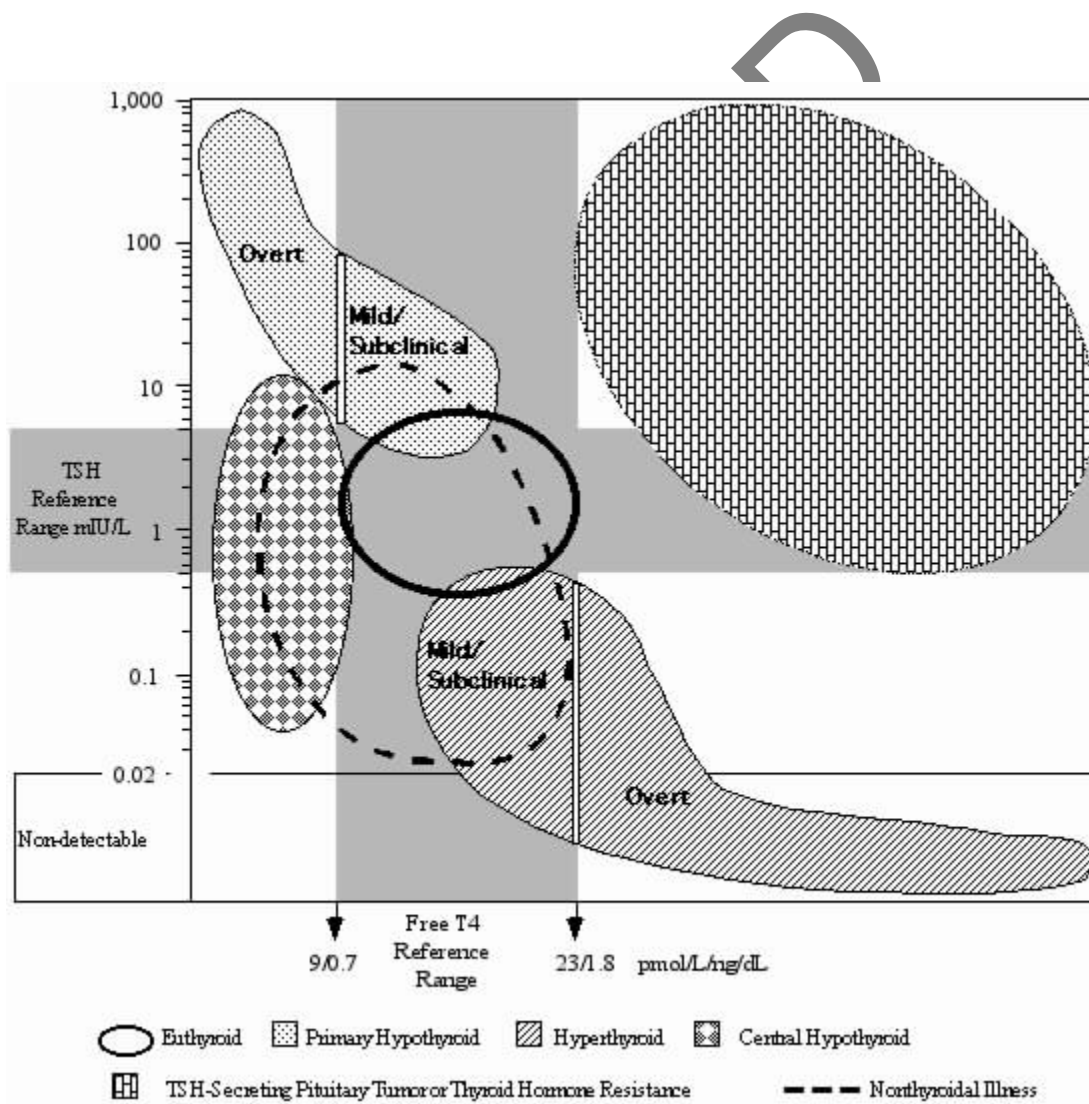


Fig 4. The serum TSH/FT4 relationship typical of different clinical conditions

(e) Serum TSH Measurement in Hospitalized Patients with NTI

Although most hospitalized patients with NTI have normal serum TSH concentrations, transient TSH abnormalities in the 0.02 – 20 mIU/L range are commonly encountered in the absence of thyroid dysfunction (20,87,92,93). It has been suggested that the use of a wider reference range (0.02 –10 mIU/L) would improve the positive predictive value of TSH measurements for evaluating the sick hospitalized patient (20,92,93,231). TSH should be used in conjunction with a FT4 estimate (or TT4) test for evaluating hospitalized patients with clinical symptoms or patients with a history of thyroid dysfunction (Guidelines 6 and 25).

Sometimes the cause of the TSH abnormality in a hospitalized patient is obvious, such as in the case of dopamine or glucocorticoid-therapy (87,92). In other cases the TSH abnormality is transient and appears to be caused by NTI per se, and resolves with recovery from the illness. It is common to see a transient minor suppression of TSH into the 0.02-0.2 mIU/L range during the acute phase of an illness, followed by a rebound to mildly elevated values during recovery (103). It is important to use a TSH assay with a functional sensitivity ≤ 0.02 mIU/L in the hospital setting in order to be able to reliably determine the degree of TSH suppression. Specifically, the extent of TSH suppression can be used to discriminate sick hyperthyroid patients with profoundly low serum TSH values (< 0.02 mIU/L) from patients with a mild transient suppression of NTI (20).

Guideline 25. TSH Measurement in Hospitalized Patients

- ☐ TSH + T4 (FT4 or TT4) is the most useful test combination to detect thyroid dysfunction in a sick hospitalized patient.
- ☐ It is more appropriate to use a widened TSH reference interval (0.05 to 10.0 mIU/L) in the hospitalized setting. Serum TSH levels may become subnormal transiently in the acute phase and become elevated in the recovery phase of an illness.
- ☐ A serum TSH value between 0.05 and 10.0 mIU/L is usually consistent with a euthyroid state, or only a minor thyroid abnormality that can be evaluated by retesting after the illness subsides. (This only applies to patients not receiving medications such as dopamine that directly inhibit pituitary TSH secretion.)
- ☐ A low-normal TSH level in the presence of a low TT4 and low TT3 may reflect central hypothyroidism as a result of a prolonged illness; whether or not this condition requires immediate treatment remains uncertain and is currently controversial.
- ☐ When thyroid dysfunction is suspected, a thyroid peroxidase antibody (TPOAb) test may be useful to differentiate autoimmune thyroid disease from NTI.

Diagnosing hyperthyroidism in NTI patients can be a challenge because current FT4 methods can give both inappropriately low and high values in euthyroid NTI patients (101,232). Serum TT4 and TT3 measurements may be useful for confirming a diagnosis of hyperthyroidism if assessed relative to the severity of the illness (Guideline 6). A suppressed serum TSH below 0.02mIU/L is less specific for hyperthyroidism in hospitalized individuals, compared with ambulatory patients. One study found that 14% of hospitalized patients with TSH < 0.005 mIU/L were euthyroid. However, such patients had a detectable TRH-stimulated TSH response whereas patients who were truly hyperthyroid with NTI did not (20).

Mild (subclinical) hypothyroidism cannot be easily diagnosed during a hospitalization, because of the frequency of high TSH abnormalities associated with NTI. Provided that the thyroid hormone (FT4 or TT4) concentration is within normal limits, any minor abnormality in serum TSH (0.02-20.0 mIU/L) arising from a mild (subclinical) thyroid condition is unlikely to affect the outcome of the hospitalization, and can be deferred for evaluation 2-3 months after discharge. In contrast, sick hypothyroid patients typically exhibit the combination of low FT4 and elevated TSH (>20 mIU/L) (92).

(f) Central Hypothyroidism

The log/linear relationship between TSH and FT4 dictates that patients with primary hypothyroidism and a subnormal FT4 should have a serum TSH value > 10 mIU/L (Figure 1) [Section-2 A1]. When the degree of TSH elevation in response to a low thyroid hormone level appears inappropriately low, pituitary insufficiency should be excluded. A diagnosis of central hypothyroidism will usually be missed using a “TSH first” strategy (19).

Guideline 26. Levothyroxine (L-T4) Replacement Therapy for Central Hypothyroidism

- ❑ A serum FT4 level in the upper third of the reference interval is the therapeutic target for the L-T4 replacement dose used to treat central hypothyroidism due to pituitary or hypothalamic dysfunction.
- ❑ When using FT4 as the therapeutic endpoint for central hypothyroidism, the daily dose of L-T4 should be withheld on the day of the FT4 measurement. (Serum FT4 is increased (~13 %) above baseline for 9 hours after ingesting L-T4).

Central hypothyroid conditions are characterized by paradoxically normal or slightly elevated serum TSH in the majority of cases (29). In one study of central hypothyroid patients, 35% had subnormal TSH values but 41% and 25% had inappropriately normal and elevated TSH values, respectively (233). It is now well documented that the paradoxically elevated serum TSH levels seen in central hypothyroid conditions is caused by the measurement of biologically inert TSH isoforms that are secreted when the pituitary is damaged or when hypothalamic TRH stimulation is deficient (197). The inappropriate TSH values arise because the monoclonal antibodies used in current TSH assays cannot distinguish between TSH isoforms of different biological activity, since TSH biological activity is determined not by the protein backbone but by the degree of glycosylation, specifically the sialylation of the TSH molecule. It appears that normal TRH secretion is essential for producing normal TSH sialylation and association of the TSH subunits to form mature, biologically active TSH molecules (29,197,234). The biological activity of TSH in central hypothyroid conditions appears to be inversely related to the degree of TSH sialylation as well as the FT4 level in the circulation (29). TRH-stimulation testing may be useful for specifically diagnosing central hypothyroidism (235). Typical TSH-responses in such conditions are blunted (<2-fold rise/ ≤ 4.0 mIU/L increment) and may be delayed (197,204,235,236). In addition, the T3-responses to TRH-stimulated TSH are blunted and correlate with TSH biological activity (197,237,238).

Guideline 27. Clinical Utility of TSH Assays (Functional Sensitivity ≤ 0.02 mIU/L)

- ❑ Serum TSH measurement is the most diagnostically sensitive test for detecting mild (subclinical), as well as overt, primary hypo- or hyperthyroidism in ambulatory patients.
- ❑ The majority (>95%) of healthy euthyroid subjects have a serum TSH concentration below 2.5 mIU/L. Ambulatory patients with a serum TSH above 2.5 mIU/L when confirmed by a repeat TSH measurement made after 3-4 weeks, may be in the early stages of thyroid failure, especially if TPOAb is detected.
- ❑ A serum TSH measurement is the therapeutic endpoint for titrating the L-T4 replacement dose for primary hypothyroidism (see Guideline 23) and for monitoring L-T4 suppression therapy for differentiated thyroid carcinoma (see Guideline 24).
- ❑ Serum TSH measurements are more reliable than FT4 in hospitalized patients with non-thyroidal illness not receiving dopamine. Serum TSH should be used in conjunction with T4 (TT4 or FT4) testing for hospitalized patients (Guidelines 6 and 26).
- ❑ TSH cannot be used to diagnose central hypothyroidism because current TSH assays measure biologically inactive TSH isoforms.
- ❑ Central hypothyroidism is characterized by an inappropriately normal or slightly elevated serum TSH level and a blunted (<2-fold rise/ ≤ 4.0 mIU/L increment) TRH response.
- ❑ When the serum FT4 is low and yet the serum TSH is only minimally elevated (<10 mIU/L), a diagnosis of central hypothyroidism should be considered.
- ❑ Serum TSH measurements are an important pre-natal and first trimester screening test to detect mild (subclinical) hypothyroidism in the mother (see Guideline 4).
- ❑ A low TSH in the setting of a multinodular goiter suggests the presence of mild (subclinical) hyperthyroidism due to thyroid autonomy.
- ❑ A serum TSH measurement is required for confirming that an elevated thyroid hormone level is due to hyperthyroidism and not a thyroid hormone binding protein abnormality (such as FDH).
- ❑ A serum TSH measurement is the primary test for detecting amiodarone – induced thyroid dysfunction (see Guideline 5).

(g) Inappropriate TSH Secretion Syndromes

As shown in Table 1, binding protein abnormalities or assay technical problems are the most common causes for a discordant FT4/TSH relationship. The apparent paradoxical dissociation between high levels of thyroid hormone and a non-suppressed serum TSH has led to the widespread use of the term “inappropriate TSH secretion syndromes” to describe these conditions. Specimens that display a discordant TSH/FT4 relationship are increasingly being identified now that sensitive TSH assays that can reliably detect subnormal TSH concentrations are available and in widespread use [Section-3 C2]. As shown in Table 1, it is critical to first exclude likely causes of a discordant TSH/FT4 ratio, i.e technical interference and/or binding protein abnormality. This confirmatory testing should be performed on a fresh specimen by measuring TSH together with free and total thyroid hormone with a different manufacturer’s method. Only after the more common causes of discordance have been eliminated should rare conditions such as a TSH-secreting pituitary tumor or thyroid hormone resistance be considered.

When the abnormal biochemical profile has been confirmed, the possibility that a TSH-secreting pituitary tumor is the cause of the paradoxical TSH should first be eliminated before assigning the diagnosis of thyroid hormone resistance (THR). It should be noted that both conditions can coexist (247). TSH-secreting pituitary tumors have similar biochemical profiles to THR but can be distinguished from THR by TSH-alpha subunit testing and radiographic imaging. Additionally, TRH-stimulation testing may occasionally be useful in developing the differential diagnosis. Specifically, a blunted TRH-stimulation test and T3-suppression test is characteristic of most TSH-secreting pituitary tumors whereas a normal response is seen in most cases of THR (245).

(i) TSH-Secreting Pituitary Tumors

Pituitary tumors that hypersecrete TSH are rare, representing <1% of cases of inappropriate TSH secretion (27,28). These tumors often present as a macroadenoma with symptoms of hyperthyroidism associated with a non-suppressed serum TSH and MRI evidence of a pituitary mass (28).

After excluding a technical reason for the paradoxically elevated TSH level (i.e. HAMA), the diagnosis of TSH-secreting pituitary tumor is usually made on the basis of:

- A lack of TSH response to TRH stimulation
- An elevated serum TSH alpha subunit
- A high alpha subunit/TSH ratio
- The demonstration of a pituitary mass on MRI

Guideline 28. For Manufacturers of TSH Tests

- ☐ Manufacturers that market TSH tests with differing sensitivities are urged to discontinue marketing the less sensitive product.
- ☐ There is no justification for the pricing of TSH assays to be based on sensitivity!
- ☐ There is no scientific justification for reflexing from a less sensitive to a more sensitive TSH test.
- ☐ Manufacturers should help laboratories independently establish functional sensitivity by providing appropriately low TSH human serum pools when requested.
- ☐ Manufacturers should indicate the use of calibration factors, especially if calibration factors are country-dependent.
- ☐ Manufacturers should quote the recovery of the TSH Reference Preparation at the claimed functional sensitivity.
- ☐ Kit Package Inserts should:
 - Document the realistic functional sensitivity of the method using the Guideline 20 protocol
 - Cite the functional sensitivity that can be achieved across a range of clinical laboratories
 - Display the typical between-run precision profile expected for a clinical laboratory
 - Recommend the use of functional sensitivity not analytical sensitivity to determine the lowest reporting limit. (Analytical sensitivity prompts laboratories to adopt an unrealistic detection limit.)

(ii) Thyroid Hormone Resistance

Thyroid hormone resistance (THR) is usually caused by a mutation of the thyroid hormone (TR), TR-beta receptor gene that occurs in 1: 50,000 live births (239-242). Although the clinical presentation can be variable, patients have a similar biochemical profile. Specifically, serum FT4 and FT3 are typically elevated (from a minimal degree to a 2-3-fold elevation above the upper normal limit) and associated with a normal or slightly elevated serum TSH that responds to TRH stimulation (242,243). However, it should be recognized that TSH secretion is not inappropriate given the fact that the tissue response to thyroid hormone is reduced, requiring higher thyroid hormone levels to maintain a normal metabolic state. THR patients typically have a goiter as a result of chronic hypersecretion of a hybrid TSH isoform that has increased increased biologic potency (199,244). The clinical manifestation of thyroid hormone excess covers a wide spectrum. Some patients appear to have a normal metabolism with a near-normal serum TSH and whose receptor defect appears to be compensated for by high levels of thyroid hormone (Generalized THR). Other patients appear to be hypermetabolic and to have a defect that selectively affects the pituitary (Pituitary THR).

The distinctive features of THR are the presence of a non-suppressed TSH, together with an appropriate response to TRH despite elevated thyroid hormone levels (242,245). Although rare, it is important to consider the diagnosis of THR when encountering a patient with elevated thyroid hormone levels associated with a paradoxically normal or elevated TSH (242,246). Such patients have often been misdiagnosed as having hyperthyroidism and have been subjected to inappropriate thyroid surgery or radioiodide gland ablation (242).



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 chemiluminescence). 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 Hashimoto's thyroiditis. In fact, when TPOAb is measured by a sensitive immunoassay, >95% of subjects with Hashimoto's 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).

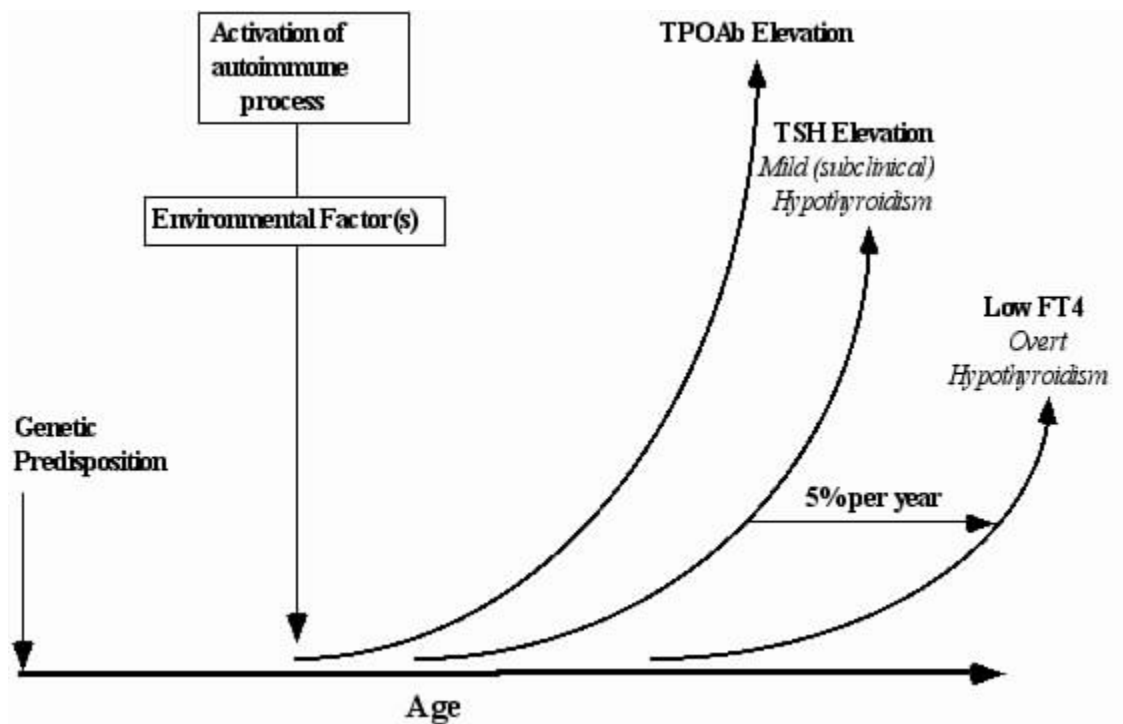


Fig 5. TPOAb Changes with Developing Autoimmune Thyroid Disease

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

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 sufficient areas, it is not usually necessary or cost-effective to order both TPOAb and TgAb, because TPOAb-negative patients with detectable TgAb rarely display thyroid dysfunction.
- ☐ In iodide deficient 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 ALL 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 surrogate 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

<i>Antibody</i>	<i>Function</i>	<i>Detection Method</i>
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
<i>TSAb: Thyroid stimulating antibodies</i> <i>TBAb/TSBAb: TSH receptor blocking antibodies</i> <i>TGI: Thyroid growth stimulating antibodies</i> <i>TSH-R: TSH receptor</i> <i>TBII: Thyroid binding inhibiting immunoglobulins</i>		

(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 ¹²⁵I-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' ophthalmopathy". 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 ($\pm$ 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 ophthalmopathy (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).

ARCHIVED

E. Thyroglobulin (Tg)

Thyroglobulin (Tg), the precursor protein for thyroid hormone synthesis is detectable in the serum of most normal individuals when a sensitive method is used. The serum Tg level integrates three major factors: (i) the mass of differentiated thyroid tissue present; (ii) any inflammation or injury to the thyroid gland which causes the release of Tg; and (iii) the amount of stimulation of the TSH receptor (by TSH, hCG or TRAb). An elevated serum Tg concentration is a non-specific indicator of thyroid dysfunction. Most patients with elevated serum Tg have benign thyroid conditions. The primary use of serum Tg measurements is as a tumor marker for patients carrying a diagnosis of differentiated thyroid cancer (DTC). Approximately two thirds of these patients have an elevated pre-operative serum Tg level that confirms the tumor's ability to secrete Tg, and validates the use of serum Tg measurements as a post-operative tumor marker (307). In contrast, when the pre-operative serum Tg concentration is not elevated above normal, there is no evidence that the tumor is capable of Tg secretion, and the value of an undetectable post-operative serum Tg value is less reassuring. In such patients a detectable post-operative serum Tg could represent a large amount of tumor. In general, changes in serum Tg post-operatively represent changes in tumor mass, provided that a constant TSH level is maintained with L-T4 therapy.

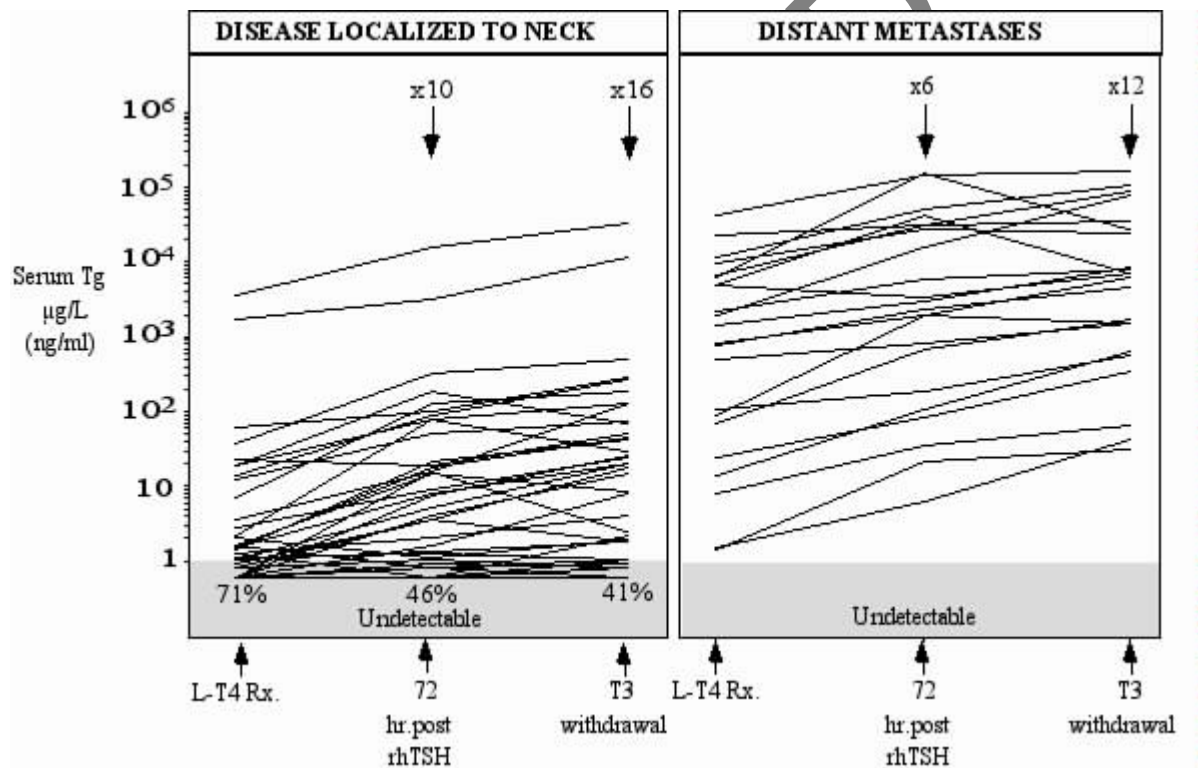


Fig 6. Serum Tg responses after rhTSH administration or T3 withdrawal. Data from ref. 308.

A serum Tg measured during TSH stimulation [endogenous TSH or recombinant human TSH (rhTSH)] is more sensitive for detecting residual or metastatic DTC than a basal Tg measurement made during L-T4 treatment (Figure 6) (308). The magnitude of the serum Tg increase in response to TSH provides a gauge of the TSH sensitivity of the tumor. Well-differentiated tumors typically display a ~10-fold stimulation of serum Tg in response to a high TSH (309). Poorly differentiated tumors that do not concentrate iodide may display a blunted response to TSH stimulation (310).

1. Current Status of Tg Methods

Thyroglobulin is usually measured in serum, but measurements can also be made in thyroid cyst fluids and material obtained by fine needle biopsy of thyroid nodules (311). The measurement of Tg in serum is

technically challenging. Currently, immunometric assays (IMA) are gaining in popularity over radioimmunoassay (RIA) methods. This is because IMA methods offer the practical advantage of a shorter incubation time, an extended dynamic range for the assay and a more stable labeled antibody reagent that is less prone to labeling damage than RIA (312). Laboratories can now choose from a range of both isotopic (immunoradiometric, IRMAs) and nonisotopic, (primarily chemiluminescence, ICMA) IMA methods. However, IMA methods are more prone to interference by thyroglobulin autoantibodies (TgAb), which cause an underestimation of serum Tg levels. This has prompted some laboratories to choose RIA methods for measuring serum Tg in TgAb-positive patients and to restrict the use of IMA methods to TgAb-negative patients only. However, no method can claim to be totally unaffected by TgAb interference that can cause either an over- or underestimation of Tg RIA measurements. Apart from the problems with TgAb interference, current Tg IMA methods are also compromised by differences in standardization and specificity and generally show poor sensitivity, sub-optimal between-run precision and the potential for high dose "hook" effects (312).

(a) Standardization

Serum Tg concentrations measured by either RIA or IMA methods, vary widely (312,313). A recent collaborative effort sponsored by the Community Bureau of Reference of the Commission of the European Communities has developed a new international Tg reference preparation, CRM-457 (298,314). This material can be obtained from Dr. Christos Profilis, BCR, Rue de la Loi 200, B 1049 Brussels, Belgium.

Guideline 42. For Manufacturers Developing Tg Methods

- ☐ The diluent used for standards should ideally be Tg-free/TgAb-free human serum. Non-serum matrices should be selected to produce a signal (radioactive counts, relative light units etc) that is identical to Tg-free/TgAb-free human serum to avoid matrix-related biases.

The bias between different Tg methods may result from differences between the Tg-free matrix used to dilute standards and patient serum, or differences in the epitope recognition by the different Tg antibodies used by individual manufacturers. Ideally, the diluent used for standards should be Tg-free/TgAb-free human serum or alternatively, a non-serum matrix that has been selected to produce a signal (radioactive counts, relative light units etc) that is identical to Tg-free/TgAb-free human serum. It is critical that physicians be informed before the laboratory changes its Tg method to allow for a re-baselining of DTC patients.

The widespread adoption of the CRM-457 standard was projected to reduce, but not eliminate the significant method-to-method variability that exists with this procedure. It was hoped that worldwide standardization would facilitate better agreement in the literature from different studies as well as improve the clinical use of serial Tg monitoring of DTC patients who sometimes have serum Tg measurements determined by different laboratories. Unfortunately, the use of the new CRM-457 standard has not eliminated the problems of between-method variability as much as initially thought. Currently, serum Tg levels determined by methods that use CRM-457 standards can differ by as much as four-fold (Figure 7). These method-to-method differences are greater than the goal for maximum imprecision required for monitoring individual patients (Table 5) and precludes the interchangeable use of different Tg methods for long-term follow-up of thyroid cancer patients.

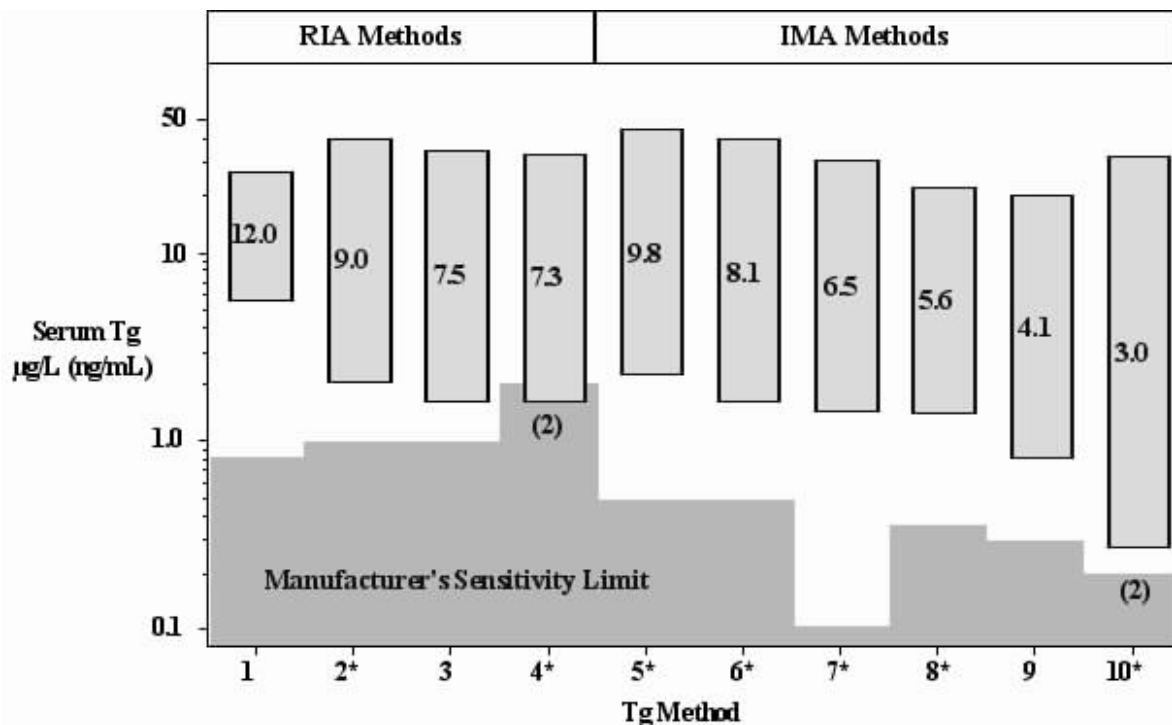


Fig 7. Mean $\pm$ 2sd values for measuring 20 TgAb-negative normal sera by 10 different Tg methods. Method #1= Diagnostic Systems Laboratories, Webster, TX, USA; Method #2=University of Southern California RIA, Los Angeles, CA, USA; RIA #3= Kronus RIA, Boise ID, USA; Method #4= Endocrine Sciences RIA, Calabasas, CA, USA; Method #5= Nichols Institute Diagnostics ICMA, San Juan Capistrano, CA, USA; Method #6= Endocrine Sciences ICMA, Calabasas, CA, USA; Method #7= Sanofi Pasteur IRMA, Marnes-La-Coquette, France; Method #8= Kronus OptiQuant IRMA, Boise ID, USA; Method #9= Brahms DynoTest TgS IRMA, Berlin, Germany; Method #10= Diagnostic Products Immulite ICMA, Los Angeles, CA, USA. An asterisk denotes assays claiming CRM-457 standardization.

Guideline 43. For Laboratories Considering Changing their Tg Method

Select a Tg method on the basis of its performance characteristics not cost or expediency. Before changing the Tg method the laboratory should consult with physician users and compare results between the old and proposed new method using specimens from both TgAb-negative and TgAb-positive patients.

- ☐ **TgAb-negative patients:** If the bias between the old and new method results is $> 10\%$, physicians should be informed and given sufficient time to re-baseline critical patients.
- ☐ **TgAb-positive patients:** The laboratory should warn physicians about the likely direction of interference in the presence of TgAb.
- ☐ If serum Tg values are to be reported for TgAb-positive specimens, an appropriate cautionary comment should be displayed on each laboratory report:

☐ **FOR IMA METHODS:**

IMA methods may give inappropriately low or underestimate serum Tg levels when TgAb is present. Undetectable serum Tg results cannot be used to indicate the absence of tumor in a TgAb-positive patient. A detectable Tg level indicates that Tg is present, but concentrations may be underestimated.

☐ **FOR RIA METHODS:**

RIA methods may give inappropriately higher- or underestimated serum Tg values when TgAb is present (depending on the method). Detectable serum Tg results should not be used as the sole factor for determining the presence of residual thyroid tissue or tumor.

(b) Sensitivity

Some Tg methods are too insensitive to detect the lower euthyroid reference limit that approximates 1-3 µg/L (ng/mL) (depending on the assay). Methods that are unable to detect Tg in normal sera are usually too insensitive for monitoring DTC patients for recurrence. As with TSH, Tg assay functional sensitivity is determined by the 20% CV between-run precision [Section-3 C2]. The protocol used to determine Tg assay functional sensitivity is the same as described for TSH (Guideline 20) with the three stipulations described in Guideline 44.

(c) Precision

Both within-run and between-run precision, expressed as percent coefficient of variation (% CV) are important parameters for validating the performance of a Tg assay. Precision should be established using TgAb-negative serum pools with target Tg values at three different levels (see Guideline 44).

The within-run precision for immunoassay methods is better than between-run precision as would be expected. This is because measurements made within a single run are not subject to the variability introduced by using batches of reagents and different instrument calibrations. Within-run precision may be the more relevant parameter when assessing the serum Tg response to rhTSH stimulation (308). In this setting, a basal and rhTSH-stimulated specimen are drawn 3 to 5 days apart and usually measured in the same run (Figure 6) (308,309). In contrast, when using Tg measurement for serial monitoring, the longer the interval between runs the greater the variability and the worse the between-run precision. Non-human matrices used to determine low-range precision may produce unrealistic functional sensitivity limits compared with measurements made in TgAb-free human serum. It is important to establish functional sensitivity and between-run precision from data spanning a 6 to 12 month period, since this is the typical clinical interval used for monitoring DTC patients.

The suggested goal for maximum imprecision of serum Tg measurements for monitoring patients should be <5% (Table 5). It is unlikely that current Tg assays can maintain such tight precision over the typical 6 to 12 month time-span used for monitoring DTC patients. This precision problem can be overcome by measuring archived, stored samples from the patient in the same run as the current specimen (9).

Guideline 44. Tg Assay Functional Sensitivity & Between –Run Precision

Functional sensitivity and between-run precision should be established using the same protocol as for TSH (Guideline 20) with three important stipulations:

- ☐ Use human serum pools that contain no TgAb, determined by a sensitive TgAb immunoassay.
- ☐ Target values are recommended for low, medium and high pools:
 - Low Pool (used to determine functional sensitivity) should have a serum Tg value that is 30 to 50 % higher than the expected functional sensitivity (FS) limit.
[If FS = 1.0 µg/L (ng/ml) the low pool target should be 1.3 to 1.5 µg/L (ng/ml)]
 - Medium Pool target = ~10 µg/L (ng/ml) i.e. close to the mid-normal range.
 - High Pool target = ~90% of the upper reportable limit suggested by manufacturer.
- ☐ The test period used for assessing between-run precision should be at least 6 months. This is more representative of the clinical interval used for monitoring DTC patients than the 6-8 week interval recommended for TSH in Guideline 20.

(d) High Dose Hook Effect

A high dose hook effect affects primarily IMA methods. Falsely low values due to a “hook effect” are especially problematic for tumor-marker tests like Tg, because it is not unusual to encounter very high values when patients have advanced metastatic disease (307,310,315). A hook effect occurs when an excessive amount of antigen overwhelms the binding capacity of the capture antibody. This results in an inappropriately low signal that translates into an inappropriately low or paradoxically normal range result for a patient with an excessively elevated serum Tg concentration (>1000 µg/L (ng/mL)) (312).

Manufacturers of IMA methods attempt to overcome the hook effect problem by one of two approaches:

- Two-step assay design. The serum specimen is first reacted with the capture antibody before unbound constituents are washed away and the labeled antibody is introduced, followed by a second incubation.
- Two dilutions (usually undiluted and 1/10) are made for each specimen To detect any “hook”.

A “hook” is suspected when the dilution tube has a higher result than the undiluted specimen. Further dilutions are made until the result in the dilution tube decreases and the serum Tg concentrations of the two dilutions are in agreement.

Guideline 45. Testing for “Hook” Effects

- ❑ A two-step design is recommended to minimize hook problems. “One-step” assays that are more prone to hook effects should measure every specimen at two dilutions (undiluted and 1:10) to check for a discrepancy in the two results.
- ❑ All assays (two-step or one-step) should be validated for a hook effect before manufacturer release.
- ❑ To check for a hook effect, measure serial 10-fold dilutions of ~ 20 different TgAb-negative specimens with serum Tg concentrations above 10,000 µg/L (ng/ml) and ~ 20 different TgAb-negative specimens with serum Tg values above 100,000 µg/L until parallelism is demonstrated.

(e) Thyroglobulin Autoantibody (TgAb) Interference

Thyroglobulin autoantibodies (TgAb) are detected in a higher percentage of DTC patients than the general population (~20 versus ~10 %, respectively) (276). Serial serum TgAb measurements may be an independent prognostic indicator of the efficacy of treatment for, or recurrence of, DTC in TgAb-positive patients (276-278,316). Any TgAb present in the specimen has the potential to interfere with any Tg method (317,318). Because TgAb is heterogeneous, neither the measured TgAb concentration nor an exogenous Tg recovery test is 100% reliable for predicting whether the TgAb in a specimen will cause interference (276,317,318)). Probably the most reliable hallmark of TgAb interference is the presence of RIA/IMA discordance. Specifically, Tg measured by RIA is typically higher than Tg measured by IMA if the specimen contains interfering TgAb (276,309). There is now consensus that Tg recovery tests are an unreliable approach for detecting TgAb and should be eliminated (276,318). Early studies that reported low recoveries in the absence of TgAb in some sera were flawed by the insensitivity of early TgAb methods. When a sensitive immunoassay is used, TgAb is always detected when recovery is low.

Non-competitive immunometric assay (IMA) methods appear to be more prone to TgAb interference than RIA methods, as evidenced by the finding of undetectable Tg values in Graves disease subjects (319,318). It appears that IMAs fail to quantify the Tg that is complexed with TgAb in some cases, and this can result in an underestimation of the total Tg concentration. In contrast, RIA methods appear capable of quantifying both the free and TgAb-bound Tg moieties in the specimen, and typically produce higher values than IMA methods when TgAb is present (276,309). The sensitivity and specificity of different TgAb tests is highly variable [Section-3 D6(b)]. It is essential that the TgAb measurement be made by the laboratory performing the Tg testing because that laboratory is responsible for selecting the TgAb method most suited for detecting TgAb interference with the Tg methods it uses.

When serum containing TgAb are measured by both an RIA and IMA method, an RIA:IMA discordance [$\text{Tg RIA} = \geq 2 \text{ } \mu\text{g/L (ng/mL)}$; $\text{Tg IMA} = \text{undetectable}$] is frequently observed. This discordance appears to characterize TgAb interference with one or both classes of method. Since the current threshold for a positive rhTSH-stimulated Tg response is 2 µg/L (ng/mL), this degree of discordance has the potential to influence clinical decision-making (308). Some believe that RIA measurements produce more clinically valid serum Tg results for TgAb-positive patients than IMA measurements, as judged by correlations with clinical status and parallelism with serial TgAb measurements (276,320). However, it should be stressed that no RIA method is immune to TgAb interference in all TgAb-positive sera and the influence of TgAb on different RIA methods is quite variable and relates to the assay components and incubation conditions. Specifically, the quality of the ^{125}I -Tg tracer, together with the specificity of the Tg polyclonal antibody reagent, determines the propensity of

the method for TgAb interference (275,321,322).

Guideline 46. TgAb Interference and Recovery Tests

- ❑ Recovery tests do not reliably detect TgAb and should be discouraged and eliminated. Previous studies have shown that low recoveries are sometimes seen in the absence of TgAb were flawed by the insensitivity of early TgAb methods. When sensitive immunoassays are used, TgAb can always be detected when recovery is low.
- ❑ Discordance between IMA and RIA Tg measurements for TgAb-positive specimens suggests TgAb interference (if values are typically concordant for TgAb-negative specimens).
- ❑ Laboratories should not report undetectable serum Tg values for TgAb-positive patients if the method produces inappropriately low or undetectable serum Tg values for TgAb-positive DTC patients with documented disease.

Although no current Tg method is guaranteed free from interference by TgAb, the underestimation of serum Tg concentrations typical of TgAb interference with IMA methodology is the most serious direction of interference, since underestimation has the potential to mask metastatic disease. It follows that laboratories should not report undetectable serum Tg values for TgAb-positive patients if that method produces inappropriately low or undetectable serum Tg values for TgAb-positive DTC patients with documented disease.

Guideline 47. For Manufacturers and Laboratories

Tg method package inserts should cite realistic performance characteristics for the method (i.e. performance that can be reproduced across a range of clinical laboratories).

- ❑ Assays should be standardized against the CRM-457 reference preparation. Assays not standardized against CRM-457 should provide a correction factor.
- ❑ The mean Tg level and the 2sd limits of the reference range for TgAb-negative normal euthyroid subjects (established using Guideline 48) should be cited in all publications to allow comparison of absolute values.
- ❑ Assays that cannot detect Tg in all normal sera have suboptimal sensitivity for monitoring DTC patients.
- ❑ The matrix used to dilute the standards should be checked for bias (Guideline 42).
- ❑ Functional sensitivity and within and between-run precision should be established using the protocols described in Guideline 44.
- ❑ TgAb interference should be assessed by checking for RIA:IMA discordances in TgAb-positive sera [TgAb levels 100 to >1000 kIU/L (IU/ml)].
- ❑ TgAb immunoassay measurements and not exogenous Tg recovery studies should be used to detect TgAb interference (see Guideline 46).
- ❑ Serum Tg values for TgAb-positive specimens should not be reported if the method gives inappropriately undetectable values in TgAb-positive DTC patients with documented disease.

2. Tg Messenger RNA (mRNA) Testing

The clinical value of Tg mRNA measurements in peripheral blood has yet to be established. Before Tg mRNA testing can be used to facilitate the therapeutic decision-making for DTC, questions regarding the sensitivity and tissue specificity of Tg mRNA in peripheral blood need to be resolved (323-325).

Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of tissue specific mRNA has been used to detect circulating cancer cells in the peripheral blood of patients with melanoma, prostate and breast malignancies (326-328). The availability of Tg-specific primers now allows the application of this technique to the detection of Tg mRNA transcripts in blood. The use of RT-PCR to detect recurrent thyroid cancer was first reported in 1996 (329). Subsequently, the technique has been applied to cervical lymph node metastases and has been found to be more sensitive than the measurement of Tg in the aspirate (330).

A number of groups have now developed quantitative RT-PCR methods to detect Tg mRNA transcripts in

blood (323-325,331-333)). These studies generally find detectable Tg mRNA in all normal subjects but with a poor correlation with serum Tg as measured by immunoassay (331,332). The correlation between Tg mRNA and tumor burden also differs. Some studies have reported that the amount of Tg mRNA correlates with the presence or absence of metastases while others report no such correlation (324,331,333). These discrepancies likely reflect differences in the sensitivity and specificity of the Tg primers and RT-PCR systems used, differences in the sensitivity of the imaging techniques and Tg immunoassays used as well as differences in the TSH status of the patient. Specificity problems (false positives) are a recognized limitation of RT-PCR methodology (328,334). Further studies are needed to determine whether the detectable Tg mRNA levels reported for athyreotic patients without known metastases reflect clinically occult disease, assay artifact or illegitimate transcription.

The correlation between Tg mRNA test results and clinical recurrence, especially in patients with positive Tg mRNA and undetectable serum Tg levels, would need to be shown before the Tg mRNA test becomes widely used in clinical practice. Since the Tg mRNA test is more expensive than a serum Tg measurement, it is likely that if Tg mRNA measurements are shown to be clinically useful, these tests will be reserved for high-risk or TgAb-positive patients in whom serum Tg measurements are diagnostically unreliable.

3. Serum Tg Reference Values

(a) Normal Euthyroid Subjects

Serum Tg concentrations are log-normally distributed in euthyroid individuals. Values tend to be slightly higher in women, but gender-related reference ranges are unnecessary (335). Cigarette smoking is a factor associated with goiter and higher serum Tg values (336). Tg reference ranges are geographically sensitive, since serum Tg is influenced by iodide availability and intake (337,338). Subject selection for the normal cohort for Tg reference range evaluation should have the following exclusion criteria:

- ☐ Goiter
- ☐ Cigarette smoking
- ☐ Personal or family history of thyroid disease
- ☐ Presence of thyroid autoantibodies (TgAb and/or TPOAb)
- ☐ Serum TSH < 0.5 mU/L or >2.0 mU/L

(b) Serum Tg Values following Thyroid Surgery

As indicated by Guideline 48, the Tg reference interval cited on laboratory reports does not apply to patients who have had thyroid surgery! In the first few weeks after surgery, the serum Tg will be determined by the completeness of the surgery, the degree of leakage of Tg from the surgical margins, and most importantly whether thyroid hormone has been given to prevent the expected rise in TSH. In fact, the serum TSH concentration is such a powerful modulator of the serum Tg level that it is usually necessary to know the TSH status of the patient before assessing the significance of any serum Tg measurement.

In the early weeks following thyroidectomy, serum Tg concentrations typically fall with a half-life approximating 2-4 days, when thyroid hormone administration prevents TSH from rising (340,341). In this setting, the relationship between the pre-operative and 6-8 week post-operative serum Tg values can provide information that could influence the treatment plan. During long-term monitoring, serum Tg concentrations measured on and off L-T4 treatment (low or high TSH, respectively) provide different information. The pattern of change in serum Tg values (on L-T4 treatment) is a better indicator of a change in tumor burden than any single serum Tg value (122). The serum Tg concentration during L-T4 treatment is a more stable indicator of tumor mass than a serum Tg measured when the TSH is high (L-T4 withdrawal or rhTSH administration) prior to a radioiodine (RAI) scan. This is because the magnitude of the TSH-stimulated serum Tg elevation is influenced by the extent and chronicity of the TSH elevation, which can vary from scan to scan. However, as shown in Figure 6, because TSH usually stimulates serum Tg more than five-fold, TSH-stimulated serum Tg measurements are more sensitive for detecting disease confined to the neck, than serum Tg levels measured

during TSH suppression (308,309). The magnitude of the TSH-stimulated serum Tg response provides a gauge of the TSH sensitivity of the tumor. Poorly differentiated metastatic tumors that are RAI-scan negative have blunted (less than three-fold) TSH-stimulated serum Tg responses (310).

Guideline 48. Serum Tg Normal Reference Intervals

- Tg reference ranges should be determined locally because serum Tg concentrations are influenced by iodide intake:

Countries with adequate iodide intake: The serum Tg reference interval for a TgAb-negative euthyroid population using CRM-457-standards approximates 3 to 40 µg/L (ng/ml).

Countries manifesting iodide deficiency: The population mean Tg value and the upper Tg reference limit may be elevated relative to the degree of iodide deficiency.

- Laboratories should validate their Tg normal reference interval independent of the manufacturer.
- Tg reference ranges should be established from the log transformed values of 120 normal, non-smoking, euthyroid (TSH 0.5 to 2.0 mIU/L) subjects less than 40 years of age with no personal or family history of thyroid disease and with no evidence of TgAb or TPOAb.
- It is misleading to cite the normal euthyroid reference range when reporting serum Tg values for thyroidectomized DTC patients. Reference values should be related to the euthyroid reference limits for the method, the thyroid mass and TSH status.

For example, the reference ranges below would be appropriate for a Tg method with a euthyroid reference range of 3-40 µg/L (ng/ml):

Tg µg/L (ng/ml)	Condition
3 – 40	Normal thyroid gland reference (TSH 0.4-4.0 mIU/L)
1.5 – 20	Normal thyroid gland reference (TSH <0.1 mIU/L)
< 10	Thyroid lobectomy (TSH < 0.1 mIU/L)
< 2	Near-total thyroidectomy (TSH < 0.1 mIU/L)

4. Clinical Uses of Serum Tg Measurement

The serum Tg concentration reflects thyroid mass, thyroid injury and TSH receptor stimulation (122). It follows that an elevated serum Tg is a non-specific finding associated with virtually any thyroid pathology.

(a) Non-Neoplastic Conditions

Serum Tg is elevated when patients have a goiter or in most hyperthyroid conditions. A low serum Tg concentration can be a useful parameter for confirming the diagnosis of thyrotoxicosis factitia and/or investigating the etiology of congenital hypothyroidism (342,343).

Guideline 49. Serum Tg Measurement for Non-Neoplastic Conditions

Abnormally high serum Tg concentrations result from abnormalities in thyroid mass, excessive thyroidal stimulation, or physical damage to the thyroid secondary to surgery, FNA or thyroiditis.. Serum Tg measurements are useful:

- For diagnosing thyrotoxicosis factitia which is characterized by a non-elevated serum Tg.
- To investigate the etiology of congenital hypothyroidism in infants detected by neonatal screening.
- To assess the activity of inflammatory thyroiditis, eg subacute thyroiditis, or amiodarone-induced thyroiditis.

Serum Tg measurements are also sometimes useful to confirm a past history of thyroiditis, in which the serum Tg concentration is typically the last biochemical parameter to normalize (up to 2 years) (344). Recent studies propose the use of serum Tg measurement as a parameter to reflect the iodide status in a given population

(337,338).

(b) Differentiated Thyroid Carcinomas (DTC)

In the setting of DTC, the serum Tg concentration reflects thyroid mass (tumor or normal remnant), thyroid injury (surgery or FNA) and TSH receptor stimulation (endogenous or rhTSH) (122). Since the TSH level is a major regulator of serum Tg concentrations, it is difficult to interpret serum Tg values without knowing the TSH status of the patient. Although there is no “normal Tg reference range” for treated DTC patients, the normal relationship between thyroid mass and serum Tg provides an important reference point. Specifically, one gram of normal thyroid tissue releases ~1 µg/L (ng/mL) Tg into the circulation when the serum TSH is normal and ~0.5 µg/L (ng/mL) when the serum TSH is suppressed below 0.1 mU/L.

Guideline 50. Serum Tg Measurements for Differentiated Thyroid Carcinoma (DTC)

TgAb-Negative patients:

- ☐ Pre-operative serum values (drawn before or >2 weeks after FNA) are useful for determining the Tg-secretion capacity of the tumor.
- ☐ The acute post-operative decline in serum Tg reflects the completeness of surgery with the serum Tg half-life of 3-4 days. (If thyroid hormone is given to prevent a rise in TSH).
- ☐ There is no “normal range” for a thyroidectomized patient! Completely athyreotic patients should have no Tg detectable in their serum, even if the TSH is elevated.
- ☐ Useful reference point: one gram of normal thyroid tissue releases ~ 1 µg/L (ng/ml) Tg into the serum when TSH is normal, and ~0.5 µg/L (ng/ml) when TSH is suppressed < 0.1 mU/L.
- ☐ When serum Tg is detectable during L-T4 treatment (stable TSH), changes in tumor burden can be monitored by serial serum Tg measurements without thyroid hormone withdrawal or rhTSH.
- ☐ When serum Tg is undetectable during L-T4 treatment (and TgAb is absent) a TSH-stimulated serum Tg is more sensitive for detecting disease localized to the neck than serum Tg measured during TSH suppression.
- ☐ There is typically a >5-fold increase in serum Tg above basal L-T4 Rx. values following TSH stimulation (endogenous or rhTSH). Paired studies show that rhTSH-stimulated Tg responses are approximately half those seen with endogenous TSH following thyroid hormone withdrawal.

TgAb-Positive Patients:

- ☐ Typically display blunted or absent TSH-stimulated serum Tg responses.
- ☐ Serial TgAb measurements (by immunoassay) are valuable as a surrogate tumor marker test

(i) Pre-operative Serum Tg

Some thyroid tumors lack the ability to secrete thyroglobulin. An elevated pre-operative serum Tg level is seen in 2/3 of patients with DTC indicating that their tumors have the capacity for Tg secretion and by inference, post-operative serum Tg monitoring can be used clinically in these patients (307). This information is key to the interpretation of post-operative serum Tg results. If the pre-operative serum Tg level is within normal limits, an undetectable post-operative serum Tg value is less reassuring because it is unclear whether the tumor originally secreted Tg. The sensitivity of post-operative serum Tg monitoring for detecting recurrence will be highest when the tumor is relatively small (≤2cm diameter) and the pre-operative serum Tg value is high. (Note: pre-operative specimens should be drawn before FNA, and held to await the cytologic diagnosis, or can be drawn >2 weeks following FNA.)

(ii) Serum Tg Measurement 1-2 months after Thyroid Surgery

Following thyroid surgery, serum Tg concentrations fall rapidly with a half-life of ~2-4 days (340). Any Tg released from surgical margins should largely resolve within the first two-month period after surgery. During this time TSH will be the dominant influence on the serum Tg level. If thyroid hormone therapy is initiated immediately after surgery to prevent the rise in TSH, the serum Tg concentration will decline to a level that

reflects the size of the normal thyroid remnant plus any residual or metastatic tumor. Since the thyroid remnant left after near-total thyroidectomy typically approximates 2 grams of tissue, a serum Tg concentration $< 2 \mu\text{g/L}$ (ng/mL) is expected when the patient has undergone successful near-total thyroidectomy and has serum TSH maintained below 0.1 mU/L.

(iii) Serum Tg Measurement during Long-term Monitoring on L-T4 Rx.

When the TSH level is stable during L-T4 therapy, any change in the serum Tg level will reflect a change in tumor mass. Clinical recurrence in tumors judged to be “poor Tg secretors” (normal range pre-operative Tg value) may be associated with low or undetectable post-operative serum Tg values. In contrast, recurrence of tumors considered as “good Tg secretors” (elevated pre-operative Tg values) is usually associated with a progressive rise in serum Tg (122). The pattern of serial serum Tg measurements, made when the patient has a stable TSH, is more clinically useful than an isolated Tg value. However, it is possible to interpret the significance of an isolated Tg value by knowing the normal reference range of the Tg assay, the extent of thyroid surgery and the serum TSH level (at steady state), as shown in Figure 8.

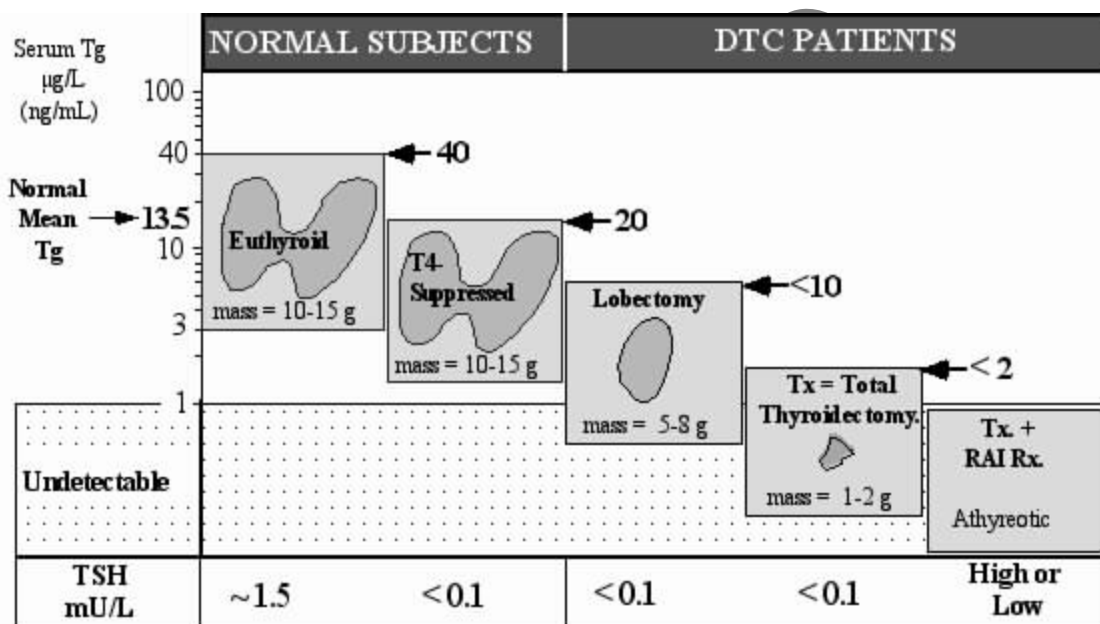


Fig 8. Expected serum Tg values relative to thyroid mass and TSH status. (For methods with a different normal reference range than shown in Figure 8, adjust the absolute values by applying a correction factor based on the mean normal value of the method. (i.e. for methods with a mean normal value of 6.2 $\mu\text{g/L}$ (ng/mL) correct the values shown by 50%).

Assumptions:

- ☐ No recent thyroid injury (surgery or FNA)
- ☐ Using Guideline 48, euthyroid normal control mean Tg = 13.5, range 3-40 (2sd) $\mu\text{g/L}$ (ng/mL)
- ☐ Mass of normal thyroid tissue = 10-15 grams
- ☐ One gram of normal thyroid tissue produces $\sim 1 \mu\text{g/L}$ (ng/mL) Tg in serum @normal TSH
- ☐ One gram of normal thyroid tissue produces $\sim 0.5 \mu\text{g/L}$ (ng/mL) Tg @ TSH < 0.1 mU/L

(iv) Serum Tg Responses to TSH Stimulation

The magnitude of the rise in serum Tg in response to either endogenous TSH (thyroid hormone withdrawal) or recombinant human TSH (rhTSH) administration, provides a gauge of the TSH sensitivity of the tumor (308,309). Typically, TSH stimulation of normal thyroid remnants or a well-differentiated tumor produces a >3 -fold increase in serum Tg above basal (TSH-suppressed) levels, in TgAb-negative patients (Figure 6). The

serum Tg response to an endogenous TSH rise is typically greater than for rhTSH (308,345). Moreover, poorly differentiated tumors, display a blunted (< 3 -fold) increase in serum Tg in response to TSH stimulation (310). It should be noted that TgAb-positive patients typically show a blunted or absent rhTSH-stimulated Tg response by most assays, even when the basal serum Tg concentration is detectable.

ARCHIVED



F. Calcitonin (CT) and RET Proto-oncogene

Medullary carcinoma of the thyroid (MTC) arises from a malignant transformation of the parafollicular C cells of the thyroid and accounts for ~5-8% of all cases of thyroid cancer. Approximately 75% are sporadic in presentation and 25% are hereditary (9,11,347). In a study of thyroid nodules, the prevalence of MTC is reported as 0.57% (348). The behavior and management of MTC differs from that of well-differentiated follicular-derived thyroid carcinomas (346). The inherited forms of MTC come under the heading of multiple endocrine neoplasia (MEN) types 2A and 2B. These are autosomal dominant inherited multiglandular syndromes with age-related penetrance and variable expression. Familial MTC (FMTC) is characterized by the occurrence of MTC without any associated endocrinopathy. In 1993, genetic mutations in the RET proto-oncogene were discovered (349,350). The gene responsible for these diseases is known to be located on the chromosome sub-band 10q11.2. The phenotypic expressions of inherited MEN are summarized in Table 7.

1. Detection of MTC by Measuring Serum Calcitonin (CT)

(a) Calcitonin Biosynthesis

The *CALC-1* gene encoding human CT is located on the tip of the short arm of chromosome 11 (11p15.3-15.5). Although the parafollicular C cells of the thyroid gland are the dominant source of circulating mature CT, several other categories of neuroendocrine cells besides the thyroid normally contain and secrete CT.

Mature CT is a 32-amino-acid polypeptide with a disulfide bridge and a carboxyterminal proline amide that play functionally important roles in mature CT (350). As shown in Figure 9, mature CT results from the post-translational modification of a larger 141 amino-acid precursor (preprocalcitonin) within the parafollicular C cells. Preprocalcitonin first undergoes cleavage of a signal peptide to form procalcitonin (proCT), a prohormone consisting of 116 amino-acid residues. At the proCT amino-terminus there is a 57-amino-acid peptide, called aminoproCT (or PAS-57), and at the carboxyl terminus, there is a 21 amino-acid peptide called calcitonin carboxyterminal peptide-1 (CCP-1 or Katakalein). The immature CT peptide consisting of 33 aminoacids is located centrally within the ProCT molecule. The mature, active, 32 aminoacid CT (which includes an amidated proline at its carboxyterminus) is produced from immature CT by the enzyme peptidylglycine-amidating monooxidase (PAM).

(b) Calcitonin (CT) Methods

Until 1988, CT assay methods were primarily based on radioimmunoassay involving the use of polyclonal antibodies that recognised both the mature CT monomer and other circulating forms (precursors and degradation products). These earlier assays lacked specificity and sensitivity. Since 1988, improvements with new immunometric techniques based on the use of monoclonal antibodies (one of which recognises the N-terminal region and the other the C-terminal region) has allowed for the development of more specific and sensitive assays that detect the mature-32 amino-acid monomer of CT. Currently two-site immunometric assays detect CT in fasting plasma samples in 83% of healthy men and 46% of healthy women (351-353). The CT values produced by different methods can differ, however leading to difficulties in the interpretation of CT results. It is important for physicians to recognize that inter-method differences do exist and can play a role in the proper interpretation and use of CT for the diagnosis and management of MCT.

(c) Basal Calcitonin (CT) Values

Basal CT values were found to be a diagnostically useful marker for MTC in 1968 (354). Currently two-site IMAs, specific to mature CT, typically report CT levels below 10 ng/L (pg/ml) for all normal healthy controls and 90 % of patients with thyroid abnormalities, other than MTC (348,355-357).

Table 7. MEN Disease Phenotypes

PHENOTYPE	CLINICAL FEATURES	
MEN2A (60%)	Medullary thyroid Carcinoma (MTC) Pheochromocytoma Hyperparathyroidism Notalgia	100% 8-60% 5-20% <5%
MEN2B (5%)	MTC Pheochromocytoma Marfanoid Habitus Mucosal neuromas and ganglioneuromatosis of the gut	100% 50% 100% 100%
FMTc (35%)	MTC	100%

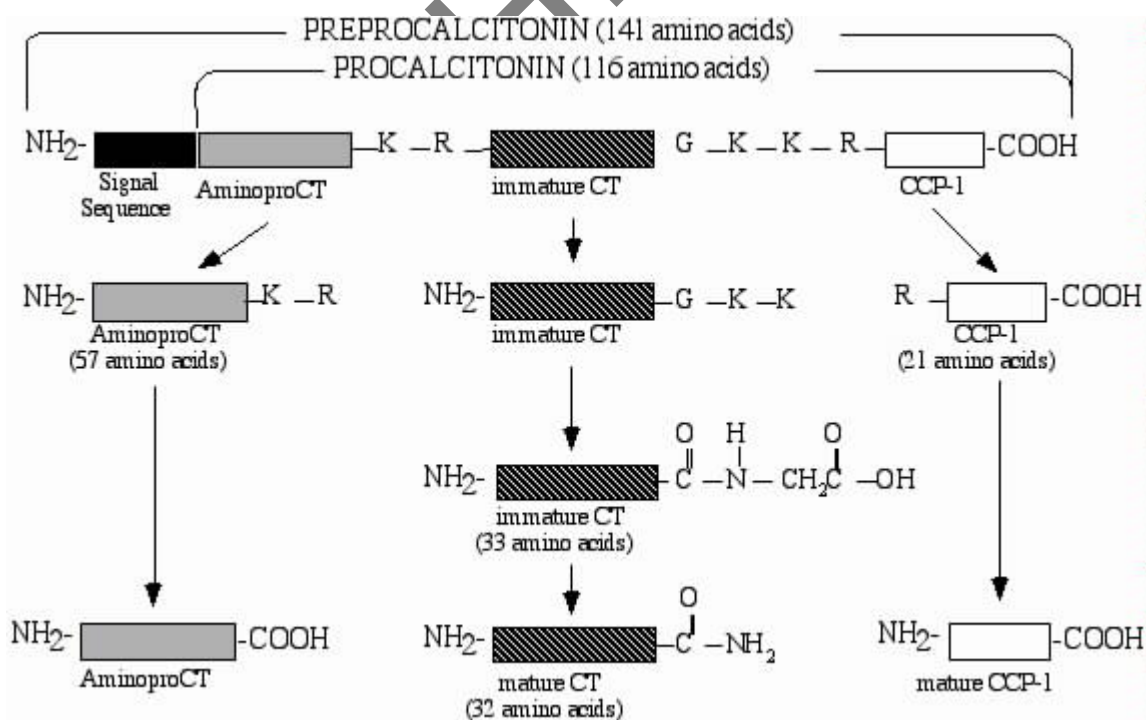


Fig 9. Post Transcriptional Calcitonin Maturation

Guideline 51. Calcitonin (CT) Assays

- ☐ Mature (32 amino acid) CT is the principal tumor marker for MTC.
- ☐ CT measurements used for the diagnosis of MTC and for monitoring purposes should be performed using two-site immunometric assays that are specific for the mature 32 amino acid monomer of CT.
- ☐ Currently, the lower normal threshold for CT is generally accepted as being under 10 pg/ml (ng/L).
- ☐ As new, more sensitive CT kits become available, the lower CT threshold should be redefined.

Patients with micro or macro forms of MTC (sporadic or familial form) have elevated CT levels that correlate with the tumor mass (358). Hyperplasia of the C cells (HCC) is the earliest histologic finding prior to the development of a microcarcinoma, when patients present with MEN2. HCC appears soon after birth, and at this stage in the disease, the basal CT levels can be normal. A normal CT result therefore cannot rule out C-cell pathology at its earliest stages.

(d) Provocative Calcitonin-Stimulation Tests Used for Diagnosing MTC

Provocative stimuli, such as calcium and pentagastrin (Pg) and when Pg is unavailable, omeprazole, have been used to expose C-cell abnormalities, since they induce an increase in the CT level at all stages of MTC (359-364). One advantage of these tests is that they are able to detect HCC before MTC appears in earnest. In countries like the United States where genetic testing is readily available, surgery for gene carriers is based on genetic testing alone and provocative tests are rarely used. In some countries Pg has become difficult to obtain and the majority of surgeries are now performed based on genetic testing alone. Provocative tests are usually employed:

- ☐ To confirm the diagnosis of MTC preoperatively when basal CT levels are only mildly elevated (less than 100 pg/ml).
 - ☐ To detect C-cell disease in *RET*-positive gene carriers
 - ☐ For pre-surgical monitoring of *RET*-positive children
 - ☐ For post-operative monitoring for tumor recurrence
- When genetic testing is not readily available

Guideline 52. Clinical Utility of Serum CT Measurements for Diagnosing MTC

- ☐ Calcitonin (CT) measurements are method-dependent. This can impact the interpretation of CT results.
- ☐ Increased levels of calcitonin in the serum can be seen for patients with autoimmune thyroid diseases (Hashimoto's thyroiditis or Graves' disease).
- ☐ Hyperplasia of the C cells (HCC) is the earliest histological finding prior to the development of a microcarcinoma. A non-elevated CT may be seen with HCC in the earliest stages of developing MTC.
- ☐ A rise in serum CT levels above 10 pg/ml (ng/L) suggests early MTC at the microcarcinoma stage
- ☐ There is a positive correlation between CT levels and tumor mass.

(i) Pentagastrin (Pg) Stimulation Test

The Pg stimulation test has been widely used for the diagnosis of MTC but is not readily available in many countries (359,365). The Pg test consists of an I.V. infusion of Pg (0.5µg/kg/body weight) over 5 seconds. Slow administration of Pg reduces transient side effects (nausea, vomiting, substernal tightness, flushing, and tingling of the extremities) and improves patient tolerance of the test. Blood samples are drawn at baseline and 1, 2, 5 and sometimes 10 minutes after starting the infusion.

The results and interpretation of Pg-stimulated CT values are tabulated in Table 8. The Pg-stimulated peak in CT is typically less than 10 ng/L (pg/ml) in 80% of healthy adult volunteers, and under 30 ng/L (pg/ml) for 95% of the general population. Normal men exhibit higher values than women. A positive test [CT peak response greater than 100 ng/L (pg/ml)] suggests the presence of MTC. When patients have the familial mutation responsible for MEN 2, a peak between 30 and 100 ng/L (pg/ml) is typically seen and suggests HCC or a microcarcinoma. Although a Pg-induced increase in CT of less than 100 ng/L (pg/ml) is known to occur in

adults with thyroid abnormalities other than MTC (see Table 9) no such results have ever been obtained in children under 12 years of age not bearing the *RET* mutation (366). The absence of an increase in CT in a young individual bearing the *RET* mutation does not rule out the possibility that MTC can occur at an older age.

Table 8. Interpretation of the Pentagastrin (Pg) Test

#	CT ng/L (pg/mL)	Interpretation
1	CT Peak < 10	Normal (80% of adults)
3	CT Peak >30 <50	5% of normal adults
4	CT Peak >50 <100	Possible CMT or other thyroid pathologies
5	CT Peak >100	Probable CMT
6	Basal or post Pg CT value > 10 pg/ml	C-cell pathology or residual tissue in MEN 2 patients and MTC after surgery

The best age to test for a C cell pathology in children bearing the *RET* mutation for MEN 2 with the Pg stimulation test has not yet been established. It varies with the type of mutation and the type of MEN 2 present in these families (367,368). Hence, young carriers of the mutation with normal basal levels of CT should have genetic or stimulation testing performed as early as possible post-natally for MEN 2B, and at 2 years of age for MEN 2A. However, it should be stressed that high CT levels are normally found in neonates followed by an age-related decline from birth to about one year of age; no data is yet available on this age-group as far as stimulation testing is concerned (369). This test should be repeated at least once a year until it becomes positive, at which time a total thyroidectomy should be performed. Given the prognosis of MTC, the low tolerance to a Pg test, and the psychological implications for the family, some physicians prefer not to repeat the Pg test until it becomes positive and opt to perform a thyroidectomy on all 4 to 5 year old carriers of the *RET* mutation.

(ii) Calcium Stimulation Test

This test consists of administering 2.5 mg/kg of calcium gluconate intravenously over 30 seconds. Blood samples are drawn at baseline and again at 1, 2 and 5 minutes after the calcium infusion. C-cell hyperplasia is suspected if the plasma CT level rises above 100 ng/L. No important adverse effects have been observed with this test, with the exception of a mild and transient generalized sensation of warmth. Calcium infusions have been reported to be less sensitive than the Pg test for the diagnosis of MTC (370-372). Furthermore, this test has not been evaluated using a CT assay specific for the mature CT monomer, and thus needs to be re-evaluated. It has been reported that calcium infusion combined with Pg test enhances the sensitivity of the Pg test (359).

(e) Basal and Post-Stimulated CT Levels in the Follow-Up of Surgery Patients

After a thyroidectomy, serum CT measurements are the accepted tumor marker for detecting residual thyroid tissue or metastases. A detectable basal or post Pg stimulated CT level constitutes proof that there is some residual tumor tissue present (373,374).

Guideline 53. Postoperative Follow-up of MTC

- ☐ Serum CT and CEA should be measured just prior to, and 6 months after, surgery for MTC. Serum CT levels fall slowly in some patients. The first post-operative CT measurement should not be made until 2 weeks after surgery.
- ☐ The presence of residual tissue or a recurrence of MTC can only be ruled out if both basal and post pentagastrin or calcium-stimulated CT levels are undetectable.

In view of the variations in the rate of disappearance of serum calcitonin, the first post-operative control sample should be taken at least 2 weeks after surgery (375). It should be noted that carcino-embryonic antigen (CEA) is also measured along with CT to detect the recurrence of MTC. In addition, CEA appears to be a useful marker of MTC de-differentiation, and is indicative of a poor prognosis.

(f) Elevated Calcitonin Levels in Conditions other than MTC

As shown in Table 9, elevated calcitonin levels have also been observed in other pathologies besides MTC and neuroendocrine tumors. Increased serum calcitonin release occurs with autoimmune thyroid diseases (Hashimoto's thyroiditis or Graves' disease) (376-378). Non-thyroid conditions where elevated CT has been noted include severe renal insufficiency, hypercalcemia and hypergastrinemia, acute pulmonary inflammatory conditions and other local or general forms of sepsis (Biermer's disease, iatrogenic disorders, etc.) (379-381).

Since, in some cases the elevated CT levels were detected by polyclonal RIA, these reports require confirmation using the current monoclonal antibody based assays that are more specific for mature CT. Studies using specific antiserum raised against ProCT, CT and CCP-1, in conjunction with HPLC and gel filtration, have shown that patients with an elevated calcitonin associated with a non-thyroid condition have markedly increased serum levels of intact ProCT, and to a lesser extent the un-cleaved form, CT-CCP-1. These patients usually have normal or only minimally elevated levels of mature CT. Using epitope-specific antiserum and isolation techniques, it has been shown that tumors other than MTC can secrete large amounts of mature CT and various CT precursors (382). This can be seen with various neuroendocrine tumors, especially small cell lung cancer and bronchial carcinoid. However, only a slight increase in the CT level, if any, is observed after the Pg test when patients with these neuroendocrine tumors are tested (383). C-cell hyperplasia occurs in lymphocytic thyroiditis and some patients with differentiated thyroid cancer (384-386). This HCC may be responsible for a slightly elevated mature CT level and for the increased CT response observed with the Pg test.

Table 9. Conditions with Elevated Calcitonin other than MTC

Neuroendocrine tumors	Lung small cell carcinoma, bronchial and intestinal carcinoid, all neuroendocrine tumors
Benign C-cell Hyperplasia (HCC)	Autoimmune thyroid diseases Differentiated thyroid cancer
Other diseases	Kidney disease Hypergastrinemia Hypercalcemia

2. Detection of MTC by Measuring the *RET* Proto-oncogene

Until 1987, the only method available for detecting subjects at risk for MTC was to perform repeated stimulated CT measurements on family members of MTC patients. The subsequent identification of the locus 10q11.2 responsible for MEN 2 on chromosome 10 then made it possible to detect at-risk subjects by genetic screening (378). It has now been established that several types of mutations on chromosome 10 can activate the proto-oncogene *RET*, that is responsible for MEN 2 (349,350). This now allows physicians to screen for the condition before the first biological signs appear. Currently in many developed countries, genetic studies are the first line approach for this diagnosis. For accurate disease prediction however, it is necessary that positive genetic screening results be followed with an exhaustive survey of both the healthy and affected members of the family.

The *RET* gene is a 21 exon gene that encodes a membrane tyrosine kinase receptor. This membrane-associated receptor is characterized by a cadherin-like region in the extra-cellular domain, a cysteine-rich region immediately external to the membrane and an intracellular tyrosine kinase domain. As shown in Figure 10, the mutations described so far in MEN2 are located in exons 8, 10, 11, 13, 14, 15 and 16 (368,387-391).

(a) Genetic Screening for MEN 2 Diagnosis

MEN2 is an autosomal, dominant familial disease, caused by the activation of missense mutations in the *RET* proto-oncogene (349). Approximately 75% of all MTCs are sporadic and solitary in origin. In 44% of such tumors, a somatic mutation at codon 918 is present (392). Screening must be performed on all collateral family members, ancestors and descendants of the index case, and then all of the descendants of members known to be affected. The screening is based on the identification of a proto-oncogene *RET* genomic mutation using genomic DNA sequence analysis of the index case and on a systematic search for this mutation in all the potentially affected members of the family (Figure 11) (393,394).

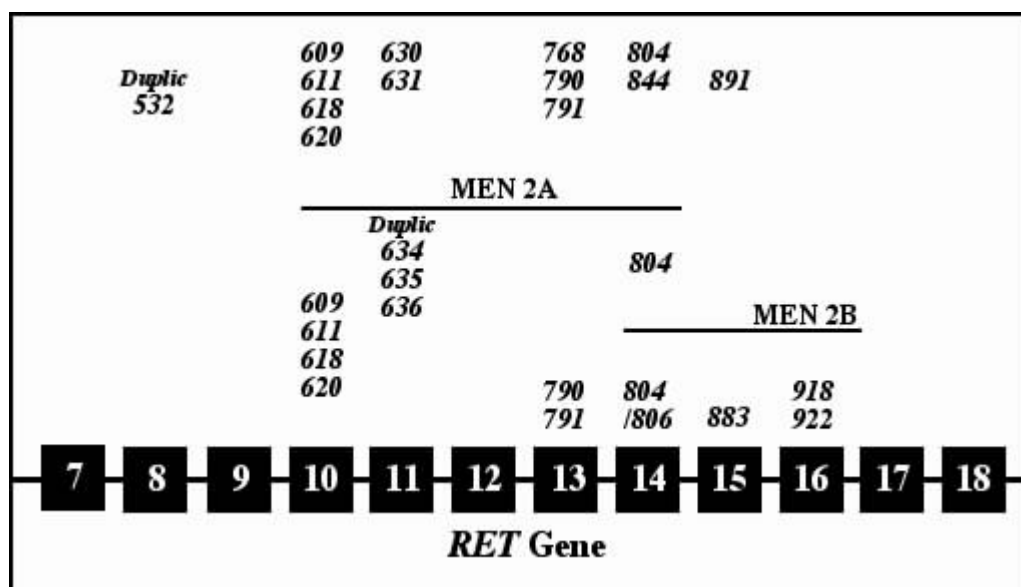


Fig 10. Most common *RET* proto-oncogene mutations

To date, five mutations of the *RET* gene are present in 97% of all cases of MEN2 (Figure 10). Mutations responsible for MEN 2A mainly affect the cysteine-rich extracellular domain, each converting a cysteine into another amino acid. Mutations also occur in the cysteine codons 609, 611, 618 and 620 of exon 10 and cysteine codon 634 (368,378). Familial Medullary Thyroid Carcinoma (FMTC) is most frequently associated with mutations in the cysteine codons in exon 10 as well as codons 768 and 804 in exons 13 and 14 (368). Most (87 %) of the mutations in codon 634 in exon 11 are associated with the multiple organ manifestations of MEN 2A (MTC, pheochromocytoma, and hyperparathyroidism) (9,378).

MEN 2B-associated tumors are caused by mutations in the intracellular TK2 domain. Most (97%) MEN 2B cases involve amino acid 918 in exon 16 with a methionine converted into a threonine. These often occur as new (de novo) germline mutations (395). A minority (5%) of MEN2B mutations affect amino acid 883 in exon 15 or 922 (378,394). A correlation between phenotype and genotype suggests that in FMTC patients with non-cysteine *RET* mutations, the onset of C-cell disease is delayed to later in life compared to patients with classical *RET* mutations in exon 10 (368,396).

Guideline 54. Genetic Risk of MTC

- ☐ In MEN 2 kindred 50% of the family members are potentially affected by the disease.
- ☐ Almost all patients bearing *RET* mutations will develop MTC. (Note: inactivating mutations of the *ret* gene also cause Hirschsprung's disease).
- ☐ 5-10% of sporadic MTC have been found to carry germline *RET* mutations. Therefore *RET* analysis is justified in all patients with apparently sporadic MTC.

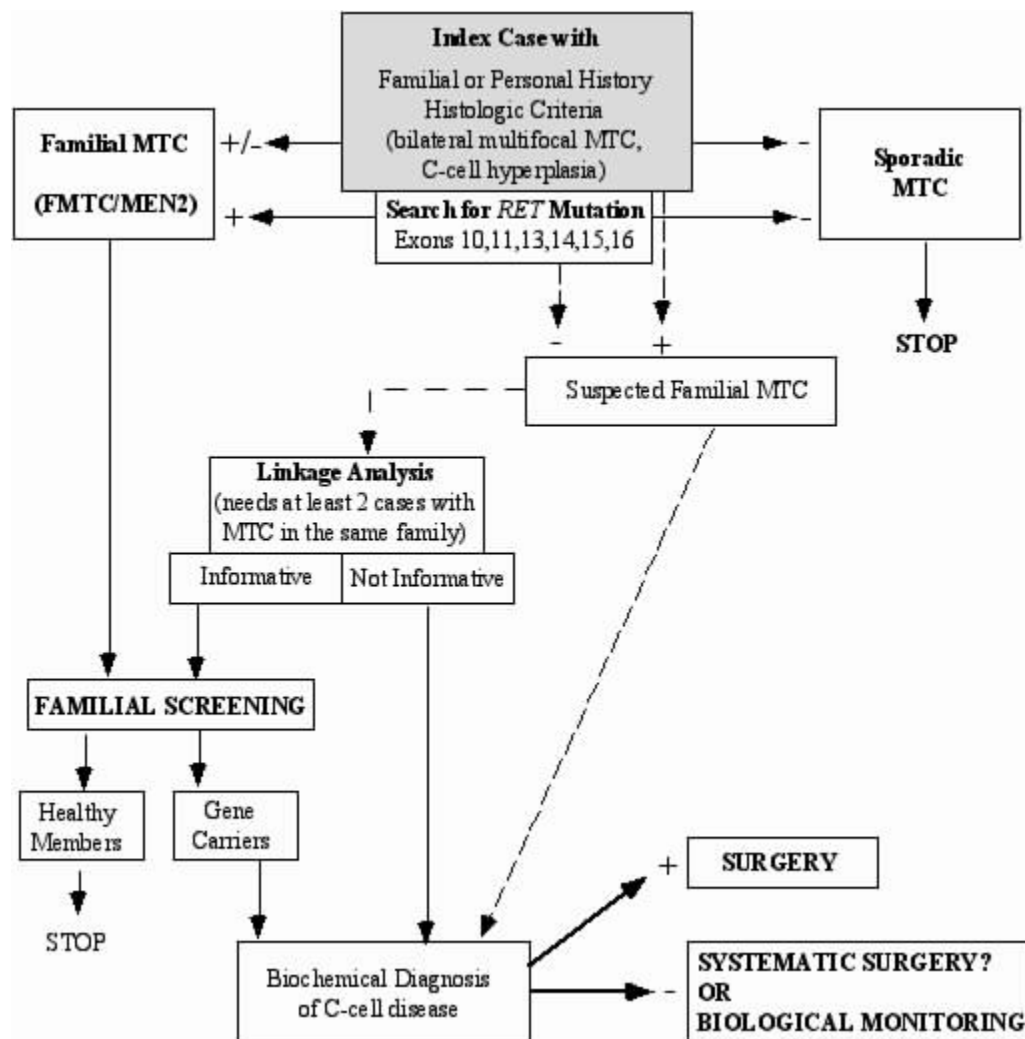


Figure 11. Diagnosis/Treatment Algorithm for MTC

When a mutation has been identified in a family, one can be certain that family members and their descendants not bearing the mutation are free of the pathology. Conversely, the subjects bearing the mutation have the pathology and will require surgical treatment to manage, or prevent the development of disease (Figure 11). If no genomic mutation is identified in the index case, as is the case in less than 3% of MEN 2A and 5% of FMTC, linkage analysis can be used to predict the risk level for the family members. If no predictions of this kind are possible because of the genealogy of the family, the detection of disease will have to be carried out by repeated clinical studies and specific biological tests at appropriate intervals.



G. Urinary Iodine Measurement

An adequate dietary intake of iodine is required for normal thyroid gland hormone production and to maintain a euthyroid state. It follows therefore that the measurement of iodine intake from foodstuffs or medications has clinical relevance. In the clinical laboratory, iodine measurements are used primarily for epidemiological studies or for research (3). To date, the major application of iodine analysis is to assess the dietary iodine intake of a given population (3,397,398). This is an issue of considerable importance, since it has been estimated that iodine deficiency disorders (IDD) potentially affect 2.2 billion people throughout the world. Even in developed countries such as the USA and Australia, a decline in dietary iodine intake has been demonstrated, while borderline dietary intake has long been characteristic in much of Europe (398,399).

As the majority of ingested iodine is excreted in the urine, the measurement of urinary iodine excretion (UI) provides an accurate approximation of dietary iodine intake (399). In most circumstances the determination of UI provides little useful information on the long-term iodine status of an individual, since the results obtained merely reflect recent dietary iodine intake. However, measuring UI in a representative cohort of individuals from a specific population provides a useful index of the iodine level endemic to that region (399,400). Besides estimating the UI concentration in people, other applications of iodine measurements include determining iodine in milk, food products and drinking water (401,402). Iodine measurement in thyroid or breast tissue has been performed as part of research studies (403). Since low inorganic iodine concentrations in serum (~1pg/dL) are associated with relatively abundant hormonal iodine, the measurement of plasma inorganic iodine (PII) has been restricted to research studies in pregnancy (404).

1. Urinary Iodine (UI) excretion

The UI-excretion level from a population study can provide a relatively accurate estimate of the dietary iodine status of that population (399,400). Iodine intake is best determined from a 24-hour urine sample, but logistics make it impractical to use such measurements for epidemiological studies. Differences in the dilution of spot urine specimens can be compensated for by expressing results normalized to urine creatinine as μg of iodine excreted/gram of creatinine (405). The diurnal and seasonal cycles of iodine and creatinine urinary excretion are different. Therefore the ratio of iodine/creatinine can vary during the day or the time of year. In addition, there is no ideal substitute for the accuracy of a 24-hour urine collection, which can be difficult to obtain. However, the UI estimation of iodine intake is most important in developing countries where the iodine/creatinine index may be less satisfactory and where there is a lower creatinine excretion rate secondary to varying degrees of malnourishment (406). It has also been shown that urinary iodine excretion can be variable even in healthy, well-nourished subjects. For these reasons, and to avoid errors introduced in the performance of different creatinine assays, the World Health Organization (WHO) has recommended that for epidemiological studies, the excretion of UI may be expressed as μg of iodine per volume (pg/dL or $\mu\text{g/l}$) of urine. Differences in urine dilution states inherent in obtaining a spot urine sample can be partially compensated for by using a large number (~50) of subjects in each study population. Recent reports suggest that the use of age and sex adjusted (UI/Cr) ratios in a fasting morning specimen comes close to the true (24 hour) iodine excretion if nutrition is generally adequate (400,407). Although seasonal variations may not be as important in warmer climates, they do affect the results in Northern Europe where dairy milk is a major source of dietary iodine. In such populations, the practice of indoor feeding of cattle with mineral rich supplements results in higher UI excretion during the winter months. More recently it has been suggested that UI has a diurnal variation, with values reaching a median in early morning or 8-12 hours after the last meal suggesting that samples should be collected at these times (408).

2. Dietary Iodine

In many countries, adequate dietary iodine intake is achieved through the iodization of salt but the availability of iodized salt is only mandatory in some developed countries and voluntary in many others. There is also evidence of a decline in iodine consumption in some industrial countries (399). Diminished iodine intake can occur with vegetarian diets, particularly in areas where the fruits and vegetables are grown in iodine deficient soil (409).

3. Units of UI Measurement

For epidemiological studies, iodine excretion is normally expressed as μg of iodine excreted. Conversion to equivalent SI units:

- $\mu\text{g/dL} = 0.07874 \mu\text{M/L}$
- $1.0 \text{ pM/L} = 12.7 \text{ pg/dL}$.

4. Applications of Iodine Measurement

(a) Epidemiologic Surveys

The major application of iodine measurements is for epidemiological surveys. The recommended daily iodine intake is: - 90 $\mu\text{g/day}$ for children, 150 $\mu\text{g/day}$ for adults and 200 $\mu\text{g/day}$ for pregnant or lactating mothers. The suggested norms for UI excretion as an index of the severity of iodine deficiency are shown in Table 10 (398).

Table 10. Urinary Iodine Excretion and Iodine Deficiency

*Iodine Deficiency	None	Mild	Moderate	Severe
UI $\mu\text{g/L}$	>100	50-99	20-49	<20
Goiter prevalence	<5%	5.0-19.9%	2~29.9%	>30%
*IDD Newsletter Aug 1999 15: 33-48				

(b) Pregnancy and the Neonate

Fortunately, the occurrence of severe iodine deficiency that leads to endemic cretinism has been reduced as a result of dietary iodine supplementation programs. However, iodine deficiency still persists in large areas of the globe. The situation where dietary iodine deficiency may have more serious consequences is in the pregnant woman, where maternal iodine deficiency can compromise the thyroid status of the fetus and newborn child (2,410). Reports on the variation in UI excretion during pregnancy differ. Some studies have reported a decline or no change, while others have shown an increase (47,411-413). These differences may reflect variations in the dietary iodine supply (414). However, the use of urinary iodine to estimate iodine sufficiency during pregnancy can be misleading, since pregnancy causes an increase in the iodine excretion rate. This results in a relative increase in the urinary iodine concentration, thus giving a false sense of adequacy of iodine nutrition (415). It has been shown when dietary iodine intake is inadequate during pregnancy, that there is evidence of thyroidal stress, an increase in both thyroid volume and serum Tg and a relative decrease in FT4 (47). Administration of iodine to pregnant mothers results in increased UI excretion and a reversal of the observed iodine deficient thyroidal changes. The importance in avoiding any compromise in thyroid function during pregnancy was recently emphasized by the report that children of even mildly hypothyroid mothers can develop defects in neuropsychological development (64,65). This finding is consistent with earlier reports that plasma inorganic iodine (PII) declines during pregnancy. Early methods of measuring (PII) were based on the administration of a tracer dose of ^{131}I to patients and measuring the specific activity of the radioisotope in serum and urine (405). Other methods depended on the ratio of iodine to creatinine in serum and urine (405,416). A recent study using perchlorate digestion and the formula $\text{PII} = \text{Total Serum Iodine} - \text{Protein Bound Iodine}$ concluded that, at least in iodine sufficient areas, there was no trend for PII values to be depressed during pregnancy (404).

(c) Excessive Iodine Intake

It is well known that excessive iodine intake may, in susceptible individuals, lead to the inhibition of thyroid hormone synthesis (the Wolff Chaikhoff effect) and can be of iatrogenic origin (417,418). A similar excess of iodine intake by previously iodine-deficient individuals with thyroid autonomy may result in hyperthyroidism (the Jod Basedow effect) (398,420). Population based dietary iodine intake programs can influence the form of

thyroid disease that occurs. This is particularly true for hyperthyroidism, with toxic nodular goiter being more prominent when iodine intake is low, and Graves' disease more prominent when iodine intake is high. However, it has been shown that a program of controlled dietary iodine intake can, after a transient increase in hyperthyroidism in the first year, cause a decrease in both toxic nodular goiter and Graves Disease if followed over a period of time (421). Differences in disease presentation can also alter the epidemiological profile of thyroid cancer with a relative increase in papillary thyroid carcinoma together with an improved prognosis when the iodine supply is increased (422).

Fear of the side effects of excess iodine has impeded the introduction of programs of iodine prophylaxis or even the possibility of administering iodine following accidental release of radioactive iodine. There is however, general agreement that the benefits of iodine administration far exceed the risks from excessive iodine exposure (398). Thus the requirement for iodine measurements in the assessment of iodine excess states may exceed that for iodine deficiency. Iodine excess can result from the use of iodine rich medications such as the commonly prescribed cardiac antiarrhythmic drug amiodarone or antiseptics containing iodine (Guideline 5) (75,418,419,421,423). The thyroidal consequences of amiodarone therapy may depend on the underlying dietary iodine status of the area where the patient resides. Hypothyroidism is more frequent where dietary iodine intake is high, such as in the USA, and hyperthyroidism is more frequent where the intake is low, such as in parts of Europe (424).

Excess dietary iodine intake has also been implicated in the increased prevalence of autoimmune thyroiditis or increase in thyroglobulin antibody positivity following iodine prophylaxis. This may be due to increased antigenicity of more highly iodinated forms of thyroglobulin (425,426). The assessment of iodine excess is usually made with a 24hr urine collection. It should be understood that organic iodine present in radiological contrast material can be taken up into body fat. The slow release of iodine from body fat stores has been associated with a high UI excretion rate that can persist for several months following the administration of this contrast material. (427).

5. Iodine Methodology

Methods that measure iodine content in biological specimens have traditionally relied on the conversion of organic iodinated compounds to inorganic iodine and the removal of potential interfering substances (eg. thiocyanate) that can interfere with the colorimetric measurement of the inorganic iodine (428). The procedure involves a preliminary digestion step followed by the colorimetric estimation of iodine through its catalytic action in the Sandell-Kolthoff (SK) reaction. In this reaction, Ce^{4+} (ceric ions) are reduced to Ce^{3+} (cerous ions) in the presence of As^{3+} (arsenious ions) that are then oxidized to As^{5+} (arsenic ions) producing a change in color from yellow to colorless. Following a short incubation period, this color change can be determined colorimetrically. As this reaction is time dependent, some reports suggest stopping the reaction with the addition of ferrous ammonium sulfate and performing the colorimetric readings at a later time. Further modifications of the SK reaction can produce a kinetic assay by altering the ratio of Ce/As ions. This kinetic method approach can increase the sensitivity of the assay (429). The problems associated with the removal of interfering substances such as thiocyanate in the SK reaction have been previously mentioned, and a report comparing 6 methods for iodine analysis attributed much of these interferences with the SK reaction to inadequate digestion procedures (428). Two major methods of sample digestion, dry ashing and wet ashing are routinely employed.

(a) Dry Ashing

The dry-ashing technique was first introduced in 1944 and subsequently modified. The method involves the preliminary drying of specimens in an oven at 100°C. The dried residue is then incinerated in the presence of strong alkali (KOH/K_2CO_3) for approximately 3 hours at 600°C. The ash is then reconstituted in distilled H_2O and the iodine content measured colorimetrically as described above. This is a somewhat time consuming and expensive method requiring thick-walled pyrex test tubes to withstand the high temperatures and a muffle furnace, ideally equipped with microchip temperature control. However, it does yield excellent results not only in urine samples but is also suitable for measuring the iodine content of foodstuffs and tissue samples that

require complete digestion. Strict temperature control is particularly useful in preventing iodine loss should the temperature drift above 600°C or if the time of incineration is extended (429,430). It is also important that the iodine standards be subjected to incineration, as the added KOH is known to reduce the sensitivity of the SK reaction based assay. These methods were developed for the determination of protein bound iodine (PBI) used to measure thyroid hormones before the advent of specific radioimmunoassays for T4 and T3. As samples are incinerated together in a muffle furnace, the dry-ashing procedure is particularly susceptible to cross-contamination by a high iodine-containing specimen. To overcome this possibility some investigators have suggested prior screening of samples to detect such specimens. The problem of cross-contamination is particularly problematical with the dry-ashing procedure but has the potential to affect all iodine quantitation methods. It is therefore desirable that the iodine measurement area be isolated and kept as far away from other laboratory activities, particularly those that might involve use of iodine-containing reagents. The aesthetics of handling and volatilizing large volumes of urine for epidemiological studies also makes the isolated laboratory desirable.

(b) Wet Ashing

The most widely used method of digestion is the wet-ashing technique first proposed in 1951, although this approach is controversial. In this method the urine specimens are digested using perchloric acid. This method has been automated. While the autoanalyser method has found widespread use, it does depend on the use of acid digestion and a dialysis module. The latter has been shown to be prone to significant interference by substances such as thiocyanate (428). Several variations of the wet ashing method for iodine measurements have been developed. These are primarily aimed at simplifying the methods, reducing the labor cost and rendering the method more suitable for on site epidemiological studies. Various methods have been described which yield similar results to established methods (431). In one such method, the authors indicate that a single technician can perform 150 tests per day at a cost of less than \$0.50 each (431). More recently, even simpler methods using either acid digestion or UV irradiation of samples have been described (432). The wet-ashing technique has drawbacks in that perchloric acid and potassium chlorate are potentially explosive and their use requires the use of a dedicated and expensive fume hood. For this reason a less hazardous method of digesting urine samples using ammonium persulfate as the oxidizing agent was proposed. However, the use of ammonium persulfate was shown not to be a very efficient means for mineralizing iodinated compounds such as T3, T4, amiodarone etc. A further modification involving the incorporation of the digestion and reaction process into a microplate technology has been reported (433). More recently an assay was developed in kit form that allows for a more rapid quantitative measurement of UI after charcoal purification. (Urojod, Merck KGaA, Darmstadt, Germany). This method appears simple to perform and has the potential to be used in the field for epidemiological studies or for occasional use in the assessment of excess iodine ingestion (434).

(c) Sensitivity and Specificity of Iodine Methods

Assays using the SK reaction yield sensitivities between 10 and 40 mg/L that is more than adequate for UI measurement. Greater sensitivity has been reported using the kinetic assay (0.01pg/L) (429). Reported sensitivities using inductively coupled plasma mass spectrometry (ICP-MS) technique is in the area of 2µg/L (413,434). Providing the initial digestion is complete, the SK assay is very specific for iodine. However incomplete digestion can lead to interference by substances such as iodine-containing medications, thiocyanates, ascorbic acid or heavy metals such as Hg or Ag (429). In expert hands the SK reaction yields excellent intra- and inter-assay precision with CV's < 5% routinely achieved. This is provided the digestion is adequately controlled so that the recovery of the iodine standard is 90 to 100% (429,430,432).

(d) Non Incineration Assays

In addition to methods based on alkaline and acid digestion, other published methods for iodine determination include the use of bromine in acid conditions as a digesting agent or the use of ultraviolet radiation (430,435). Iodine selective electrodes and mass spectrometry have been used to measure iodine in various fluids including urine (436,437). In this case the iodine activity that is measured approximates the iodine concentration. A drawback to this method is that the electrodes become coated and require frequent polishing and other ions such as sulphite interfere. This approach is therefore not ideally suited for measurements in urine but can be used to

measure iodine in other fluids and extracts of foodstuffs. Although not suitable for routine UI measurement, the technique can be applied to the assessment of iodine overload in urine in patients treated with amiodarone or other iodine rich compounds (437). As the electrode only responds to iodine and not to iodinated compounds, it can be a useful means of specifically measuring iodine in the presence of other iodinated compounds. Many other techniques that are clearly unsuitable for routine clinical use include nuclear activation analysis, or HPLC. One method that has been widely reported is the use of (ICP-MS) (432,438). This method has been shown to have good agreement with conventional digestion techniques using SK quantitation (432,433). However, the required equipment is expensive and not readily available. Isotope dilution analysis has been applied to the analysis of both urine and drinking water (402). In vivo measurement of intrathyroidal iodine content has been achieved using X-ray fluorescence that can have relevance to the assessment of patients with amiodarone induced hyperthyroidism (419).

Guideline 55. Urinary Iodine Measurement

- The Technicon Autoanalyser is generally no longer commercially available, with the result that laboratories seeking to commence iodide measurement will need to develop manual in-house methods.
- Mass spectrometry is a simple and reproducible method which can be recommended if such equipment is already on site.
- Many simplified digestion methods incorporating SK colorimetry have been described.
- Wet-ashing reagents perchloric acid and potassium chlorate are potentially explosive and their use requires availability of an expensive fume hood. A less hazardous system using ammonium persulfate may be preferable
- Measurement of iodide in samples other than urine (eg tissues, foodstuffs) may still require the more conventional dry or wet-ashing techniques.
- Inter and intra assay CV should be < 10% and recovery of added iodide should be between 90 and 100%.
- In industrialized countries, clinical laboratories are most frequently requested to perform urinary iodide measurements to investigate iodide overload. One of the simplified methods outlined above, or a semi-quantitative kit is the method of choice.
- To facilitate uniformity in concentration units used to report urinary iodide excretion, UI should be expressed as µg Iodide /L of urine (µg/L).

6. Summary

Measurement of iodine in tissues and biological fluids is unlikely to play a key role in routine clinical biochemistry laboratories in the immediate future. However in view of the large number of individuals with IDD worldwide (2.2 billion affected) and recent reports that dietary iodine intake is declining in the United States and Australia, the assessment of UI as part of epidemiological studies will continue to be of considerable interest and importance. Reference laboratories will no doubt continue to use dry- or wet-ashing techniques, depending on availability of equipment and space. Recent recommendations that laboratories " have several different methods available to allow the user to select the one best suited to specific needs" would seem a prudent course for centers specializing in iodine measurements.



H. Thyroid Fine Needle Aspiration (FNA) and Cytology

The prevalence of palpable thyroid nodules in adults increases with age (average 4 -7% for the United States population) with thyroid nodules being more common in women than men (439-441). In adults, 95% of these nodules are benign. In contrast, although rare (0.22% to 1.8%) patients with thyroid nodules who are under 21 years of age have a higher incidence of malignancy (33% versus 5%, children versus adults, respectively) (442-445). The methods currently used for assessing thyroid nodules include, fine needle aspiration (FNA), thyroid scan and ultrasound. Practice guidelines suggest that an initial FNA is more diagnostically useful and cost effective than other forms of investigation (446). Despite such guidelines, a recent United States study reported that in 1996, FNA was only used as the initial procedure in 53% of thyroid nodule cases (447). Despite the fact that isotopically “cold” thyroid nodules are considered suspicious for carcinoma, most benign thyroid nodules (cysts, colloid nodules, benign follicular lesions, hyperplastic nodules and nodules of Hashimoto’s thyroiditis) also present as “cold” nodules. In addition, “warm” or iso-functioning nodules that do not result in a completely suppressed TSH and thus surrounding normal thyroid tissue is not suppressed, can be malignant. Logistic regression analysis indicates that adequate cytologic material significantly increases with the size of the nodule (448). Although ultrasound can be used to detect non-palpable nodules, ultrasound cannot differentiate between benign and malignant lesions. In general, ultrasound is typically used for evaluating complex cystic masses and nodules that are difficult to palpate (449). Ultrasound is also used to determine the size of nodules and to monitor nodule growth, as well as verify the presence of non-palpable nodules that have been incidentally detected by other imaging procedures. Ultrasound-guided FNA should be used for hypoechoic nodules and when aspiration cytology fails to yield adequate cellular material (450,451).

1. Indications for FNA

All solitary or dominant nodules ≥ 1 cm in diameter should be evaluated by FNA. FNA is preferred to thyroid scanning or ultrasonography as the initial diagnostic test for evaluating patients with thyroid nodules (452). Since FNA became popular in the 1970s, the number of thyroid surgical procedures has decreased by 50% whereas the percent yield of cancers for patients undergoing surgery for thyroid nodules has increased from 10-15% to 20-50% (453). The frequency of false negative FNA reports is related to the skill of the operator and the experience of the cytopathologist (454). False negative rates appear to be less than 2 % (455).

Guideline 56. Use of Fine Needle Aspiration (FNA) of the Thyroid

- FNA is recommended for all palpable solitary or dominant nodules, independent of size.
- FNA is preferred over thyroid scan or ultrasonography as the initial diagnostic test for thyroid nodules. However, a previous ultrasound may aid the physician performing the aspiration.
- When TSH is suppressed or the patient is thyrotoxic, a nuclear scan maybe indicated before FNA. However, the result of the scan should not exclude the necessity for FNA.
- “Hot” nodules detected by nuclear scan are less likely to be malignant than “cold” nodules.

2. Factors Suggesting a Higher Risk for Thyroid Cancer

A number of factors are associated with an increased risk for thyroid carcinoma (456-458). These are:

- Age, < 20 or > 40 years
- Nodule size > 2cm diameter
- Regional adenopathy
- Presence of distant metastases
- Prior head or neck irradiation
- Rapidly growing lesion
- Development of hoarseness, progressive dysphagia, or shortness of breath
- Family history of papillary thyroid cancer
- Family history of medullary cancer or MEN Type 2

Some of these risk factors are included in tumor risk-assessment protocols. The TNM classification protocol (tumor size, presence of lymph nodes, distant metastases) and age is the general tumor risk assessment algorithm. A number of thyroid-specific staging protocols have been developed (12). These protocols are used to provide objective information necessary for establishing an appropriate treatment plan for the projected outcome. Although the TNM classification protocol is in general use, it can be misleading when applied to thyroid tumors. Specifically, with non-thyroid cancers, the presence of lymph node metastases is a heavily weighted factor that negatively impacts on mortality. In contrast, differentiated thyroid cancers often arise in young patients in whom the presence of lymph node metastases may or may not have a minimal effect on mortality, but increase the risk of recurrence.

Guideline 57. For Physicians

- It is important that the endocrinologist, surgeon, nuclear medicine physician and cytopathologist act in concert to integrate the staging information into a long-term treatment plan and thereby ensure continuity of care.
- Preferably, the physicians responsible for the long-term management of the patient should review the slides with the cytopathologist and understand the cytopathologic interpretation to establish meaningful treatment strategies for the patient.

3. Factors Suggesting a Lower Risk for Thyroid Cancer

FNA may be deferred in low-risk patients with the following characteristics:

- Autonomous “hot” nodules (serum TSH < 0.1 mIU/L).
- Incidental nodules < 1 cm, detected by ultrasound.
- Pregnant patients presenting with a solitary nodule. FNA of nodules detected during pregnancy can be deferred until after delivery without increasing the risk of morbidity from DTC (459). If it is necessary to surgically remove a nodule during pregnancy, surgery during the 2nd trimester minimizes the risk to the fetus.
- Multinodular thyroid glands with nodules < 1 cm.
- Fluctuating or soft nodules.
- Hashimoto’s thyroiditis. Indications include firm, “rubbery” gland on physical examination without dominant nodules and an associated elevation in TPOAb.

4. Follow-up of Patients with Deferred FNA

The follow-up frequency (i.e. every 6 to 24 months) should be appropriate for the degree of diagnostic certainty that the nodule is benign. The efficacy of L-T4 therapy to suppress TSH can be variable. The goal of follow-up is to identify patients with undiagnosed or subsequent malignancy and to specifically recognize any progressive enlargement that could result in local compressive complications and cosmetic concerns by monitoring nodule size preferably with ultrasound. If ultrasound is not available, a careful physical examination should be made. This may be accomplished by:

- Placing a tape over the nodule and outlining the borders with a pen, then pasting the tape into the patient’s chart.
- Using a ruler to record the nodule diameter in two dimensions
- Palpating for enlarged adjacent lymph nodes
- Diagnosing any associated clinical or mild (subclinical) thyroid dysfunction by periodic serum TSH and TPOAb measurements.
- Evaluating patients for signs of undiagnosed or subsequent malignancy such as:
 - progressive nodule or goiter enlargement
 - rising serum Tg level.
 - local compression and invasive symptoms (i.e. dysphagia, dyspnea, cough, pain)

- hoarseness
- tracheal deviation
- regional lymphadenopathy

5. Guidelines for Who Should Perform FNA

Experience with aspiration cytology is essential. If the cytologist or ultrasonographer performs the FNA, there must be an exchange of appropriate information with the clinician (460). Physicians performing FNA should be able to request a review of the slides with the cytopathologist and understand the cytology results in order to recommend appropriate therapy based on the tissue diagnosis. Ideally, the physician performing the FNA should also be the physician responsible for the long-term management of the patient in order to assure continuity of care.

Guideline 58. Selection of Physicians to Perform FNA

Thyroid gland aspirations should be performed by physicians who:

- ☐ Are skilled in the technique and perform thyroid aspirations frequently.
- ☐ Can understand the interpretation of the cytology results.
- ☐ Are able to recommend appropriate therapy depending on the results of the aspiration.

6. Technical Aspects of Performing FNA

It is recommended that aspirin or other agents that affect coagulation be discontinued for several days before the procedure. FNA is typically performed using 22 to 25 gauge needles and 10 or 20 ml syringes that may, or may not be attached to a “pistol-grip” device. Aspiration should be as minimally traumatic as possible. Some physicians favor administering topical local anesthetic (1% lidocaine) while others do not. It is recommended that a minimum of two passes be made into various portions of the nodule to decrease sampling error. Slides are typically fixed in Papanicolaou’s fixative and stained. It is imperative to fix immediately and avoid drying and drying artifacts to preserve nuclear detail. It is also useful to use a rapid stain, such as Diff-Quik and examine the slides at the time of aspiration to assess adequacy of specimen for cytologic evaluation. Other slides may be air-dried for alcohol fixation and subsequent staining (excellent for detecting colloid). Any additional material can be combined with material rinsed from the needle and spun down to form a cell block which can then be embedded in agar. Cell-blocks can provide histologic information and be used for special staining studies. It is important to adequately protect the slides for transport to the laboratory. Slides should be submitted to the cytopathologist with clinical details together with the nodule size, location and consistency.

Firm nodules are usually suspicious for carcinoma whereas fluctuant or soft nodules suggest a benign process. When cyst fluid is aspirated the volume, color and presence of blood should be recorded together with a record of any residual mass left after aspiration. If there is a residual mass after cyst aspiration it should be re-aspirated. Clear, colorless fluid suggests a parathyroid cyst, whereas yellow fluid is more typical of a cyst of thyroid follicular origin. After aspiration, local pressure should be applied to the site of the aspiration for 10-15 minutes to minimize the likelihood of swelling. The patient can be discharged with a small bandage over the aspiration site with instructions to apply ice should discomfort occur later.

Often the FNA cytology information can be augmented by submitting the material for flow cytometry or immunoperoxidase staining [Section-3 H8]. Any thyroid tissue in a lateral neck node is thyroid cancer (99%) unless proven otherwise!

7. Cytologic Evaluation

If a cytopathologist experienced with the thyroid is not available locally, it may be essential that the slides be sent to an outside expert for review. In the future, electronic review of cytopathology specimens will become increasingly available as tele-cytopathology technology develops.

Guideline 59. Selection of the Cytopathologist

- ☐ The cytopathologist should have an interest and experience in reading thyroid cytology. If an experienced cytopathologist is not available locally, the slides should be sent for review by a cytopathologist with thyroid expertise outside the institution.
- Cytopathologists should be willing to review the slides with the patient's physician on request.

8. Special Tissue Stains

Special tissue stains can be helpful in the following situations:

- ☐ When there is a mass of questionable malignancy or thyroid origin - Use specific antibody stains for Tg, TPO (MoAb 47) Galectin-3 and CEA (461-466).
- ☐ For questionable lymphoma, use B-cell immunotyping
- ☐ Undifferentiated/anaplastic thyroid cancer - stains for vimentin, P53, keratin
- ☐ Questionable medullary thyroid cancer - stains for calcitonin, neuron-specific enolase, chromogranin and/or somatostatin.

9. Diagnostic Categories

Some cytopathologists believe that there must be at least six clusters of follicular cells of 10 to 20 cells each on two different slides in order to accurately report a thyroid lesion as benign (466-468). A cytologic diagnosis of malignancy can be made from fewer cells, provided that the characteristic cytologic features of malignancy are present.

Guideline 60. Cytopathologic Characteristics

Thyroid cytology interpretation can be difficult and challenging. The amount of tissue contained on the slides may depend on the method of aspiration (ultrasound versus manual).

The evaluation should assess:

- The presence or absence of follicles (microfollicles versus variable-sized follicles)
- Cell size (uniform versus variable)
- Staining characteristics of the cells
- Tissue polarity (cell block only)
- Presence of nuclear grooves and/or nuclear clearing
- Presence of nucleoli
- Presence and type of colloid (watery and free versus thick and viscous)
- Monotonous population of either Follicular or Hurthle cells
- Presence of lymphocytes

(a) Benign Lesions (~ 70% of cases)

Clinical presentations that suggest a benign condition (but not necessarily exclude FNA)

- Sudden onset of pain or tenderness suggests hemorrhage into a benign adenoma or cyst, or subacute granulomatous thyroiditis, respectively. (However, hemorrhage into a cancer can also present with sudden pain).
- Symptoms suggesting hyperthyroidism or autoimmune thyroiditis (Hashimoto's).
- Family history of benign nodular disease, Hashimoto's thyroiditis or other autoimmune disease.

- Smooth, soft easily mobile nodule.
- Multi-nodularity (no dominant nodule).
- Mid-line nodule over hyoid bone that moves up and down with protrusion of tongue is likely to be a thyroglossal duct cyst.

• ***Cytologic and/or laboratory analyses that suggest a benign condition include:***

- presence of abundant watery colloid
- foamy macrophages
- cyst or cyst degeneration of a solid nodule
- hyperplastic nodule
- abnormal serum TSH
- lymphocytes and/or high TPOAb (suggests Hashimoto's thyroiditis or rarely lymphoma)

Guideline 61. For Laboratories & Physicians

- ☐ In addition to routine cytology, the laboratory should provide access to special immunoperoxidase staining for CT, Tg, TPO or Galectin-3 for special cases. (Send out to a different laboratory if necessary).
- ☐ Laboratories should archive all slides and tissue blocks "in trust" for the patient and make materials available for a second opinion when requested.
- ☐ Cytopathology laboratories should use standardized reporting of FNAs. The simplest approach uses four diagnostic categories: (1) Benign, (2) Malignant, (3) Indeterminate/Suspicious, and (4) Unsatisfactory/Inadequate. This should help achieve meaningful comparisons among different laboratories regarding outcomes.
- ☐ Cytopathology laboratories should share their analysis of FNA results with clinicians by citing their rates for true and false positives and negatives

Guideline 62. Follow-up of Patients with Benign Disease

- Some advocate performing a second FNA several months later to confirm the test.
- Others do not recommend a repeat FNA if the first yielded adequate tissue, provided that the nodule was less than 2 cm and has been stable in size during a year of follow up. In this case, follow-up with an annual physical examination and measurement of the nodule size, preferably with ultrasound is recommended. If ultrasound is not available, changes in nodule size may be detected by measurements made by a tape and/or ruler.
- It is recommended that enlarging lesions or any clinically suspicious nodules should be re-aspirated.

Benign conditions include, but are not be limited to, the following:

- ☐ simple goiter
- ☐ multinodular goiter
- ☐ colloid nodule*
- ☐ colloid cyst*
- ☐ simple cyst*
- ☐ degenerating colloid nodule
- ☐ Hashimoto's thyroiditis
- ☐ hyperplastic nodule

**often have inadequate cytologic specimen due to lack of follicular cells*

(b) Malignant Lesions (~ 5-10% of cases)

There are differences of opinion regarding the optimal degree of surgery for thyroid malignancies. In most

centers in the United States, near-total or total thyroidectomy, performed by an experienced surgeon, is the favored opinion. In Europe, other opinions exist (469). The risk of complications is lower when a surgeon is selected who performs thyroid operations frequently.

(i) Papillary Carcinoma (~ 80% of malignancies)

This classification includes mixed papillary and follicular and variants such as the tall cell variant and the sclerosing variant (a histological diagnosis)

Cytologic/Histologic. Two or more of the features below suggest a papillary malignancy:

- nuclear inclusions, “cleared-out”, “ground glass” or “orphan annie” nuclei.
- nuclear “grooves” (not just a few)
- overlapping nuclei
- psammoma bodies (rare)
- papillary projections with fibrovascular core
- “ropey” colloid

(ii) Follicular or Hurthle Cell Neoplasms (~20% of malignancies)

Lesions in this diagnostic category display cytologic evidence that may be compatible with malignancy but are not diagnostic (457,470). Factors suggesting malignancy include male gender, nodule size ≥ 3 cm and age >40 years (470). Definitive diagnosis requires histologic examination of the nodule to demonstrate the presence of capsular or vascular invasion. Re-aspiration is usually discouraged as it rarely provides useful information. There are currently no genetic, histologic or biochemical tests that are routinely used to differentiate between benign and malignant lesions in this category. Appropriate markers would need to be shown to distinguish between benign and malignant neoplasms in FNA specimens by multiple investigators. A number of studies suggest that TPO expression, measured by the monoclonal antibody MoAb 47, improves the specificity of correctly diagnosing histologically benign lesions over FNAB cytology alone (83 versus 55%, TPOAb immunodetection versus cytology alone, respectively) (461,462). More recently, Galectin-3, a beta-galactoside binding protein has been found to be highly and diffusely expressed in all thyroid malignancies of follicular cell origin (including papillary, follicular, hurthle and anaplastic carcinomas) but minimally in benign conditions (463-466,471). Most surgeons believe that an intra-operative frozen section offers minimal value in differentiating malignant from benign lesions when patients have follicular or Hurthle cell neoplasms (472). Sometimes a staged lobectomy is performed followed by a completion thyroidectomy within 4 to 12 weeks if capsular or vascular invasion in the histologic specimen indicates malignancy. A recent study found that the prognosis for patients with Hurthle cell carcinoma is predicted by well-defined histomorphologic characteristics (473).

Cytologic/Histologic. Features suggesting a Follicular or Hurthle malignancy include:

- minimal amounts of free colloid
- high density cell population of either follicular or Hurthle cells
- microfollicles

Cytology. These lesions may be reported as:

- ☐ “Hurthle cell neoplasm”
- ☐ “Suspicious for follicular neoplasm”
- ☐ “Follicular neoplasm/lesion”
- ☐ “Indeterminate” or “non-diagnostic”

(iii) Medullary Carcinoma (1-5% of thyroid malignancies)

This type of thyroid cancer should be suspected when patients have a family history of medullary cancer or multiple endocrine neoplasia (MEN) Type 2 [Section-3 F].

Cytologic/Histologic Features suggesting this type of malignancy include:

- spindle-type cells with eccentric nuclei
- positive calcitonin stain
- presence of amyloid
- intranuclear inclusions (common)

(iv) Anaplastic Carcinoma (< 1% of thyroid malignancies)

This type of thyroid cancer usually only occurs in elderly patients who present with a rapidly growing thyroid mass. Such patients may have had a previous indolent thyroid mass present for many years. It is necessary to differentiate between anaplastic carcinoma for which there is very limited therapy and thyroid lymphoma for which treatments are available.

Cytologic/Histologic Features that suggest this malignancy include:

- ☐ extreme cellular pleomorphism
- ☐ multinucleated cells
- ☐ giant cells

(v) Thyroid Lymphoma (rare)

Suggested by rapid growth of a mass in an elderly patient, often with Hashimoto's thyroiditis.

Cytologic/Histologic Features suggesting this malignancy include:

- ☐ monomorphic pattern of lymphoid cells
- ☐ positive B-cell immunotyping

10. Inadequate / Nondiagnostic FNA (~ 5 to 15 %)

A cytologic diagnosis cannot be reached if there is poor specimen handling and preparation or if inadequate cellular material was obtained at the time of FNA. The principal reasons for insufficient material for diagnosis may be inexperience on the part of the physician performing the procedure, insufficient number of aspirations done during the procedure, the size of the mass, or the presence of a cystic lesion. Adequate FNA specimens are defined as containing six groups of follicular cells of 10 to 20 cells each on two different slides(467). When small nodules are of concern, the repeat FNA should be done with ultrasound guidance. FNA using ultrasound guidance reduces the incidence of inadequate specimens from 15-20% down to 3-4% in such patients (215,450,451,474,475). Ultrasound guided FNA is also indicated for nodules <1.5 cm, cystic (complex) nodules to assure sampling of the solid component, posterior or high substernal nodules or any nodule difficult to palpate, especially in the obese, muscular or large frame patient (215,450,451). FNA should be made on dominant nodules within a multinodular goiter using ultrasound guidance in order to focus the procedure on the more clinically suspicious nodule(s).

Guideline 63. Patients with Inadequate or Non-diagnostic FNA

- ☐ Repeat FNA for small nodules often yields adequate cellular material for a diagnosis. Preferably, the repeat FNA should be done with ultrasound guidance. FNA using ultrasound guidance reduces the incidence of inadequate specimens from 15-20% down to 3-4%.
- ☐ Ultrasound guided FNA is also indicated for nodules <1.5 cm, cystic (complex) nodules to assure sampling of the solid component, posterior or high substernal nodules or any nodule greater than 1.0 cm that is

difficult to palpate, especially in the obese, muscular or large frame patient. The principal (i.e dominant) nodule(s) in a multinodular goiter should be biopsied using ultrasound guidance.

ARCHIVED



I. Screening for Congenital Hypothyroidism

The prevalence of congenital primary hypothyroidism (CH) (approximately 1:3500 births) is greater than that of central hypothyroidism (hypothalamic or pituitary) CH (approximately 1:100,000). The prevalence is higher in some ethnic groups and increased in iodine deficient regions of the world (476,477). Over the last 25 years, screening for CH has been performed on whole blood spotted on filter paper, using either TT4 or TSH as the primary screening test. Such testing has become established practice in the developed world as part of screening programs for a variety of genetic conditions. In order to maximize efficiency, screening programs are frequently centralized or regionalized and operated according to strict guidelines and licensure requirements. Guidelines for CH screening have been published by the American Academy of Pediatrics in 1993, by the European Society for Pediatric Endocrinology in 1993, and updated in 1999 (478-480).

Participating testing laboratories may be from the private sector or run by State governments, but must have in place an acceptable quality assurance program and participate in proficiency testing.

Thyroid dysgenesis resulting from aplasia, hypoplasia or an ectopic thyroid gland is the most common cause of congenital hypothyroidism and accounts for approximately 85% of presenting cases (12). Inactivating mutations in the TSH receptor have been reported from a number of screening centers, but the prevalence is still unknown. The phenotype associated with TSH resistance is variable but appears to be of two types, partial or severe. Those with a TSH elevation due to partial TSH resistance are euthyroid, have a normal TT4 and may not require L-T4 replacement therapy. There is some evidence for the secretion of TSH isoforms with enhanced bioactivity in syndromes of thyroid hormone resistance [Section-3 C4(g)ii] (244). Another rare cause of CH (six patients) is a mutation of one of the genes encoding for the thyroid transcription factors, TTF-1, TTF-2 and PAX-8. These factors play a key role in controlling thyroid gland morphogenesis, differentiation and the normal development of the thyroid gland in the fetus. They bind Tg and TPO promoters to regulate thyroid hormone production.

Guideline 64. Laboratories Performing Neonatal Screening for Congenital Hypothyroidism

- ☐ Only laboratories with experience in automated immunoassay procedures, information technology and with computer back-up, and appropriately trained staff, should undertake high volume screening for Congenital Hypothyroidism.

The proper interpretation of newborn thyroid function requires some understanding of the interaction between mother and fetus. Iodine, thyrotropin releasing hormone, antithyroid medications and IgG antibodies readily cross the placenta. There is no trans-placental passage of TSH or triiodothyronine. In contrast, contrary to previous thought it is now recognized that thyroxine crosses the placenta in sufficient quantities to protect the hypothyroid fetus from the consequences of thyroxine deficiency until detection by neonatal screening programs after birth (481). Immediately following delivery there is a surge of TSH in the neonate during the first 24 hours, presumably in response to cooling. In the term newborn, circulating thyroxine increases 2 to 3-fold higher than adult levels during the first 48 hours then stabilizes and returns to cord levels by 5-6 days. The response in the premature infant is less marked and inversely related to immaturity. Both circulating T4 and TSH concentrations remain above adult levels throughout infancy and decrease during childhood to reach adult concentrations after puberty (Table 3) (42).

1. Criteria needed for CH screening laboratories

Only laboratories with experience in automated immunoassay procedures, information technology and computer back-up with appropriately trained staff should undertake screening for CH. Neonatal screening programs rely on large numbers of samples coming from a relatively wide area. The logistics of sample transport, i.e. postal transit time, delays in posting at maternity wards and delays in taking action after the result is produced, are more significant time limiting factors in identifying infants at risk for CH than the speed of analytic testing. Screening should take place on a daily basis so that the results can be immediately available and acted upon. Treatment should begin as soon as possible, preferably within the first two weeks of life.

The minimum number of newborns that should be screened per year is debatable and relies on the fact that analytical proficiency is best accomplished when reasonable numbers of positive cases are encountered and cost efficiency is realized with higher volumes of testing. The screening program should ensure that follow-up testing is done on infants with positive screening results and that access to experienced diagnostic expertise is available. Laboratories should follow up and tightly control the rates of false negative and false positive results. A referral pediatric endocrinologist should be available for follow up testing to ensure that the correct diagnosis and treatment is achieved.

2. Screening Strategies

Screening methods should have low costs and be easy to perform.

Most screening programs for congenital hypothyroidism rely on tests that elute blood from filter paper spots, collected from infants by heel stick. The analytical reagents for measuring thyroid hormone in the filter paper eluates usually require some modification to run on the different automated immunoassay platforms used for this testing. Two different approaches for thyroid hormone screening of blood spot specimens have evolved – either measuring TT4 or TSH levels. In either case results should be interpreted using age-adjusted reference ranges (see Table 3 and Guideline 3).

Guideline 65. For Laboratories Performing Thyroid Testing of Neonates and Infants

- Thyroid test results in neonates must be reported with gestation and age-specific reference intervals, respectively.
- Each Laboratory should establish its own cut off levels according to the method used.

(a) Primary TT4 with reflex TSH measurement

Most North American screening programs use an initial TT4 measurement, with reflex TSH testing of specimens with low TT4 levels (usually less than the 10th percentile). Historically, this approach was adopted because the turnaround time of the earlier TT4 assays was much shorter than for TSH, test kits for TT4 were more reliable, the screening was performed earlier in the neonatal period (usually at 1-2 days of age) and the cost for TT4 testing was less than that for TSH. Although the measurement of FT4 in serum is readily available, FT4 methods are not usually employed for screening because of sensitivity limitations due to the small sample taken from filter paper blood spots and the high dilution that results from the elution of the specimen (482). The TT4-first screening approach has some advantages, particularly in programs where samples need to be collected early in the neonatal period. TT4 is also less influenced by the TSH surge that follows the cutting of the umbilical cord and lasts for the first 24 hours. Both of these factors suggest that TT4 screening will result in fewer false-positives when early (< 24 hour) testing is necessary. Furthermore, the TT4-first approach can detect the rare case of central hypothyroidism that would be missed with a TSH-first approach.

The disadvantages of TT4-first screening relates to the difficulties in setting the TT4 cut-off value low enough to minimize false-positives, but high enough to detect CH in infants with ectopic thyroid glands who may have TT4 concentrations above the 10th percentile. In addition, a low TT4 and normal TSH can be encountered in a number of other conditions: (a) hypothalamic-pituitary hypothyroidism (b) thyroxine binding globulin (TBG) deficiency (c) prematurity (d) illness or (e) a delayed rise in TSH. In programs where the follow up of infants with secondary or tertiary hypothyroidism has been carried out, only 8 of 19 cases were detected by TT4 screening, seven were diagnosed clinically before screening and four, although having low TT4 concentrations on screening, were not followed up (483-485). TBG deficiency has no clinical consequence such that the treatment of this condition is contraindicated. TT4 screening may also be useful in the very low birth weight infants (< 1500g) in whom TSH is normal at the usual time of screening, and only begins to rise weeks later. However, significantly lower TT4 values are typically seen in pre-term versus full-term infants (482).

(b) Primary TSH Measurement

Europe and much of the rest of the world have adopted TSH as the primary CH screening assay. Primary TSH screening has advantages over TT4 screening in areas of iodine deficiency, since neonates are more susceptible to the effects of iodine deficiency than adults and these infants have an increased frequency of high blood spot TSH levels. TSH screening makes it possible to monitor the iodine supply in the newborn population, especially since many European countries are still iodine deficient (486). Additionally, there is now little difference in cost between TSH and TT4 test reagents.

The TSH cutoff level used for recall varies between programs. In one program a two-tiered approach was adopted (487). Specifically, if the infant is more than 48 hours old and the initial blood spot TSH result is <10 mIU/L whole blood units, no further follow up is done. If the TSH is between 10 and 20 mIU/L whole blood units, a second blood spot is collected from the infant. TSH is normal in most of these repeat specimens. However, if the TSH is >20 mIU/L whole blood units the infant is recalled to be evaluated by a consultant pediatrician and other thyroid function tests are performed on the serum sample. For specimens drawn earlier than 48 hours, appropriate cut off values should be used (482). This approach ensures that the mildest forms of hypothyroidism characterized by only a modest increase in TSH are followed up, although it produces a higher number of false positives that must be followed through the system. Although most results above 20 mIU/L are due to CH, it is important to rule out maternal ingestion of antithyroid drugs or the use of iodine antiseptic solutions at delivery as a cause of transient TSH elevations.

Guideline 66. Pre-term and Early Discharge of Neonates

The TSH surge that follows the cutting of the umbilical cord and lasts for the first 24 hours may be delayed in pre-term infants and may lead to more false-positive TSH results when infants are tested within 24 hours of birth

- When using TSH to screen pre-term infants, a second sample collected 2 to 4 weeks after birth is recommended, since in some cases there is a delayed rise in TSH, perhaps due to immaturity of the pituitary-thyroid feedback mechanism.
- The TT4 –first approach may offer advantages for very low birth weight infants or when screening can only be performed within 24 hours of birth.

3. Blood Spot Assays for TSH

TSH measurements made on blood spot specimens are either reported in serum units, by relating the whole blood calibrators to serum values as in North American programs, or are reported in whole blood units, as in European programs. The absolute TSH values are significantly lower with the latter approach, because part of the volume of the spot is occupied by red cells. This difference in reporting has created confusion in the past and is still not resolved. It is necessary to increase the whole blood units by 30-50% to approximate the serum units.

Guideline 67. Countries with Iodine Deficiency

- ☐ Primary TSH testing is recommended in preference to primary TT4 with reflex TSH in countries that have mild or moderate iodine deficiency.

Screening assays for CH require TSH to be measured in blood spots as small as 3-4 mm in diameter. The new “third generation” TSH IMAs with functional sensitivities down to 0.02 mIU/L are well suited for this purpose [Section-2 C]. However, not all manufacturers have developed blood spot TSH assays since it is considered a specialized and limited market. Microtitre-plate assays using non-isotopic signals, such as time resolved fluorescence, are well suited for blood spot specimens and are in widespread use. An advantage of these systems is that as elution of the blood spot is carried out in the microtitre plate well, all of the TSH in the

sample is available for binding to the monoclonal antibody on the wall of the microtitre plate well.

Other automated systems that do not use a microtitre plate format however, can be successfully used for blood spot TSH assays. These usually require off-line elution of the TSH from the blood spot and a sampling of the eluate by the automated immunoassay analyser. Some of these systems have the advantage of results within 20 minutes and have a high throughput rate of 180 test results per hour. Additionally, these systems incorporate positive identification of the sample, making the identification of an increased blood spot result from the correct patient more secure. An automated punch of the filter paper containing the blood spot has been designed so that bar-coded labels with a unique number, placed on the elution tubes or microtiter plates, are read before punching. The same identification number is then printed on the patient's filter paper card. The automated immunoassay analyzer reads the same bar-coded label on the elution tubes and results are printed or downloaded to the laboratory host computer against the unique patient identification number and demographics if these have been previously entered. For those laboratories without automation, TSH assays utilizing antibody coated tube assays are still suitable, but are not amenable to high throughput automation.

Guideline 68. Performance Criteria for Blood Spot TSH Screening of Newborns

- Functional sensitivity of the TSH assay should be at least 1.0 mIU/L.
- Between run coefficient of variation should ideally be <10% and not more than 20%.
- Internal quality control samples should cover the reportable range and must be included in every run.
- At least one of the quality controls materials should be supplied by a different manufacturer from the TSH reagent manufacturer.
- Standards should be made in blood, i.e. be identical to specimens tested.
- Use the same filter paper for the samples, standards and controls.
- Participation in National and/or International external quality control programs is essential (see Appendix B).

4. Sample Collection

The technique for collecting blood samples by heel stick on filter-paper is of the utmost importance. Only filter-paper that meets NCCLS standards should be used ["Blood on Filter Paper For Neonatal Screening Programs" Approved Standard – Third Edition. LA4-A3, Vol 17 N° 16, October 1997. National Committee for Clinical Laboratory Standards] (488). This requires a continuous training program, well-written protocols and establishing criteria for adequacy of specimen collection.

Guideline 69. TSH Cut-off values for the Screening of Neonates > 48 hours of age

Reported values should be identified in whole blood or serum units. It is necessary to increase the whole blood units by 30-50% to approximate serum units.

- Initial blood spot TSH < 10 mIU/L whole blood units – no further action
- Initial blood spot TSH 10-20 mIU/L whole blood units – repeat the test on a second blood spot
- Initial blood spot TSH >20 mIU/L whole blood units – recall infant for evaluation by pediatric endocrinologist

The decision as to when to obtain the sample is determined by the requirements of other newborn screening protocols and whether the sample is taken in the hospital or at home. In Europe, samples are usually taken between 48 hours and 8 days after birth, depending on local practice. In many of the screening programs in the United States, economic pressures that prompt early discharge dictate that specimens be drawn before 48 hours. Sample collection time impacts on the TSH-first strategy more than TT4-first because a TSH surge occurs at the time the umbilical cord is cut. In the majority of infants the increase in TSH returns to normal within 24 hr, but in some infants, TSH can remain elevated for up to 3 days. For pre-term infants, a second sample, collected 2 to 4 weeks after the first sample, is advisable since in some cases there is a delayed rise in TSH, perhaps due to

immaturity of the pituitary-thyroid feedback mechanism (489).

5. Confirmation Testing

Measurements performed on filter paper eluates are not diagnostic but are of screening value only and abnormal results must be confirmed with routine quantitative methods! Confirmatory blood samples should be drawn by venipuncture. In some countries a blood sample is also collected from the mother at the same time to check maternal thyroid function. Specifically, TSH receptor blocking antibodies (TBAb/TSBAb) present in mothers carrying a diagnosis of hypothyroidism (even when receiving adequate L-T4 replacement) can cause transient hypothyroidism in the infant (in 1:180,000 neonates) (301,490).

Guideline 70. Filter Paper Eluate Measurements

- Measurements made on filter paper eluates are not diagnostic. Values are at best only semi-quantitative and help identify individuals likely affected by congenital hypothyroidism. Any abnormal newborn screening result must be confirmed with quantitative serum thyroid tests.

Some programs in Europe advocate follow-up testing with serum FT4, TSH and TPOAb in the mother as well as the infant. It is important to note that serum FT4 and TT4 levels are higher in the neonatal period so that borderline results in infants with mild hypothyroidism should be compared with age-related reference intervals for the particular thyroid test used (Table 3).

The aim of CH screening programs is to detect CH and expedite thyroid hormone replacement therapy as early as possible (within 14 days). However, additional tests to determine the etiology of CH should also be carried in order to determine whether the condition is transient, permanent or due to genetic causes (needed for genetic counseling) (Table 11). Some of these tests need to be performed before L-T4 replacement treatment begins, while others can be performed during therapy. In the case of transient hypothyroidism due to transplacental passage of TBAb/TSBAb from mother to infant, treatment with L-T4 is indicated since the presence of blocking antibody in the neonate inhibits the actions of TSH resulting in a lowered FT4 concentration (301,491). Once the antibodies have been degraded over a period of three to six months, depending on the amount of antibody present, then L-T4 therapy can be gradually discontinued. The mother's thyroid antibody status should be monitored in any subsequent pregnancies as thyroid antibodies can persist for many years (492).

In many cases, at the time of the diagnosis of CH, it is impossible to determine whether the hypothyroidism is permanent or transient. Clues that are associated with transient conditions include a TSH level below 100 mIU/L, male sex, pseudohypoparathyroidism, prematurity, iodine exposure, or dopamine administration (484). In such instances it is best to manage the patient as if he/she has permanent hypothyroidism (493). If the diagnosis has not become apparent by the age of 2 years, L-T4 therapy should be discontinued for one month and the infant monitored with serial determinations of FT4 and TSH.

Guideline 71. Confirmation Testing for Abnormal Screening Tests (TT4 or TSH)

- Confirmatory blood samples from the neonate should be drawn by venipuncture.
- Some programs in Europe advocate follow-up testing of only the infant and in some cases the thyroid status of the mother is also investigated using serum FT4, TSH and TPOAb testing.
- Check the mother for TSH receptor blocking antibodies.
- Use method and age-specific reference intervals for TT4 and TSH testing of neonates.

6. Tests for the Etiology of Congenital Hypothyroidism

Tests that can be used to establish the diagnosis of CH and investigate its etiology are shown in Table 11. The ordering of such tests is usually the responsibility of a pediatric endocrinologist and not the screening program. Thyroid scintigraphy is useful to document the presence of any thyroid tissue present and its location. Serum thyroglobulin measurements are more sensitive than scintigraphy for detecting residual functioning thyroid

tissue and may be normal in cases where scintigraphy shows no uptake. The presence of a thyroid gland is best determined by ultrasonography that can be performed after the start of therapy since ^{123}I scintigraphy is not available everywhere. Many cases show no uptake with scintigraphy and clearly present thyroid tissue by sonography. In these cases, testing should be directed towards determining an inborn error of T4 synthesis (~10% of cases) or a transient cause such as acquired TSH receptor blocking antibodies derived via trans-placental passage (301,491).

A perchlorate discharge test response of >15% suggests an inborn error of metabolism. Specialist centers offer tests that include urinary iodine measurement, tests for a specific gene mutation such as the sodium/iodine symporter, TPO or thyroglobulin (494). More commonly, defects in the oxidation and organification of iodine and coupling defects resulting from mutation in TPO can occur. Mutations in the thyroglobulin gene give rise to abnormal thyroglobulin synthesis that can result in defective proteolysis and secretion of T4. Deiodinase gene mutations give rise to deiodinase defects as well.

Guideline 72. Detection of Transient Congenital Hypothyroidism (CH)

Since CH may be transient as a result of transplacental passage of TSH receptor blocking antibodies, it is recommended that the diagnosis be re-evaluated in all cases at 2 years of age.

- At 2 years of age a blood specimen should be obtained for basal serum FT4/TSH measurements. Discontinue L-T4 treatment and retest serum FT4/TSH after 2 weeks and again after 3 weeks. Almost 100% of children with true CH have elevated TSH levels after 2 weeks off of treatment.

Table 11. Diagnostic Tests in the Evaluation of Congenital Hypothyroidism (CH)

To Establish the Diagnosis:			
• Infant:		• Mother:	
TSH FT4		TSH FT4 TPOAb	
To Establish Etiology:			
• Infant:		• Determine size and position of thyroid by either: -Ultrasonography (in newborn) - Scintigraphy – either $^{99\text{m}}\text{Tc}$ or ^{123}I • Functional studies: - ^{123}I uptake - Serum thyroglobulin (Tg) • Inborn error of T4 production is suspected: - ^{123}I uptake and perchlorate discharge test • If iodine exposure or deficiency is suspected: -Urinary iodine determination	
• Mother:		• If autoimmune disease present: -TSH Receptor antibody (TRAb) (also in infant, if present in mother)	

7. Long-term Monitoring of Congenital Hypothyroid Patients

Most CH infants and children have normal pituitary-thyroid negative feedback control although T4 and TSH thresholds are set higher (Table 3) (43). Infants and children diagnosed with congenital hypothyroidism should be monitored frequently in the first two years of life using serum TSH as the primary monitoring test with FT4 as the secondary parameter employing age-appropriate reference intervals (Table 3) (40). In the United States, the L-T4 replacement dose is adjusted to bring the TSH below 20 mIU/L and produce a circulating T4 level in the upper half of the reference range ($>10 \mu\text{g/dl}/129 \text{ nmol/L}$) within the first two weeks after starting treatment. Infants are usually maintained on a dose of 10-15 μg L-T4/kg body weight with the monitoring of TSH and T4 every 1-2 months. In Europe, a flat L-T4 dose of 50 $\mu\text{g/day}$ is used with the T4 and TSH measurement made after 2 weeks and monthly thereafter if possible. Experience has shown that with this dosage, the therapy does not need any adjustment for the first 2 years. Frequent dose changes designed to keep a maximal dose per Kg body weight can lead to over-treatment (493).

A minority of infants treated for CH appear to have variable pituitary-thyroid hormone resistance, with relatively elevated serum TSH levels for their prevailing serum free T4 concentration. This resistance appears to improve with age (43). In rare cases, transient hypothyroidism may result from the transplacental passage of TSH-receptor blocking antibodies (282,301). It is recommended that the diagnosis of CH be re-evaluated in all cases after 2 years of age. Specifically, after a basal FT4/TSH measurement is made, L-T4 treatment is discontinued and FT4/TSH re-tested after 2 weeks and again after 3 weeks. Almost 100% of children with true CH have clearly elevated TSH after 2 weeks off treatment.

8. Missed Cases

No biochemical test is 100% diagnostic and technically accurate. One study in which screening checks were made after two-weeks of age revealed that 7% of cases of CH were missed using the TT4-first strategy, and 3% were missed with the TSH-first, approach. Recommendations are needed to address the clinical, financial and legal ramifications of false-negative screening tests and whether mandated retesting at 2 weeks such as practiced in some programs is desirable.

Guideline 73. Treatment and Follow-up of Infants with Congenital Hypothyroidism

- In Europe, a flat L-T4 dose of 50 $\mu\text{g/day}$ is used to minimize the risk of overtreatment as compared with more frequent dose changes.
- In the USA, treatment is typically initiated with L-T4 at a dose of 10-15 $\mu\text{g/kg/day}$. The goal is to raise the circulating T4 above 10 $\mu\text{g/dl}$ by the end of the first week.
- During the first year of life, TT4 is usually maintained in the upper half of the normal reference range (therapeutic target 10-16 $\mu\text{g/dl}/127-203 \text{ nmol/L}$) or if FT4 is used, the therapeutic target is between 1.4 and 2.3 ng/dl (18 and 30 pmol/L) depending on the reference range (Table 3).
- Infants and children diagnosed with congenital hypothyroidism should be monitored frequently in the first two years of life using serum TSH as the primary monitoring test with FT4 as the secondary parameter, employing age-appropriate reference intervals.
- Monitoring should be every 1-2 months during the first year or life, every 1-3 months during the second and third years and every 3-6 months until growth is complete.
- If circulating T4 levels remain persistently low and the TSH remains high despite progressively larger replacement doses of L-T4, it is important to first eliminate the possibility of poor compliance.
- The most frequent reason for failure to respond to replacement therapy has been interference with adsorption by soy-based formulas. L-T4 should not be administered in combination with any soy-based substances or with medications that contain iron.

9. Quality Assurance

All screening programs should have a continuous system for audit and publish an annual report of the outcome of the audit. By this means, an appraisal can be made of each aspect of the screening procedure against

nationally agreed upon quality standards. Although laboratories generally comply with quality standards in that they routinely participate in quality assurance programs, the pre-analytical and post-analytical phases of screening typically receive less attention. Quality assurance programs should address each of the following phases:

- *Preanalytical*
 - training for personnel conducting the sample collection
 - storage and timely transport of filter papers to the laboratory
 - linking the identification of the filter paper sample to the analytic result
- *Analytical*
 - equipment maintenance and service
 - internal quality control of filter paper results
 - national and international external quality control participation
- *Post-analytical*
 - co-ordination of follow-up of abnormal tests
 - confirmatory testing where applicable
 - appropriate storage and archiving of specimens for later testing

10. Annual Reporting

This should include items identified by audit and be a comprehensive report of CH screening over the previous twelve months. The report should monitor the distribution of increased blood spot TSH concentrations, and there should be a system to report all cases of true CH and record cases of transient elevations in TSH. The system could also provide information on any missed cases. An efficient screening program depends on a close collaboration between the screening laboratory, pediatricians, endocrinologists and all concerned in the screening process.

Guideline 74. For Physicians

- ☐ Repeat tests when the clinical picture conflicts with the laboratory test results!
- ☐ Potential pitfalls in screening are ubiquitous and no laboratory is immune!
- ☐ Maintain a high degree of vigilance. Despite all safeguards and automated systems, screening programs will occasionally miss infants with congenital hypothyroidism. Do not be lulled into a false sense of security by a laboratory report bearing normal thyroid function values.



Section 4. The Importance of the Laboratory – Physician Interface

Physicians need quality laboratory support for the accurate diagnosis and cost-effective management of patients with thyroid disorders. Laboratories need to offer analytical methods that are both diagnostically accurate and cost effective. These latter qualities are sometimes in conflict. Cost-effectiveness and quality care require that the laboratory serve not only the needs of the majority, but also meet the needs of the minority of patients who have unusual thyroid problems that challenge the diagnostic accuracy of the different thyroid tests available. Most studies on “cost effectiveness” fail to take into account the human and financial costs resulting from inappropriate management, needless duplication of effort and the unnecessary testing of patients with unusual thyroid disease presentations. These atypical presentations account for a disproportionately large expenditure of laboratory resources to come up with the correct diagnosis (191). Some of these unusual presentations include: binding protein abnormalities that affect the FT4 estimate tests; the presence of Tg autoantibodies that interfere with serum Tg measurements; medications that compromise the in vivo and in vitro metabolism of thyroid hormones and severe forms of NTI that have a myriad of effects on thyroid test results.

Guideline 75. For Laboratories and Physicians

- It is essential that clinical laboratory scientists develop an active collaboration with the physicians using their laboratory services in order to select thyroid tests with the most appropriate characteristics to serve the patient population in question.
- An active laboratory-physician interface ensures that high quality, cost-effective assays are used in a logical sequence, to assess abnormal thyroid disease presentations and to investigate discordant thyroid test results.

It is essential that clinical laboratory scientists develop an active collaboration with physicians using their laboratory services and to select thyroid tests with appropriate characteristics to serve the patient population in question. For instance, the effect of nonthyroidal illness (NTI) on the FT4 method is not as important if the laboratory serves primarily an ambulatory patient population.

In contrast, it is very important for a hospital laboratory to accurately exclude thyroid dysfunction in sick hospitalized patients. Drugs and other interferences can affect the interpretation of more than 10% of laboratory results in general, and thyroid testing is no exception (67,68,98). It follows that discordant thyroid results are often encountered in clinical practice. These discordant thyroid test results need to be interpreted with considerable care using a collaborative approach between the clinical laboratory scientist who generates the thyroid test result and the physician who manages the patient with suspected or established thyroid disease.

A. What Physicians Should Expect from Their Clinical Laboratory

Physicians depend on the laboratory to provide accurate test results and to help investigate discordant results, whether the tests are performed locally or by a reference laboratory. It is particularly important that the laboratory provide readily available data on drug interactions, reference intervals, functional sensitivities and detection limits as well as interferences that affect the methods in use. The laboratory should avoid frequent or unannounced changes in assay methods and interact closely with physicians before a change in a thyroid method is initiated. The laboratory should also be prepared to collaborate with physicians to develop clinical validation data with the implementation of any new method, as well as provide data showing a favorable relationship between the old and the proposed new test method as well as provide a conversion factor, if required. The diagnostic value and cost-savings of reflex testing strategies (i.e. adding FT3 when FT4 is high, or FT4 when TSH is abnormal) are usually site-specific (495). In the United States, laboratories by law can only implement reflex-testing after consultation with the physicians using the laboratory.

Physicians should expect their clinical laboratory to establish a relationship with a reference laboratory and/or another local laboratory that performs thyroid testing by a different manufacturer's methods. Re-measurement of the specimen by an alternative method is the cornerstone of investigating whether a discordant result is caused by a technical problem, an interfering substance in the specimen or a rare clinical condition (Guideline 7

and Table 1).

Guideline 76. Patients “Bill of Rights”

- Physicians should have the right to send specimens for testing to non-contracted laboratories when they can show that the contracted laboratory thyroid test results are not diagnostically valid or relevant.
- Physicians should have the right to request their laboratory to send a specimen to another laboratory for testing by a different manufacturer’s method if the test results are in disagreement with the clinical presentation.

The laboratory should establish and maintain an active relationship with specialized reference laboratories to ensure the availability of high-quality specialized thyroid tests. These specialized tests may include assays for Tg, TPOAb and TRAb tests. In addition, a reference laboratory offering FT4 measurements using a physical separation technique such as equilibrium dialysis should be available. The use of equilibrium dialysis for FT4 testing may be necessary under special circumstances for diagnosing thyroid disease in select patients with thyroid hormone binding protein abnormalities that interfere with the diagnostic accuracy of the automated FT4 estimate test performed in most clinical laboratories. In rare cases, it may be necessary to collaborate with a molecular diagnostic laboratory that has the expertise to identify genetic mutations characteristic of thyroid hormone resistance or medullary thyroid disease.

As shown in Table 1 and Figure 11, a number of clinical conditions, medications and specimen interferences can give rise to a diagnostically inaccurate test result that has the potential to prompt excess testing, inappropriate treatment, or in the case of central hypothyroidism mask the need for treatment. Some of the misinterpretations that can lead to serious errors are listed in Guideline 79.

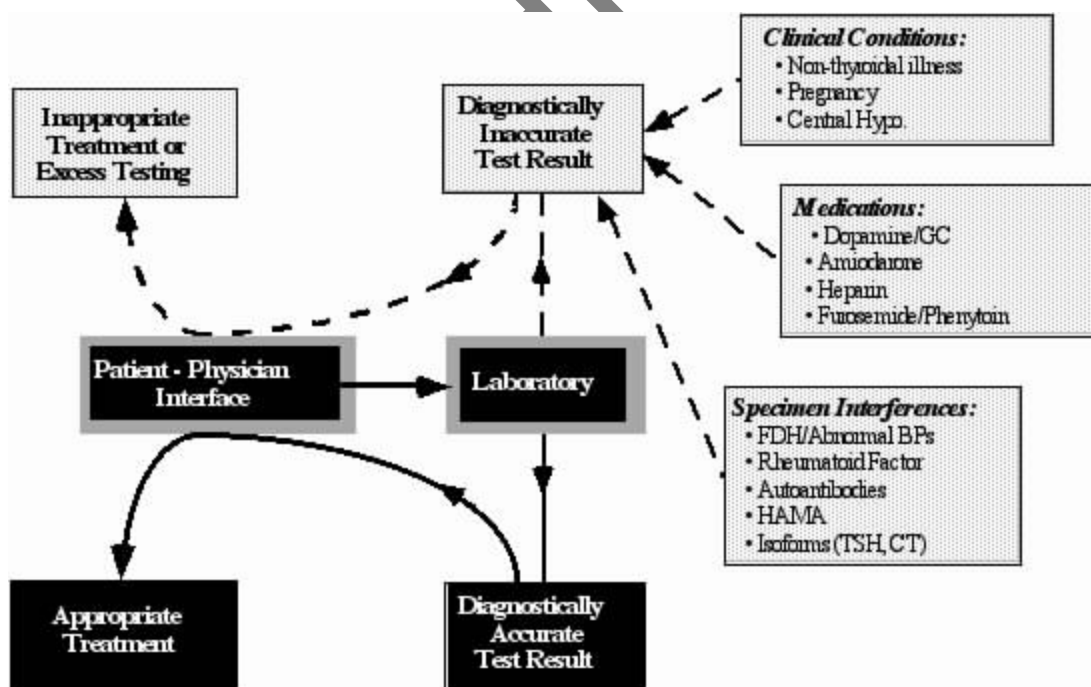


Fig 11. Consequences of Diagnostically Inaccurate Thyroid Tests

Manufacturers have the responsibility to thoroughly evaluate their methods and cooperate closely with laboratories using their products. Specifically, manufacturers should immediately inform all users of reagent problems that develop or method interferences when known and make recommendations as to how to minimize

the clinical impact of the problem. They should refrain from changing the composition of assay kits, even if the goal is to minimize interference, without informing customers and allowing sufficient time to perform correlation studies with the previous method. If the procedure has to be changed this should be indicated on the label of the kit i.e. by a version number.

Guideline 77. For Manufacturers

Manufacturers should cooperate closely with laboratories using their products. Manufacturers should:

- Rapidly inform all users of reagent problems and method interferences and recommend how to minimize their clinical impact.
- The composition of assay kits should not be changed, even if the goal is to reduce interference, without first informing customers. If the procedure has to be changed, the change should be indicated on the label of the kit (i.e. by a version number).

B. What Laboratories Should Expect of Physicians

Clinical laboratory scientists should ideally expect physicians to provide relevant clinical information with the submission of the test specimen and have a realistic understanding of the limitations of thyroid tests. For example, in some conditions, the physician should appreciate that the immunologic and biologic activity of TSH may be disconnected when patients have central hypothyroidism. This can result from pituitary dysfunction in which the immunoreactive form of TSH has impaired bioactivity (197,238).

Guideline 78. For Laboratories

- Every clinical laboratory should develop a relationship with another laboratory that uses a different manufacturer's method. Re-measurement of specimens with discordant results by an alternative method is the cornerstone of investigating whether a discordant result is caused by an interfering substance present in the specimen or as a result of "true" disease (Table 1).
- ☐ Laboratories should be able to provide physicians with the details of the thyroid method principles underpinning the test being used together with functional sensitivity, between-run precision, interferences and any bias relative to the method or other methods, and whether the tests are performed locally or sent to a reference laboratory.

Guideline 79. Misinterpretations that Can Lead to Serious Errors

When physicians or laboratorians are not aware of the limitations of test methods, serious medical errors can result:

- Inappropriate thyroid ablation because high thyroid hormone levels were reported as a result of FDH, the presence of thyroid hormone autoantibodies or thyroid hormone resistance.
- A missed diagnosis of T3-toxicosis in a frail elderly patient with NTI.
- Inappropriate treatment of a hospitalized patient for hypo- or hyperthyroidism on the basis of abnormal thyroid tests caused by NTI or a drug-related interference.
- A missed diagnosis of central hypothyroidism because the immunoreactive TSH level was reported as normal due to the measurement of biologically inactive TSH isoforms.
- Failure to recognize recurrent or metastatic disease in a thyroid cancer patient because serum Tg was inappropriately low or undetectable due to TgAb interference or a "hook" effect with an IMA measurement.
- Inappropriate treatment for DTC on the basis of an abnormally elevated serum Tg caused by TgAb interference with a Tg RIA method.
- Failure to recognize that neonatal thyrotoxicosis can be masked by transplacental passage of antithyroid drugs given to the mother for Graves' disease.

The physician should understand that anomalous laboratory thyroid test results can occur with certain medications and that the diagnostic accuracy of thyroid tests used for patients with NTI is method dependent. Without clinical feedback, it is not possible for the laboratory to appreciate the consequences of a diagnostic error (191). Misinterpretation of test results, as a consequence of a transient disequilibrium between serum TSH and FT4 following recent therapy for hypo-or hyperthyroidism can have significant consequences.

Without a strong, collegial laboratory-physician interface, the quality of laboratory support will undoubtedly be suboptimal. This is especially true in countries like the United States where laboratories rarely receive relevant clinical and medication information on the paperwork that accompanies the specimen. The inability of the laboratory to perform the final “sanity check” on the reported result(s) – i.e. relate the result to the patient’s clinical and medication history, can lead to errors, especially when physicians are unfamiliar with the technical limitations and interferences affecting the test.

ARCHIVED



Appendix A: Monograph Reviewers

Robert Adler, M.D.

Medical College of Virginia, VA, USA

Gisah Amaral de Carvalho, MD, Ph.D

Hospital de Clinicas, Universidade Federal do Parana, Brazil

Nobuyuki Amino, M.D.

Osaka University Graduate School of Medicine, Japan

Claudio Aranda, M.D.

Hospital Carlos G. Durand, Buenos Aires, Argentina

Jack H. Baskin M.D., F.A.C.E

Florida Thyroid & Endocrine Clinic, Orlando, FL, USA

Graham Beastall, Ph.D

Edinburgh Royal Infirmary NHS Trust, Scotland, UK

Geoff Beckett Ph.D., F.R.C.Path

Edinburgh Royal Infirmary NHS Trust, Scotland, UK

Liliana Bergoglio, BSc.,

Hoapital N. de Clinicas, Cordoba, Argentina

Roger Bertholf, Ph.D., DABCC, FACB

University of Florida Health Science Center, Jacksonville, FL, USA

Thomas Bigos, M.D., Ph.D.

Maine Medical Center, USA

Manfred Blum, M.D.

New York University Medical Center, New York, NY, USA

Gustavo Borrajo, M.D.

Detección de Errores Congénitos, Fundación Bioquímica Argentina, La Plata, Argentina

Irv Bromberg, M.D., C.M.

Mount Sinai Hospital, Toronto, Ontario, Canada

Rosalind Brown, M.D.

University of Massachusetts Medical School, Worcester, MA, USA

Bo Youn Cho

Asan Medical Center, Seoul, Korea

Nic Christofides, PhD.,

Ortho-Clinical Diagnostics, Cardiff CF14 7YT, Wales, UK.

Orlo Clark, M.D.

UCSF/ Mount Zion Medical Center, San Francisco, CA, USA

Rhonda Cobin, M.D.

Midland Park, NJ, USA

David Cooper, M.D.

Sinai Hospital of Baltimore, Baltimore, MD, USA

Gilbert Cote, M.D.

UT MD Anderson Cancer Center, Houston, TX, USA

Marek Czarkowski, M.D.

Warsaw, Poland

Gilbert Daniels, M.D.

Massachusetts General Hospital, Boston, MA, USA

Catherine De Micco, M.D.

University of the Mediterranean Medical School, Marseille, France

D.Robert.Dufour, M.D.

VA Medical Center, Washington DC, USA

John Dunn, M.D.

University of Virginia Health Sciences Center, Charlottesville, VA, USA

Joel Ehrenkranz, M.D.

Aspen, CO, USA

David Endres, PhD,

University of Southern California, Los Angeles, USA

Carol Evans, BSc., MSc., Ph.D, MRcPath.

University Hospital of Wales, UK

Shireen Fatemi, M.D.

Kaiser Permanente of Southern California, Panorama City, CA, USA

J. Douglas Ferry, Ph.D.,

Beaumont Hospital, Southfield, MI, USA

Jayne Franklyn, M.D. Ph.D. F.R.C.P.

Queen Elizabeth Hospital, Birmingham, UK

Jeffery Garber M.D.

Harvard Vanguard Medical Associates, Boston, MA, USA

Daniel Glinoe, M.D.

University Hospital St.Pierre, Bruxelles, Belgium

Timothy Greaves, M.D., F.A.C.P.

LAC-USC Medical Center, Los Angeles, CA, USA

B.J. Green

Abbott Laboratories, Abbott Park, IL, USA

Ian Hanning, BSc., MSc.,MRCPath

Hull Royal Infirmary, Hull, UK

Charles D. Hawker, Ph.D., MBA

Salt Lake City, Utah, USA

Georg Hennemann, M.D.

Erasmus University, Rotterdam, The Netherlands

Tien-Shang Huang, M.D.

College of Medicine, National Taiwan University, Taiwan

James Hurley, M.D.

New York Presbyterian Hospital, New York, NY, USA

William L Isley, MD

University of Missouri, Kansas City, MO, USA

Lois Jovanovic, MD

Sansum Medical Research Institute, Santa Barbara, CA, USA

George Kahaly M.D.

Gutenberg University Hospital, Mainz, Germany

Laurence Kaplan, Ph.D.

Bellevue Hospital, New York, USA

Elaine Kaptein, M.D.

University of Southern California, Los Angeles, USA

J. H. Keffer, M.D.

Melbourne Beach, FL, USA

Pat Kendall-Taylor, M.D.

Newcastle on Tyne, England, UK

Leonard Kohn, M.D.

Ohio University College of Osteopathic Medicine Athens, Ohio, USA

Annie Kung, M.D.

The University of Hong Kong, Hong Kong

Paul Ladenson, M.D.

Johns Hopkins Hospital, Baltimore, MD, USA

Peter Laurberg, M.D.

University of Aalborg, Aalborg, Denmark

P. Reed Larsen, M.D. FACP, FRCP

Harvard Medical School, Boston, MA, USA

John Lazarus, M.A. M.D., F.R.C.P.

University of Wales College of Medicine, Cardiff, Wales, UK

Charles Lewis, Jr., Ph.D.

Abbott Laboratories, Abbott Park, IL, USA

Jon LoPresti, M.D., Ph.D.

University of Southern California, Los Angeles, CA, USA

Gustavo Maccallini, Ph.D.

Hospital Carlos G. Durand, Buenos Aires, Argentina

Rui Maciel, M.D., Ph.D.

Department of Medicine, Federal University of San Paulo, Sao Paulo, Brasil

Susan J. Mandel, MD, MPH

Hospital of the University of Pennsylvania, Pennsylvania, USA

Geraldo Medeiros-Neto, M.D.

Hospital das Clinicas, San Paulo, Brazil

Jorge Mestman, M.D.

University of Southern California, Los Angeles, CA, USA

Greg Miller M.D.

Virginia Commonwealth University, Richmond, VA, USA

James J. Miller, Ph.D., DABCC, FACB

University of Louisville, Kentucky, USA

Marvin Mitchell, M.D.

University Massachusetts Medical Center, Jamaica Plain, MA, USA

John Morris, M.D.

Mayo Clinic, Rochester, MN, USA

Jerald C. Nelson, M.D.

Loma Linda University, California, USA

Hugo Niepomniszcze, M.D.

Hospital de Clinicas, University Buenos Aires, Buenos Aires, Argentina

Ernst Nystrom, M.D.

University of Goteborg, Sweden

Richard Pikner, M.D.

Charles University, Plzen, Czech Republic

Frank Quinn, Ph.D.

Abbott Laboratories, Abbott Park, IL, USA

Peter Raggatt, M.D.

Addenbrooke's Hospital, Cambridge, UK

Robert Rude, M.D.

University of Southern California, Los Angeles, CA, USA

Jean Ruf, M.D.

Department of Biochemistry & Molecular Biology, Marseille, France

Remy Sapin, Ph.D.

Institut de Physique Biologique, Strasbourg, France

Gerardo Sartorio, M.D.

Hospital J.M. Ramos Mejia, Buenos Aires, Argentina

Steven I. Sherman, M.D.

MD Anderson Cancer Center, Houston, TX, USA

Peter A. Singer, M.D.

University of Southern California, Los Angeles, CA, USA

Stephen Spalding, M.D.

VA Medical Center, Buffalo, NY, USA

Martin I. Surks, M.D.

Montefiore Medical Center, Bronx, NY, USA

Brad Therrell, Ph.D.

National Newborn Screening and Genetics Resource Center, Austin, TX, USA

Anthony D. Toft, M.D.

Edinburgh Royal Infirmary NHS Trust, Scotland, UK

Toni Torresani M.D.

University Children's Hospital, Zürich, Switzerland
R. Michael Tuttle, M.D.,
Memorial Sloan Kettering Cancer Center, New York, NY, USA
Hidemasa Uchimura, M.D.
Department of Clinical Pathology, Kyorin University, Japan
Greet Van den Berghe M.D., Ph.D.
Department of Intensive Care Medicine, University of Leuven, Leuven, Belgium
Lester Van Middlesworth, M.D., Ph.D.
University of Tennessee, Memphis, TN, USA
Paul Verheecke, M.D.
Centraal Laboratorium, Hasselt, Belgium
Paul Walfish, C.M., M.D.,
University of Toronto, Ontario, Canada
John P. Walsh, F.R.A.C.P. Ph.D.,
Sir Charles Gairdner Hospital, Nedlands, WA, Australia
Barry Allen Warner, DO
University of South Alabama College of Medicine, Mobile, AL, USA
Joseph Watine PharmD,
Laboratoire de biologie polyvalente, Hôpital Général, Rodez, France
Anthony P. Weetman, M.D.
Northern General Hospital, Sheffield, UK
Thomas Williams, M.D.
Methodist Hospital, Omaha, NE, USA
Ken Woeber, M.D.
UCSF, Mount Zion Medical Center, San Francisco, CA, USA
Nelson G. Wohllk MD,
Hospital del Salvador, Santiago, Chile

Appendix B. - Newborn Screening Quality Assurance Programs

- Australasia - Australasian Quality Assurance Program, National Testing Center 2nd Floor, National Women's Hospital, Claude Road, Epsom, Auckland, New Zealand.
- Europe - Deutsche Gesellschaft für Klinische Chemie eV, Im Mühlenbach 52a, D-53127 Bonn, Germany.
- Latin America - Programa de Evaluación Externa de Calidad para Pesquisa Neonatal (PEEC). Fundación Bioquímica Argentina. Calle 6 # 1344. (1900) La Plata, Argentina
- United Kingdom External Quality Assurance Scheme, Wolfson EQA laboratory, PO Box 3909, Birmingham, B15 2UE, UK.
- USA- Centers for Disease Control and Prevention (CDC), 4770 Buford Highway NE, Atlanta, GA 30341-3724, USA.

(The UK NEQAS program has a charge to participants, but for the other programs there is no charge).

Appendix C – Glossary of Abbreviations

AIH=	Amiodarone-Induced Hyperthyroidism
AITD =	Autoimmune Thyroid Disease
ANS =	8-Anilino-1-Napthalene-Sulphonic Acid
ATD =	Anti-Thyroid Drug Treatment
CT =	Calcitonin
CV =	% Coefficient of Variation
DTC =	Differentiated Thyroid Carcinoma
FDH =	Familial Dysalbuminemic Hyperthyroxinemia
FFA =	Free Fatty Acids

FMTC =	Familial Medullary Thyroid Carcinomas
FNA =	Fine Needle Aspiration
FT3 =	Free T3
FT4 =	Free T4
HCC=	C-cell Hyperplasia
HCG =	Human chorionic gonadotropin
IMA =	Immunometric Assay
L-T4 =	Levothyroxine
MEN =	Multiple Endocrine Neoplasia
MTC =	Medullary Thyroid Carcinoma
NTI =	Nonthyroidal Illness
PBI=	Protein-bound Iodine
Pg=	Pentagastrins
RT3=	Reverse T3
RET =	<i>RET</i> Proto-oncogene
RIA =	Radioimmunoassay
T4 =	Thyroxine
T3 =	Triiodothyronine
TBG =	Thyroxine Binding Globulin
TBPA=	Thyroxine Binding Prealbumin
TT4 =	Total Thyroxine
TT3 =	Total Triiodothyronine
TTR=	Transthyretin
Tg =	Thyroglobulin
TgAb =	Thyroglobulin Autoantibody
TPO =	Thyroid Peroxidase
TPOAb =	Thyroid Peroxidase Autoantibody
TBAb/TSBAb =	TSH Receptor Blocking Antibody
TBII =	TSH Binding Inhibitory Immunoglobulins
TRAb =	TSH Receptor Antibody
TSAb =	Thyroid Stimulating Antibody
TSH =	Thyroid Stimulating Hormone (Thyrotropin)
WHO=	World Health Organization (WHO)



References

1. Nohr SB, Laurberg P, Borlum KG, Pedersen KM, Johannesen PL, Damm P. Iodine deficiency in pregnancy in Denmark. Regional variations and frequency of individual iodine supplementation. *Acta Obstet Gynecol Scand* 1993;72:350-3.
2. Glinioer D. Pregnancy and iodine. *Thyroid* 2001;11:471-81.
3. Hollowell JG, Staehling NW, Hannon WH, Flanders DW, Gunter EW, Maberly GF et al. Iodine nutrition in the United States. Trends and public health implications: iodine excretion data from National Health and Nutrition Examination Surveys I and III (1971-1974 and 1988-1994). *J Clin Endocrinol Metab* 1998;83:3398-400.
4. Wartofsky L, Glinioer D, Solomon D, Nagataki S, Lagasse R, Nagayama Y et al. Differences and similarities in the diagnosis and treatment of Graves disease in Europe, Japan and the United States. *Thyroid* 1990;1:129-35.
5. Singer PA, Cooper DS, Levy EG, Ladenson PW, Braverman LE, Daniels G et al. Treatment guidelines for patients with hyperthyroidism and hypothyroidism. *JAMA* 1995;273:808-12.
6. Singer PA, Cooper DS, Daniels GH, Ladenson PW, Greenspan FS, Levy EG et al. Treatment Guidelines for Patients with Thyroid Nodules and Well-differentiated Thyroid Cancer. *Arch Intern Med* 1996;156:2165-72.
7. Vanderpump MPJ, Ahlquist JAO, Franklyn JA and Clayton RN. Consensus statement for good practice and audit measures in the management of hypothyroidism and hyperthyroidism. *Br Med J* 1996;313:539-44.
8. Laurberg P, Nygaard B, Glinioer D, Grussendorf M and Orgiazzi J. Guidelines for TSH-receptor antibody measurements in pregnancy: results of an evidence-based symposium organized by the European Thyroid Association. *Eur J Endocrinol* 1998;139:584-6.
9. Cobin RH, Gharib H, Bergman DA, Clark OH, Cooper DS, Daniels GH et al. AACE/AAES Medical/Surgical Guidelines for Clinical Practice: Management of Thyroid Carcinoma. *Endocrine Pract* 2001;7:203-20.
10. Ladenson PW, Singer PA, Ain KB, Bagchi N, Bigos ST, Levy EG et al. American Thyroid Association Guidelines for detection of thyroid dysfunction. *Arch Intern Med* 2000;160:1573-5.
11. Brandi ML, Gagel RJ, Angel A, Bilezikian JP, Beck-Peccoz P, Bordi C et al. Consensus Guidelines for Diagnosis and Therapy of MEN Type 1 and Type 2. *J Clin Endocrinol Metab* 2001;86:5658-71.
12. Werner and Ingbar's "The Thyroid". A Fundamental and Clinical Text. Lippincott-Raven, Philadelphia 2000. Braverman LE and Utiger RD eds.
13. DeGroot LJ, Larsen PR, Hennemann G, eds. *The Thyroid and Its Diseases*. (www.thyroidmanager.org) 2000.
14. Piketty ML, D'Herbomez M, Le Guillouez D, Lebtahi R, Cosson E, Dumont A et al. Clinical comparison of three labeled-antibody immunoassays of free triiodothyronine. *Clin Chem* 1996;42:933-41.
15. Sapin R, Schlienger JL, Goichot B, Gasser F and Grucker D. Evaluation of the Elecsys free triiodothyronine assay; relevance of age-related reference ranges. *Clin Biochem* 1998;31:399-404.
16. Robbins J. Thyroid hormone transport proteins and the physiology of hormone binding. In "Hormones in Blood". Academic Press, London 1996. Gray CH, James VHT, eds. pp 96-110.
17. Demers LM. Thyroid function testing and automation. *J Clin Ligand Assay* 1999;22:38-41.
18. Hollowell JG, Staehling NW, Hannon WH, Flanders WD, Gunter EW, Spencer CA et al. Serum thyrotropin, thyroxine and thyroid antibodies in the United States population (1988 to 1994): NHANES III. *J Clin Endocrinol Metab* 2002;87:489-99.
19. Wardle CA, Fraser WD and Squire CR. Pitfalls in the use of thyrotropin concentration as a first-line thyroid-function test. *Lancet* 2001;357:1013-4.
20. Spencer CA, LoPresti JS, Patel A, Guttler RB, Eigen A, Shen D et al. Applications of a new chemiluminometric thyrotropin assay to subnormal measurement. *J Clin Endocrinol Metab* 1990;70:453-60.
21. Meikle, A. W., J. D. Stringham, M. G. Woodward and J. C. Nelson. Hereditary and environmental influences on the variation of thyroid hormones in normal male twins. *J Clin Endocrinol Metab* 1988;66:588-92.

22. Andersen S, Pedersen KM, Bruun NH and Laurberg P. Narrow individual variations in serum T4 and T3 in normal subjects: a clue to the understanding of subclinical thyroid disease. *J Clin Endocrinol Metab* 2002;87:1068-72.
23. Cooper, D. S., R. Halpern, L. C. Wood, A. A. Levin and E. V. Ridgway. L-thyroxine therapy in subclinical hypothyroidism. *Ann Intern Med* 1984;101:18-24.
24. Biondi B, Fazio E, Palmieri EA, Carella C, Panza N, Cittadini A et al. Left ventricular diastolic dysfunction in patients with subclinical hypothyroidism. *J Clin Endocrinol Metab* 1999;2064-7.
25. Hak AE, Pols HAP, Visser TJ, Drexhage HA, Hofman A and Witteman JCM. Subclinical Hypothyroidism is an independent risk factor for atherosclerosis and myocardial infarction in elderly women: the Rotterdam Study. *Ann Intern Med* 2000;132:270-8.
26. Michalopoulou G, Alevizaki M, Piperigos G, Mitsibounas D, Mantzos E, Adamopoulos P et al. High serum cholesterol levels in persons with 'high-normal' TSH levels: should one extend the definition of subclinical hypothyroidism? *Eur J Endocrinol* 1998;138:141-5.
27. Beck-Peccoz P, Brucker-Davis F, Persani L, Smallridge RC and Weintraub BD. Thyrotropin-secreting pituitary tumors. *Endocrine Rev* 1996;17:610-38.
28. Brucker-Davis F, Oldfield EH, Skarulis MC, Doppman JL and Weintraub BD. Thyrotropin-secreting pituitary tumors: diagnostic criteria, thyroid hormone sensitivity and treatment outcome in 25 patients followed at the National Institutes of Health. *J Clin Endocrinol Metab* 76 1999;:1089-94.
29. Oliveira JH, Persani L, Beck-Peccoz P and Abucham J. Investigating the paradox of hypothyroidism and increased serum thyrotropin (TSH) levels in Sheehan's syndrome: characterization of TSH carbohydrate content and bioactivity. *J Clin Endocrinol Metab* 2001;86:1694-9.
30. Uy H, Reasner CA and Samuels MH. Pattern of recovery of the hypothalamic-pituitary thyroid axis following radioactive iodine therapy in patients with Graves' disease. *Amer J Med* 1995;99:173-9.
31. Hershman JM, Pekary AE, Berg L, Solomon DH and Sawin CT. Serum thyrotropin and thyroid hormone levels in elderly and middle-aged euthyroid persons. *J Am Geriatr Soc* 1993;41:823-8.
32. Fraser CG. Age-related changes in laboratory test results. Clinical applications. *Drugs Aging* 1993;3:246-57.
33. Fraser CG. 2001. Biological Variation: from principles to practice. AACC Press, Washington DC.
34. Drinka PJ, Siebers M and Voeks SK. Poor positive predictive value of low sensitive thyrotropin assay levels for hyperthyroidism in nursing home residents. *South Med J* 1993;86:1004-7.
35. Vanderpump MPJ, Tunbridge WMG, French JM, Appleton D, Bates D, Rodgers H et al. The incidence of thyroid disorders in the community; a twenty year follow up of the Whickham survey. *Clin Endocrinol* 1995;43:55-68.
36. Sawin CT, Geller A, Kaplan MM, Bacharach P, Wilson PW, Hershman JM et al. Low serum thyrotropin (thyroid stimulating hormone) in older persons without hyperthyroidism. *Arch Intern Med* 1991;151:165-8.
37. Parle JV, Maisonneuve P, Sheppard MC, Boyle P and Franklyn JA. Prediction of all-cause and cardiovascular mortality in elderly people from one low serum thyrotropin result: a 10-year study. *Lancet* 2001;358:861-5.
38. Nelson JC, Clark SJ, Borut DL, Tomei RT and Carlton EI. Age-related changes in serum free thyroxine during childhood and adolescence. *J Pediatr* 1993;123:899-905.
39. Adams LM, Emery JR, Clark SJ, Carlton EI and Nelson JC. Reference ranges for newer thyroid function tests in premature infants. *J Pediatr* 1995;126:122-7.
40. Lu FL, Yau KI, Tsai KS, Tang JR, Tsao PN and Tsai WY. Longitudinal study of serum free thyroxine and thyrotropin levels by chemiluminescent immunoassay during infancy. *T'aiwan Erh K'o i Hseh Hui Tsa Chih* 1999;40:255-7.
41. Zurawski D, Di Canzio J and Majzoub JA. Pediatric reference intervals for serum thyroxine, triiodothyronine, thyrotropin and free thyroxine. *Clin Chem* 1999;45:1087-91.
42. Fisher DA, Nelson JC, Carlton EI and Wilcox RB. Maturation of human hypothalamic-pituitary-thyroid function and control. *Thyroid* 2000;10:229-34.
43. Fisher DA, Schoen EJ, La Franchi S, Mandel SH, Nelson JC, Carlton EI and Goshi JH. The hypothalamic-pituitary-thyroid negative feedback control axis in children with treated congenital hypothyroidism. *J Clin Endocrinol Metab* 2000;85:2722-7.

44. Penny R, Spencer CA, Frasier SD and Nicoloff JT. Thyroid stimulating hormone (TSH) and thyroglobulin (Tg) levels decrease with chronological age in children and adolescents. *J Clin Endocrinol Metab* 1983;56:177-80.
45. Verheecke P. Free triiodothyronine concentration in serum of 1050 euthyroid children is inversely related to their age. *Clin Chem* 1997;43:963-7.
46. Glinioer D, De Nayer P, Bourdoux P, Lemone M, Robyn C, van Steirteghem A et al. Regulation of maternal thyroid function during pregnancy. *J Clin Endocrinol Metab* 1990;71:276-87.
47. Glinioer D. The regulation of thyroid function in pregnancy: pathways of endocrine adaptation from physiology to pathology. *Endocrinol Rev* 1997;18:404-33.
48. Weeke J, Dybkjaer L, Granlie K, Eskjaer Jensen S, Kjaerulff E, Laurberg P et al. A longitudinal study of serum TSH and total and free iodothyronines during normal pregnancy. *Acta Endocrinol* 1982;101:531-7.
49. Pedersen KM, Laurberg P, Iversen E, Knudsen PR, Gregersen HE, Rasmussen OS et al. Amelioration of some pregnancy associated variation in thyroid function by iodine supplementation. *J Clin Endocrinol Metab* 1993;77:1078-83.
50. Nohr SB, Jorgensen A, Pedersen KM and Laurberg P. Postpartum thyroid dysfunction in pregnant thyroid peroxidase antibody-positive women living in an area with mild to moderate iodine deficiency: Is iodine supplementation safe? *J Clin Endocrinol Metab* 2000;85:3191-8.
51. Panesar NS, Li CY and Rogers MS. Reference intervals for thyroid hormones in pregnant Chinese women. *Ann Clin Biochem* 2001;38:329-32.
52. Nissim M, Giorda G, Ballabio M, D'Alborton A, Bochiechio D, Orefice R et al. Maternal thyroid function in early and late pregnancy. *Horm Res* 1991;36:196-202.
53. Talbot JA, Lambert A, Anobile CJ, McLoughlin JD, Price A, Weetman AP et al. The nature of human chorionic gonadotropin glycoforms in gestational thyrotoxicosis. *Clin Endocrinol* 2001;55:33-9.
54. Jordan V, Grebe SK, Cooke RR, Ford HC, Larsen PD, Stone PR et al. Acidic isoforms of chorionic gonadotrophin in European and Samoan women are associated with hyperemesis gravidarum and may be thyrotrophic. *Clin Endocrinol* 1999;50:619-27.
55. Goodwin TM, Montoro M, Mestman JH, Pekary AE and Hershman JM. The role of chorionic gonadotropin in transient hyperthyroidism of hyperemesis gravidarum. *J Clin Endocrinol Metab* 1992;75:1333-7.
56. Hershman JM. Human chorionic gonadotropin and the thyroid: hyperemesis gravidarum and trophoblastic tumors. *Thyroid* 1999;9:653-7.
57. McElduff A. Measurement of free thyroxine (T4) in pregnancy. *Aust NZ J Obst Gynecol* 1999;39:158-61.
58. Christofides N, Wilkinson E, Stoddart M, Ray DC and Beckett GJ. Assessment of serum thyroxine binding capacity-dependent biases in free thyroxine assays. *Clin Chem* 1999;45:520-5.
59. Roti E, Gardini E, Minelli R, Bianconi L, Flisi M. Thyroid function evaluation by different commercially available free thyroid hormone measurement kits in term pregnant women and their newborns. *J Endocrinol Invest* 1991;14:1-9.
60. Stockigt JR. Free thyroid hormone measurement: a critical appraisal. *Endocrinol Metab Clin N Am* 2001;30:265-89.
61. Mandel SJ, Larsen PR, Seely EW and Brent GA. Increased need for thyroxine during pregnancy in women with primary hypothyroidism. *NEJM* 1990;323:91-6.
62. Burrow GN, Fisher DA and Larsen PR. Maternal and fetal thyroid function. *N Engl J Med* 1994;331:1072-8.
63. Pop VJ, De Vries E, Van Baar AL, Waelkens JJ, De Rooy HA, Horsten M et al. Maternal thyroid peroxidase antibodies during pregnancy: a marker of impaired child development? *J Clin Endocrinol Metab* 1995;80:3561-6.
64. Haddow JE, Palomaki GE, Allan WC, K. G. Williams JR, Gagnon J, O'Heir CE et al. Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *NEJM* 1999;341:549-55.
65. Pop VJ, Kuijpers JL, van Baar AL, Verkerk G, van Son MM, de Vijlder JJ et al. Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in infancy. *Clin Endocrinol* 1999;50:147-8.

66. Radetti G, Gentili L, Paganini C, Oberhofer R, Deluggi I and Delucca A. Psychomotor and audiological assessment of infants born to mothers with subclinical thyroid dysfunction in early pregnancy. *Minerva Pediatr* 2000;52:691-8.
67. Surks MI and Sievert R. Drugs and thyroid function. *NEJM* 1995;333:1688-94.
68. Kailajarvi M, Takala T, Gronroos P, Tryding N, Viikari J, Irjala K et al. Reminders of drug effects on laboratory test results. *Clin Chem* 2000;46:1395-1400.
69. Brabant A, Brabant G, Schuermeyer T, Ranft U, Schmidt FW, Hesch RD et al. The role of glucocorticoids in the regulation of thyrotropin. *Acta Endocrinol* 1989;121:95-100.
70. Samuels MH and McDaniel PA. Thyrotropin levels during hydrocortisone infusions that mimic fasting-induced cortisol elevations: a clinical research center study. *J Clin Endocrinol Metab* 1997;82:3700-4.
71. Kaptein EM, Spencer CA, Kamiel MB and Nicoloff JT. Prolonged dopamine administration and thyroid hormone economy in normal and critically ill subjects. *J Clin Endocrinol Metab* 1980;51:387-93.
72. Geffner DL and Hershman JM. Beta-adrenergic blockade for the treatment of hyperthyroidism. *Am J Med* 1992;93:61-8.
73. Meurisse M, Gollogly MM, Degauque C, Fumal I, Defechereux T and Hamoir E. Iatrogenic thyrotoxicosis: causal circumstances, pathophysiology and principles of treatment- review of the literature. *World J Surg* 2000;24:1377-85.
74. Martino E, Aghini-Lombardi F, Mariotti S, Bartelena L, Braverman LE and Pinchera A. Amiodarone: a common source of iodine-induced thyrotoxicosis. *Horm Res* 1987;26:158-71.
75. Martino E, Bartelena L, Bogazzi F and Braverman LE. The effects of amiodarone on the Thyroid. *Endoc Rev* 2001;22:240-54.
76. Daniels GH. Amiodarone-induced thyrotoxicosis. *J Clin Endocrinol Metab* 2001;86:3-8.
77. Harjai KJ and Licata AA. Effects of amiodarone on thyroid function. *Ann Intern Med* 1997;126:63-73.
78. Caron P. Effect of amiodarone on thyroid function. *Press Med* 1995;24:1747-51.
79. Bartelena L, Grasso L, Brogioni S, Aghini-Lombardi F, Braverman LE and Martino E. Serum interleukin-6 in amiodarone-induced thyrotoxicosis. *J Clin Endocrinol Metab* 1994;78:423-7.
80. Eaton SE, Euinton HA, Newman CM, Weetman AP and Bennet WM. Clinical experience of amiodarone-induced thyrotoxicosis over a 3-year period: role of colour-flow Doppler sonography. *Clin Endocrinol* 2002;56:33-8.
81. Lazarus JH. The effects of lithium therapy on thyroid and thyrotropin-releasing hormone. *Thyroid* 1998;8:909-13.
82. Kusalic M and Engelsmann F. Effect of lithium maintenance therapy on thyroid and parathyroid function. *J Psych Neurosci* 1999;24:227-33.
83. Oakley PW, Dawson AH and Whyte IM. Lithium: thyroid effects and altered renal handling. *Clin Toxicol* 2000;38:333-7.
84. Mendel CM, Frost PH, Kunitake ST and Cavalieri RR. Mechanism of the heparin-induced increase in the concentration of free thyroxine in plasma. *J Clin Endocrinol Metab* 1987;65:1259-64.
85. Iitaka M, Kawasaki S, Sakurai S, Hara Y, Kuriyama R, Yamanaka K et al. Serum substances that interfere with thyroid hormone assays in patients with chronic renal failure. *Clin Endocrinol* 1998;48:739-46.
86. Bowie LJ, Kirkpatrick PB and Dohnal JC. Thyroid function testing with the TDx: Interference from endogenous fluorophore. *Clin Chem* 1987;33:1467.
87. DeGroot LJ and Mayor G. Admission screening by thyroid function tests in an acute general care teaching hospital. *Amer J Med* 1992;93:558-64.
88. Kaptein EM. Thyroid hormone metabolism and thyroid diseases in chronic renal failure. *Endocrinol Rev* 1996;17:45-63.
89. Van den Berghe G, De Zegher F and Bouillon R. Acute and prolonged critical illness as different neuroendocrine paradigms. *J Clin Endocrinol Metab* 1998;83:1827-34.
90. Van den Berhe G. Novel insights into the neuroendocrinology of critical illness. *Eur J Endocrinol* 2000;143:1-13.
91. Wartofsky L and Burman KD. Alterations in thyroid function in patients with systemic illness: the "euthyroid sick syndrome". *Endocrinol Rev* 1982;3:164-217.

92. Spencer CA, Eigen A, Duda M, Shen D, Qualls S, Weiss S et al. Sensitive TSH tests - specificity limitations for screening for thyroid disease in hospitalized patients. *Clin Chem* 1987;33:1391-1396.
93. Stockigt JR. Guidelines for diagnosis and monitoring of thyroid disease: nonthyroidal illness. *Clin Chem* 1996;42:188-92.
94. Nelson JC and Weiss RM. The effects of serum dilution on free thyroxine (T₄) concentration in the low T₄ syndrome of nonthyroidal illness. *J Clin Endocrinol Metab* 1985;61:239-46.
95. Chopra IJ, Huang TS, Beredo A, Solomon DH, Chua Teco GN. Serum thyroid hormone binding inhibitor in non thyroidal illnesses. *Metabolism* 1986;35:152-9.
96. Wang R, Nelson JC and Wilcox RB. Salsalate administration - a potential pharmacological model of the sick euthyroid syndrome. *J Clin Endocrinol Metab* 1998;83:3095-9.
97. Sapin R, Schliener JL, Kaltenbach G, Gasser F, Christofides N, Roul G et al. Determination of free triiodothyronine by six different methods in patients with non-thyroidal illness and in patients treated with amiodarone. *Ann Clin Biochem* 1995;32:314-24.
98. Docter R, van Toor H, Krenning EP, de Jong M and Hennemann G. Free thyroxine assessed with three assays in sera of patients with nonthyroidal illness and of subjects with abnormal concentrations of thyroxine-binding proteins. *Clin Chem* 1993;39:1668-74.
99. Wilcox RB, Nelson JC and Tomei RT. Heterogeneity in affinities of serum proteins for thyroxine among patients with non-thyroidal illness as indicated by the serum free thyroxine response to serum dilution. *Eur J Endocrinol* 1994;131:9-13.
100. Liewendahl K, Tikanoja S, Mahonen H, Helenius T, Valimäki M and Tallgren LG. Concentrations of iodothyronines in serum of patients with chronic renal failure and other nonthyroidal illnesses: role of free fatty acids. *Clin Chem* 1987;33:1382-6.
101. Sapin R, Schlienger JL, Gasser F, Noel E, Lioure B, Grunenberger F. Intermethod discordant free thyroxine measurements in bone marrow-transplanted patients. *Clin Chem* 2000;46:418-22.
102. Chopra IJ. Simultaneous measurement of free thyroxine and free 3,5,3'-triiodothyronine in undiluted serum by direct equilibrium dialysis/radioimmunoassay: evidence that free triiodothyronine and free thyroxine are normal in many patients with the low triiodothyronine syndrome. *Thyroid* 1998;8:249-57.
103. Hamblin PS, Dyer SA, Mohr VS, Le Grand BA, Lim C-F, Tuxen DB, Topliss DJ and Stockigt JR. Relationship between thyrotropin and thyroxine changes during recovery from severe hypothyroxinemia of critical illness. *J Clin Endocrinol Metab* 1986;62:717-22.
104. Brent GA and Hershman JM. Thyroxine therapy in patients with severe nonthyroidal illnesses and low serum thyroxine concentrations. *J Clin Endocrinol Metab* 1986;63:1-8.
105. De Groot LJ. Dangerous dogmas in medicine: the nonthyroidal illness syndrome. *J Clin Endocrinol Metab* 1999;84:151-64.
106. Burman KD and Wartofsky L. Thyroid function in the intensive care unit setting. *Crit Care Clin* 2001;17:43-57.
107. Behrend EN, Kemppainen RJ and Young DW. Effect of storage conditions on cortisol, total thyroxine and free thyroxine concentrations in serum and plasma of dogs. *J Am Vet Med Assoc* 1998;212:1564-8.
108. Oddie TH, Klein AH, Foley TP and Fisher DA. Variation in values for iodothyronine hormones, thyrotropin and thyroxine binding globulin in normal umbilical-cord serum with season and duration of storage. *Clin Chem* 1979;25:1251-3.
109. Koliakos G, Gaitatzi M and Grammaticos P. Stability of serum TSH concentration after non refrigerated storage. *Minerva Endocrinol* 1999;24:113-5.
110. Waite KV, Maberly GF and Eastman CJ. Storage conditions and stability of thyrotropin and thyroid hormones on filter paper. *Clin Chem* 1987;33:853-5.
111. Levinson SS. The nature of heterophilic antibodies and their role in immunoassay interference. *J Clin Immunoassay* 1992;15:108-15.
112. Norden AGM, Jackson RA, Norden LE, Griffin AJ, Barnes MA and Little JA. Misleading results for immunoassays of serum free thyroxine in the presence of rheumatoid factor. *Clin Chem* 1997;43:957-62.
113. Covinsky M, Laterza O, Pfeifer JD, Farkas-Szallasi T and Scott MG. Lambda antibody to *Escherichia coli* produces false-positive results in multiple immunometric assays. *Clin Chem* 2000;46:1157-61.

114. Martel J, Despres N, Ahnadi CE, Lachance JF, Monticello JE, Fink G, Ardemagni A, Banfi G, Tovey J, Dykes P, John R, Jeffery J and Grant AM. Comparative multicentre study of a panel of thyroid tests using different automated immunoassay platforms and specimens at high risk of antibody interference. *Clin Chem Lab Med* 2000;38:785-93.
115. Howanitz PJ, Howanitz JH, Lamberson HV and Ennis KM. Incidence and mechanism of spurious increases in serum Thyrotropin. *Clin Chem* 1982;28:427-31.
116. Boscatto, L. M. and M. C. Stuart. Heterophilic antibodies: a problem for all immunoassays. *Clin Chem* 1988;34:27-33.
117. Kricka LJ. Human anti-animal antibody interference in immunological assays. *Clin Chem* 1999;45:942-56.
118. Sapin R and Simon C. False hyperprolactinemia corrected by the use of heterophilic antibody-blocking agent. *Clin Chem* 2001;47:2184-5.
119. Feldt-Rasmussen U, Petersen PH, Blaabjerg O and Horder M. Long-term variability in serum thyroglobulin and thyroid related hormones in healthy subjects. *Acta Endocrinol (Copenh)* 1980;95:328-34.
120. Browning MCK, Ford RP, Callaghan SJ and Fraser CG. Intra-and interindividual biological variation of five analytes used in assessing thyroid function: implications for necessary standards of performance and the interpretation of results. *Clin Chem* 1986;32:962-6.
121. Lum SM and Nicoloff JT. Peripheral tissue mechanism for maintenance of serum triiodothyronine values in a thyroxine-deficient state in man. *J Clin Invest* 1984;73:570-5.
122. Spencer CA and Wang CC. Thyroglobulin measurement- Techniques, clinical benefits and pitfalls. *Endocrinol Metab Clin N Amer* 1995;24:841-63.
123. Weeke J and Gundersen HJ. Circadian and 30 minute variations in serum TSH and thyroid hormones in normal subjects. *Acta Endocrinol* 1978;89:659-72.
124. Brabant G, Prank K, Hoang-Vu C and von zur Muhlen A. Hypothalamic regulation of pulsatile thyrotropin secretion. *J Clin Endocrinol Metab* 1991;72:145-50.
125. Fraser CG, Petersen PH, Ricos C and Haeckel R. Proposed quality specifications for the imprecision and inaccuracy of analytical systems for clinical chemistry. *Eur J Clin Chem Biochem* 1992;30:311-7.
126. Rodbard, D. Statistical estimation of the minimal detectable concentration ("sensitivity") for radioligand assays. *Anal Biochem* 1978;90:1-12.
127. Ekins R and Edwards P. On the meaning of "sensitivity". *Clin Chem* 1997;43:1824-31.
128. Fuentes-Arderiu X and Fraser CG. Analytical goals for interference. *Ann Clin Biochem* 1991;28:393-5.
129. Petersen PH, Fraser CG, Westgard JO and Larsen ML. Analytical goal-setting for monitoring patients when two analytical methods are used. *Clin Chem* 1992;38:2256-60.
130. Fraser CG and Petersen PH. Desirable standards for laboratory tests if they are to fulfill medical needs. *Clin Chem* 1993;39:1453-5.
131. Stockl D, Baadenhuijsen H, Fraser CG, Libeer JC, Petersen PH and Ricos C. Desirable routine analytical goals for quantities assayed in serum. Discussion paper from the members of the external quality assessment (EQA) Working Group A on analytical goals in laboratory medicine. *Eur J Clin Chem Clin Biochem* 1995;33:157-69.
132. Plebani M, Giacomini A, Beghi L, de Paoli M, Roveroni G, Galeotti F, Corsini A and Fraser CG. Serum tumor markers in monitoring patients: interpretation of results using analytical and biological variation. *Anticancer Res* 1996;16:2249-52.
133. Browning MC, Bennet WM, Kirkaldy AJ and Jung RT. Intra-individual variation of thyroxine, triiodothyronine and thyrotropin in treated hypothyroid patients: implications for monitoring replacement therapy. *Clin Chem* 1988;34:696-9.
134. Harris EK. Statistical principles underlying analytic goal-setting in clinical chemistry. *Am J Clin Pathol* 1979;72:374-82.
135. Nelson JC and Wilcox RB. Analytical performance of free and total thyroxine assays. *Clin Chem* 1996;42:146-54.
136. Evans SE, Burr WA and Hogan TC. A reassessment of 8-anilino-1-naphthalene sulphonic acid as a thyroxine binding inhibitor in the radioimmunoassay of thyroxine. *Ann Clin Biochem* 1977;14:330-4.
137. Karapitta CD, Sotiroidis TG, Papadimitriou A and Xenakis A. Homogeneous enzyme immunoassay for triiodothyronine in serum. *Clin Chem* 2001;47:569-74.

138. De Brabandere VI, Hou P, Stockl D, Theinpont LM and De Leenheer AP. Isotope dilution-liquid chromatography/electrospray ionization-tandem mass spectrometry for the determination of serum thyroxine as a potential reference method. *Rapid Commun Mass Spectrom* 1998;12:1099-103.
139. Tai SSC, Sniegowski LT and Welch MJ. Candidate reference method for total thyroxine in human serum: Use of isotope-dilution liquid chromatography-mass spectrometry with electrospray ionization. *Clin Chem* 2002;48:637-42.
140. Thienpont LM, Fierens C, De Leenheer AP and Przywara L. Isotope dilution-gas chromatography/mass spectrometry and liquid chromatography/electro-spray ionization-tandem mass spectrometry for the determination of triiodo-L-thyronine in serum. *Rapid Commun Mass Spectrom* 1999;13:1924-31.
141. Sarne DH, Refetoff S, Nelson JC and Linarelli LG. A new inherited abnormality of thyroxine-binding globulin (TBG-San Diego) with decreased affinity for thyroxine and triiodothyronine. *J Clin Endocrinol Metab* 1989;68:114-9.
142. Schussler GC. The thyroxine-binding proteins. *Thyroid* 2000;10:141-9.
143. Beck-Peccoz P, Romelli PB, Cattaneo MG, Faglia G, White EL, Barlow JW et al. Evaluation of free T4 methods in the presence of iodothyronine autoantibodies. *J Clin Endocrinol Metab* 1984;58:736-9.
144. Sakata S, Nakamura S and Miura K. Autoantibodies against thyroid hormones or iodothyronine. *Ann Intern Med* 1985;103:579-89.
145. Despres N and Grant AM. Antibody interference in thyroid assays: a potential for clinical misinformation. *Clin Chem* 1998;44:440-54.
146. Hay ID, Bayer MF, Kaplan MM, Klee GG, Larsen PR and Spencer CA. American Thyroid Association Assessment of Current Free Thyroid Hormone and Thyrotropin Measurements and Guidelines for Future Clinical Assays. *Clin Chem* 1991;37:2002-2008.
147. Ekins R. The science of free hormone measurement. *Proc UK NEQAS Meeting* 1998;3:35-59.
148. Wang R, Nelson JC, Weiss RM and Wilcox RB. Accuracy of free thyroxine measurements across natural ranges of thyroxine binding to serum proteins. *Thyroid* 2000;10:31-9.
149. Nelson JC, Wilcox BR and Pandian MR. Dependence of free thyroxine estimates obtained with equilibrium tracer dialysis on the concentration of thyroxine-binding globulin. *Clin Chem* 1992;38:1294-1300.
150. Ekins R. The free hormone hypothesis and measurement of free hormones. *Clin Chem* 1992;38:1289-93.
151. Ekins RP. Ligand assays: from electrophoresis to miniaturized microarrays. *Clin Chem* 1998;44:2015-30.
152. Ekins R. Analytic measurements of free thyroxine. *Clin Lab Med* 1993;13:599-630.
153. Nusynowitz, M. L. Free-thyroxine index. *JAMA* 1975;232:1050.
154. Larsen PR, Alexander NM, Chopra IJ, Hay ID, Hershman JM, Kaplan MM et al. Revised nomenclature for tests of thyroid hormones and thyroid-related proteins in serum. *J Clin Endocrinol Metab* 1987;64:1089-94.
155. Burr WA, Evans SE, Lee J, Prince HP, Ramsden DB. The ratio of thyroxine to thyroxine-binding globulin measurement in the evaluation of thyroid function. *Clin Endocrinol* 1979;11:333-42.
156. Attwood EC and Atkin GE. The T4: TBG ratio: a re-evaluation with particular reference to low and high serum TBG levels. *Ann Clin Biochem* 1982;19:101-3.
157. Szpunar WE, Stoffer SS and DiGiulio W. Clinical evaluation of a thyroxine binding globulin assay in calculating a free thyroxine index in normal, thyroid disease and sick euthyroid patients. *J Nucl Med* 1987;28:1341-3.
158. Nelson JC and Tomei RT. Dependence of the thyroxin/thyroxin-binding globulin (TBG) ratio and the free thyroxin index on TBG concentrations. *Clin Chem* 1989;35:541-4.
159. Sterling K and Brenner MA. Free thyroxine in human serum: Simplified measurement with the aid of magnesium precipitation. *J Clin Invest* 1966;45:153-60.
160. Schulssler GC and Plager JE. Effect of preliminary purification of ¹³¹I-Thyroxine on the determination of free thyroxine in serum. *J Clin Endocrinol* 1967;27:242-50.
161. Nelson JC and Tomei RT. A direct equilibrium dialysis/radioimmunoassay method for the measurement of free thyroxin in undiluted serum. *Clin Chem* 1988;34:1737-44.
162. Tikanoja SH. Ultrafiltration devices tested for use in a free thyroxine assay validated by comparison with equilibrium dialysis. *Scand J Clin Lab Invest* 1990;50:663-9.

163. Ellis SM and Ekins R. Direct measurement by radioimmunoassay of the free thyroid hormone concentrations in serum. *Acta Endocrinol (Suppl)* 1973;177:106-110.
164. Weeke J and Orskov H. Ultrasensitive radioimmunoassay for direct determination of free triiodothyronine concentration in serum. *Scand J Clin Lab Invest* 1975;35:237-44.
165. Surks MI, Hupart KH, Chao P and Shapiro LE. Normal free thyroxine in critical nonthyroidal illnesses measured by ultrafiltration of undiluted serum and equilibrium dialysis. *J Clin Endocrinol Metab* 1988;67:1031-9.
166. Holm SS andreasen L, Hansen SH, Faber J and Staun-Olsen P. Influence of adsorption and deproteinization on potential free thyroxine reference methods. *Clin Chem* 2002;48:108-114.
167. Jaume JC, Mendel CM, Frost PH, Greenspan FS, Loughton CW. Extremely low doses of heparin release lipase activity into the plasma and can thereby cause artifactual elevations in the serum-free thyroxine concentrations as measured by equilibrium dialysis. *Thyroid* 1996;6:79-83.
168. Stevenson HP, Archbold GP, Johnston P, Young IS, Sheridan B. Misleading serum free thyroxine results during low molecular weight heparin treatment. *Clin Chem* 1998;44:1002-7.
169. Laji K, Rhidha B, John R, Lazarus J and Davies JS. Artifactual elevations in serum free thyroxine and triiodothyronine concentrations during heparin therapy. *QJM* 2001;94:471-3.
170. Lim CF, Bai Y, Topliss DJ, Barlow JW and Stockigt JR. Drug and fatty acid effects on serum thyroid hormone binding. *J Clin Endocrinol Metab* 1988;67:682-8.
171. Czako G, M. H. Zweig, C. Benson and M. Ruddel. On the albumin-dependence of measurements of free thyroxine. II Patients with non-thyroidal illness. *Clin Chem* 1987;33:87-92.
172. Csako G, Zweig MH, Glickman J, Ruddel M and K. J. Direct and indirect techniques for free thyroxine compared in patients with nonthyroidal illness. II. Effect of prealbumin, albumin and thyroxine-binding globulin. *Clin Chem* 1989;35:1655-62.
173. Csako G, Zweig MH, Glickman J, Kestner J and Ruddel M. Direct and indirect techniques for free thyroxine compared in patients with nonthyroidal illness. I. Effect of free fatty acids. *Clin Chem* 1989;35:102-9.
174. Ross HA and Benraad TJ. Is free thyroxine accurately measurable at room temperature? *Clin Chem* 1992;38:880-6.
175. Van der Sluijs Veer G, Vermes I, Bonte HA and Hoorn RKJ. Temperature effects on Free Thyroxine Measurement: Analytical and Clinical Consequences. *Clin Chem* 1992;38:1327-31.
176. Fisher DA. The hypothyroxinemia of prematurity. *J Clin Endocrinol Metab* 1997;82:1701-3.
177. Stockigt JR, Stevens V, White EL and Barlow JW. Unbound analog radioimmunoassays for free thyroxine measure the albumin-bound hormone fraction. *Clin Chem* 1983;29:1408-10.
178. Aravelo G. Prevalence of familial dysalbuminemic hyperthyroxinemia in serum samples received for thyroid testing. *Clin Chem* 1991;37:1430-1.
179. Sapin R and Gasser F. Anti-solid phase antibodies interfering in labeled-antibody assays for free thyroid hormones. *Clin Chem* 1995;45:1790-1.
180. Inada M and Sterling K. Thyroxine transport in thyrotoxicosis and hypothyroidism. *J Clin Invest* 1967;46:1442-50.
181. Lueprasitsakul W, Alex S, Fang SL, Pino S, Imscher K, Kohrle J et al. Flavonoid administration immediately displaces thyroxine (T₄) from serum transthyretin, increases serum free T₄ and decreases serum thyrotropin in the rat. *Endocrinol* 1990;126:2890-5.
182. Stockigt JR, Lim CF, Barlow J, Stevens V, Topliss DJ, Wynne KN. High concentrations of furosemide inhibit plasma binding of thyroxine. *J Clin Endocrinol Metab* 1984;59:62-6.
183. Hawkins RC. Furosemide interference in newer free thyroxine assays. *Clin Chem* 1998;44:2550-1.
184. Wang R, Nelson JC and Wilcox RB. Salsalate and salicylate binding to and their displacement of thyroxine from thyroxine-binding globulin, transthyrin and albumin. *Thyroid* 1999;9:359-64.
185. Munro SL, Lim C-F, Hall JG, Barlow JW, Craik DJ, Topliss DJ and Stockigt JR. Drug competition for thyroxine binding to transthyretin (prealbumin): comparison with effects on thyroxine-binding globulin. *J Clin Endocrinol Metab* 1989;68:1141-7.
186. Stockigt JR, Lim C-F, Barlow JW and Topliss DJ. 1997. *Thyroid hormone transport*. Springer Verlag, Heidelberg. 119 pp.
187. Surks MI and Defesi CR. Normal free thyroxine concentrations in patients treated with phenytoin or carbamazepine: a paradox resolved. *JAMA* 1996;275:1495-8.

188. Ross HA. A dialysis method for the measurement of free iodothyronine and steroid hormones in blood. *Experientia* 1978;34:538-9.
189. Sapin R. Serum thyroxine binding capacity-dependent bias in five free thyroxine immunoassays: assessment with serum dilution experiments and impact on diagnostic performance. *Clin Biochem* 2001;34:367-71.
190. Law LK, Cheung CK and Swaminathan R. Falsely high thyroxine results by fluorescence polarization in sera with high background fluorescence. *Clin Chem* 1988;34:1918.
191. Kricka LJ. Interferences in Immunoassay - still a threat. *Clin Chem* 2000;46:1037-8.
192. McBride JH, Rodgerson DO and Allin RE. Choriogonadotrophin interference in a sensitive assay for Thyrotropin. *Clin Chem* 1987;33:1303-4.
193. Ritter D, Stott R, Grant N and Nahm MH. Endogenous antibodies that interfere with Thyroxine fluorescence polarization assay but not with radioimmunoassay or EMIT. *Clin Chem* 1993;39:508-11.
194. DeGroot LJ, Larsen PR, Refetoff S and Stanbury JB. *The Thyroid and its Diseases*. Fifth Edition, 1984; John Wiley & Sons, Inc., New York:266-7.
195. Beck-Peccoz P, Amr S, Menezes-Ferreira NM, Faglia G and Weintraub BD. Decreased receptor binding of biologically inactive thyrotropin in central hypothyroidism: effect of treatment with thyrotropin-releasing hormone. *N Engl J Med* 1985;312:1085-90.
196. Beck-Peccoz P and Persani L. Variable biological activity of thyroid-stimulating hormone. *Eur J Endocrinol* 1994;131:331-40.
197. Persani L, Ferretti E, Borgato S, Faglia G and Beck-Peccoz P. Circulating thyrotropin bioactivity in sporadic central hypothyroidism. *J Clin Endocrinol Metab* 2000;85:3631-5.
198. Rafferty B and Gaines Das R. Comparison of pituitary and recombinant human thyroid-stimulating hormone (rhTSH) in a multicenter collaborative study: establishment of the first World Health Organization reference reagent for rhTSH. *Clin Chem* 1999;45:2207-15.
199. Persani L, Borgato S, Romoli R, Asteria C, Pizzocaro A and Beck-Peccoz P. Changes in the degree of sialylation of carbohydrate chains modify the biological properties of circulating thyrotropin isoforms in various physiological and pathological states. *J Clin Endocrinol Metab* 1998;83:2486-92.
200. Gershengorn MC and Weintraub BD. Thyrotropin-induced hyperthyroidism caused by selective pituitary resistance to thyroid hormone. A new syndrome of "inappropriate secretion of TSH". *J Clin Invest* 1975;56:633-42.
201. Faglia G, Beck-Peccoz P, Piscitelli G and Medri G. Inappropriate secretion of thyrotropin by the pituitary. *Horm Res* 1987;26:79-99.
202. Spencer CA, Takeuchi M and Kazarosyan M. Current status and performance goals for serum thyrotropin (TSH) assays. *Clinical Chemistry* 1996;42:141-145.
203. Laurberg P. Persistent problems with the specificity of immunometric TSH assays. *Thyroid* 1993;3:279-83.
204. Spencer CA, Schwarzbein D, Guttler RB, LoPresti JS and Nicoloff JT. TRH stimulation test responses employing third and fourth generation TSH assays. *J Clin Endocrinol Metab* 1993;76:494-498.
205. Vogeser M, Weigand M, Fraunberger P, Fischer H and Cremer P. Evaluation of the ADVIA Centaur TSH-3 assay. *Clin Chem Lab Med* 2000;38:331-4.
206. Spencer CA, Takeuchi M, Kazarosyan M, MacKenzie F, Beckett GJ and Wilkinson E. Interlaboratory/intermethod differences in functional sensitivity of immunometric assays for thyrotropin (TSH): impact on reliability of measurement of subnormal concentration. *Clin Chem* 1995;41:367-74.
207. Tunbridge WM, Evered DC, Hall R, Appleton D, Brewis M, Clark F, Evans JG, Young E, Bird T and Smith PA. The spectrum of thyroid disease in a community: the Whickham survey. *Clin Endocrinol* 1977;7:481-93.
208. Rago T, Chiovato L, Grasso L, Pinchera A and Vitti P. Thyroid ultrasonography as a tool for detecting thyroid autoimmune diseases and predicting thyroid dysfunction in apparently healthy subjects. *J Endocrinol Invest* 2001;24:763-9.
209. Hershman JM and Pittman JA. Utility of the radioimmunoassay of serum thyrotropin in man. *Ann Intern Med* 1971;74:481-90.
210. Becker DV, Bigos ST, Gaitan E, Morris JC, Rallison ML, Spencer CA, Sugawara M, Middlesworth LV and Wartofsky L. Optimal use of blood tests for assessment of thyroid function. *JAMA* 1993;269:2736.

211. Canaris GJ, Manowitz NR, Mayor G and Ridgway EC. The Colorado Thyroid Disease Prevalence Study. *Arch Intern Med* 2000;160:19-27.
212. Skamene A and Patel YC. Infusion of graded concentrations of somatostatin in man: pharmacokinetic and differential inhibitory effects on pituitary and islet hormones. *Clin Endocrinol* 1984;20:555-64.
213. Berghout A, Wiersinga WM, Smits NJ and Touber JL. Interrelationships between age, thyroid volume, thyroid nodularity and thyroid function in patients with sporadic nontoxic goiter. *Am J Med* 1990;89:602-8.
214. Parle JV, Franklyn JA, Cross KW, Jones SC and Sheppard MC. Prevalence and follow-up of abnormal thyrotropin (TSH) concentrations in the elderly in the United Kingdom. *Clin Endocrinol* 1991;34:77-83.
215. Danese D, Sciacchitano S, Farsetti A, Andreoli M and Pontecorvi A. Diagnostic accuracy of conventional versus sonography-guided fine-needle aspiration biopsy of thyroid nodules. *Thyroid* 1998;8:15-21.
216. McDermott MT and Ridgway EC. Subclinical hypothyroidism is mild thyroid failure and should be treated. *J Clin Endocrinol Metab* 2001;86:4585-90.
217. Chu JW and Crapo LM. The treatment of subclinical hypothyroidism is seldom necessary. *J Clin Endocrinol Metab* 2001;86:4591-9.
218. Lewis GF, Alessi CA, Imperial JG and Refetoff S. Low serum free thyroxine index in ambulating elderly is due to a resetting of the threshold of thyrotropin feedback suppression. *JCEM* 1991;73:843-9.
219. Pearce CJ and Himsworth RL. Total and free thyroid hormone concentrations in patients receiving maintenance replacement treatment with thyroxine. *Br Med J* 1984;288:693-5.
220. Fish LH, Schwarz HL, Cavanaugh MD, Steffes MW, Bantle JP, Oppenheimer JH. Replacement dose, metabolism and bioavailability of levothyroxine in the treatment of hypothyroidism. *N Engl J Med* 1987;316:764-70.
221. Sawin CT, Herman T, Molitch ME, London MH and Kramer SM. Aging and the thyroid. Decreased requirement for thyroid hormone in older hypothyroid patients. *Amer J Med* 1983;75:206-9.
222. Davis FB, LaMantia RS, Spaulding SW, Wemann RE and Davis PJ. Estimation of a physiologic replacement dose of levothyroxine in elderly patients with hypothyroidism. *Arch Intern Med* 1984;144.
223. Arafah BM. Estrogen therapy may necessitate an increase in thyroxine dose for hypothyroidism. *NEJM* 2001;344:1743-9.
224. Scheithauer BW, Kovacs K, Randall RV and Ryan N. Pituitary gland in hypothyroidism. Histologic and immunocytologic study. *Arch Pathol Lab Med* 1985;109:499-504.
225. Ain KB, Pucino F, Shiver T and Banks SM. Thyroid hormone levels affected by time of blood sampling in thyroxine-treated patients. *Thyroid* 1993;3:81-5.
226. Chorazy PA, Himelhoch S, Hopwood NJ, Greger NG and Postellon DC. Persistent hypothyroidism in an infant receiving a soy formula: case report and review of the literature. *Pediatrics* 1995;96:148-50.
227. Dulgeroff AJ and Hershman JM. Medical therapy for differentiated thyroid carcinoma. *Endocrinol Rev* 1994;15:500-15.
228. Pujol P, Daures JP, Nsakala N, Baldet L, Bringer J and Jaffiol C. Degree of thyrotropin suppression as a prognostic determinant in differentiated thyroid cancer. *J Clin Endocrinol Metab* 1996;81:4318-23.
229. Cooper DS, Specker B, Ho M, Sperling M, Ladenson PW, Ross DS, Ain KB, Bigos ST, Brierley JD, Haugen BR, Klein I, Robbins J, Sherman SI, Taylor T and Maxon HR 3rd. Thyrotropin suppression and disease progression in patients with differentiated thyroid cancer: results from the National thyroid Cancer Treatment Cooperative Registry. *Thyroid* 1999;8:737-44.
230. Hurley DL and Gharib H. Evaluation and management of multinodular goiter. *Otolaryngol Clin North Am* 1996;29:527-40.
231. Bayer MF, Macoviak JA and McDougall IR. Diagnostic performance of sensitive measurements of serum thyrotropin during severe nonthyroidal illness: Their role in the diagnosis of hyperthyroidism. *Clin Chem* 1987;33:2178-84.
232. Lum SM, Kaptein EM and Nicoloff JT. Influence of nonthyroidal illnesses on serum thyroid hormone indices in hyperthyroidism. *West J Med* 1983;138:670-5.
233. Faglia G, Bitensky L, Pinchera A, Ferrari C, Paracchi A, Beck-Peccoz P, Ambrosi B and Spada A. Thyrotropin secretion in patient with central hypothyroidism: Evidence for reduced biological activity of immunoreactive thyrotropin. *J Clin Endocrinol Metab* 1979;48:989-98.

234. Faglia G, Beck-Peccoz P, Ballabio M and Nava C. Excess of beta-subunit of thyrotropin (TSH) in patients with idiopathic central hypothyroidism due to the secretion of TSH with reduced biological activity. *J Clin Endocrinol Metab* 1983;56:908-14.
235. Faglia G. The clinical impact of the thyrotropin-releasing hormone test. *Thyroid* 1998;8:903-8.
236. Trejbal D, Sulla I, Trejbalova L, Lazurova I, Schwartz P and Machanova Y. Central hypothyroidism - various types of TSH responses to TRH stimulation. *Endocr Regul* 1994;28:35-40.
237. Faglia G, Ferrari C, Paracchi A, Spada A and Beck-Peccoz P. Triiodothyronine response to thyrotropin releasing hormone in patients with hypothalamic-pituitary disorders. *Clin Endocrinol* 1975;4:585-90.
238. Horimoto M, Nishikawa M, Ishihara T, Yoshikawa N, Yoshimura M and Inada M. Bioactivity of thyrotropin (TSH) in patients with central hypothyroidism: comparison between in vivo 3,5,3'-triiodothyronine response to TSH and in vitro bioactivity of TSH. *J Clin Endocrinol Metab* 1995;80:1124-8.
239. Refetoff S, Weiss RE and Usala SJ. The syndromes of resistance to thyroid hormone. *Endocr Rev* 1993;14:348-99.
240. Weiss RE, Hayashi Y, Nagaya T, Petty KJ, Murata Y, Tunca H, Seo H and Refetoff S. Dominant inheritance of resistance to thyroid hormone not linked to defects in the thyroid hormone receptors alpha or beta genes may be due to a defective co-factor. *J Clin Endocrinol Metab* 1996;81:4196-203.
241. Snyder D, Sesser D, Skeels M et al. Thyroid disorders in newborn infants with elevated screening T4. *Thyroid* 1997;7 (Suppl 1):S1-29 (abst).
242. Refetoff S. 2000. Resistance to Thyroid Hormone. *In The Thyroid*. Braverman LE and Utiger RD, editor. Lippincott Williams & Wilkins, Philadelphia. 1028-43.
243. Beck-Peccoz P and Chatterjee VKK. The variable clinical phenotype in thyroid hormone resistance syndrome. *Thyroid* 1994;4:225-32.
244. Persani L, Asteria C, Tonacchera M, Vitti P, Krishna V, Chatterjee K and Beck-Peccoz P. Evidence for the secretion of thyrotropin with enhanced bioactivity in syndromes of thyroid hormone resistance. *J Clin Endocrinol Metab* 1994;78:1034-9.
245. Sarne DH, Sobieszczek S, Ain KB and Refetoff S. Serum thyrotropin and prolactin in the syndrome of generalized resistance to thyroid hormone: responses to thyrotrophin-releasing hormone stimulation and triiodothyronine suppression. *J Clin Endocrinol Metab* 1990;70:1305-11.
246. Ercan-Fang S, Schwartz HL, Mariash CN and Oppenheimer JH. Quantitative assessment of pituitary resistance to thyroid hormone from plots of the logarithm of thyrotropin versus serum free thyroxine index. *J Clin Endocrinol Metab* 2000;85:2299-303.
247. Safer JD, Colan SD, Fraser LM and Wondisford FE. A pituitary tumor in a patient with thyroid hormone resistance: a diagnostic dilemma. *Thyroid* 2001;11:281-91.
248. Marcocci C and Chiovato L. 2000. Thyroid -directed antibodies. *In Thyroid*. B. L. a. U. RD, editor. Lippincott Williams and Wilkins, Philadelphia. 414-31.
249. Chiovato L, Bassi P, Santini F, Mammoli C, Lapi P, Carayon P and Pinchera A. Antibodies producing complement-mediated thyroid cytotoxicity in patients with atrophic or goitrous autoimmune thyroiditis. *J Clin Endocrinol Metab* 1993;77:1700-5.
250. Guo J, Jaume JC, Rapoport B and McLachlan SM. Recombinant thyroid peroxidase-specific Fab converted to immunoglobulin G (IgG) molecules: evidence for thyroid cell damage by IgG1, but not IgG4, autoantibodies. *J Clin Endocrinol Metab* 1997;82:925-31.
251. Doullay F, Ruf J, Codaccioni JL and Carayon P. Prevalence of autoantibodies to thyroperoxidase in patients with various thyroid and autoimmune diseases. *Autoimmunity* 1991;9:237-44.
252. Radetti G, Persani L, Moroder , Cortelazzi D, Gentili L, Beck-Peccoz P. Transplacental passage of anti-thyroid autoantibodies in a pregnant woman with auto-immune thyroid disease. *Prenatal Diagnosis* 1999;19:468-71.
253. Heithorn R, Hauffa BP and Reinwein D. Thyroid antibodies in children of mothers with autoimmune thyroid disorders. *Eur J Pediatr* 1999;158:24-8.
254. Feldt-Rasmussen. Anti-thyroid peroxidase antibodies in thyroid disorders and non thyroid autoimmune diseases. *Autoimmunity* 1991;9:245-51.
255. Mariotti S, Chiovato L, Franceschi C and Pinchera A. Thyroid autoimmunity and aging. *Exp Gerontol* 1999;33:535-41.

256. Ericsson UB, Christensen SB and Thorell JI. A high prevalence of thyroglobulin autoantibodies in adults with and without thyroid disease as measured with a sensitive solid-phase immunosorbent radioassay. *Clin Immunol Immunopathol* 1985;37:154-62.
257. Feldt-Rasmussen U, Hoier-Madsen M, Rasmussen NG, Hegedus L and Hornnes P. Anti-thyroid peroxidase antibodies during pregnancy and postpartum. Relation to postpartum thyroiditis. *Autoimmunity* 1990;6:211-4.
258. Premawardhana LD, Parkes AB, AMMARI F, John R, Darke C, Adams H and Lazarus JH. Postpartum thyroiditis and long-term thyroid status: prognostic influence of Thyroid Peroxidase Antibodies and ultrasound echogenicity. *J Clin Endocrinol Metab* 2000;85:71-5.
259. Johnston AM and Eagles JM. Lithium-associated clinical hypothyroidism. Prevalence and risk factors. *Br. J Psychiatry* 1999;175:336-9.
260. Bell TM, Bansal AS, Shorthouse C, Sandford N and Powell EE. Low titre autoantibodies predict autoimmune disease during interferon alpha treatment of chronic hepatitis C. *J Gastroenterol Hepatol* 1999;14:419-22.
261. Ward DL and Bing-You RG. Autoimmune thyroid dysfunction induced by interfereon-alfa treatment for chronic hepatitis C: screening and monitoring recommendations. *Endoc Pract* 2001;7:52-8.
262. Carella C, Mazziotti G, Morisco F, Manganella G, Rotondi M, Tuccillo C, Sorvillo F, Caporaso N and Amato G. Long-term outcome of interferon-alpha-induced thyroid autoimmunity and prognostic influence of thyroid autoantibody pattern at the end of treatment. *J Clin Endocrinol Metab* 2001;86:1925-9.
263. Feldt-Rasmussen U, Schleusener H and Carayon P. Meta-analysis evaluation of the impact of thyrotropin receptor antibodies on long term remission after medical therapy of Graves' disease. *J Clin Endocrinol Metab* 1994;78:98-103.
264. Estienne V, Duthoit C, Di Costanzo, Lejeune PJ, Rotondi M, Kornfeld S et al. Multicenter study on TGPO autoantibodies prevalence in various thyroid and non-thyroid diseases: relationships with thyroglobulin and thyroperoxidase autoantibody parameters. *Eur J Endocrinol* 1999;141:563-9.
265. Czarnocka B, Ruf J, Ferrand M et al. Purification of the human thyroid peroxidase and its identification as the microsomal antigen involved in autoimmune thyroid diseases. *FEBS Lett* 1985;190:147-52.
266. Mariotti S, Caturegli P, Piccolo P, Barbesino G and Pinchera A. Antithyroid peroxidase autoantibodies in thyroid diseases. *J Clin Endocrinol Metab* 1990;71:661-9.
267. Rubello D, Pozzan GB, Casara D, Girelli ME, Boccato s, Rigon F, Baccichetti C, Piccolo M, Betterle C and Busnardo B. Natural course of subclinical hypothyroidism in Down's syndrome: prospective study results and therapeutic considerations. *J Endocrinol Invest* 1995;18:35-40.
268. Karlsson B, Gustafsson J, Hedov G, Ivarsson SA and Anneren G. Thyroid dysfunction in Down's syndrome: relation to age and thyroid autoimmunity. *Arch Dis Child* 1998;79:242-5.
269. Bussen S, Steck T and Dietl J. Increased prevalence of thyroid antibodies in euthyroid women with a history of recurrent in-vitro fertilization failure. *Hum Reprod* 2000;15:545-8.
270. Phan GQ, Attia P, Steinberg SM, White DE and Rosenberg SA. Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma. *J Clin Oncol* 2001;19:3477-82.
271. Durelli L, Ferrero B, Oggero A, Verdun E, Ghezzi A, Montanari E and Zaffaroni M. Thyroid function and autoimmunity during interferon-Beta-1b Treatment: a Multicenter Prospective Study. *J Clin Endocrinol Metab* 2001;86:3525-32.
272. Roti E, Minelli R, Giuberti T, Marchelli C, Schianchi C, Gardini E, Salvi M, Fiaccadori F, Ugolotti G, Neri TM and Braverman LE. Multiple changes in thyroid function in patients with chronic active HCV hepatitis treated with recombinant interferon-alpha. *Am J Med* 1996;101:482-7.
273. Ruf J, Carayon P and Lissitzky S. Various expression of a unique anti-human thyroglobulin antibody repertoire in normal state and autoimmune disease. *Eur J Immunol* 1985;15:268-72.
274. Ruf J, Toubert ME, Czarnocka B, Durand-Gorde JM, Ferrand M, Carayon P. Relationship between immunological structure and biochemical properties of human thyroid peroxidase. *Endocrinol* 1989;125:1211-8.
275. Feldt-Rasmussen U and Rasmussen A K. Serum thyroglobulin (Tg) in presence of thyroglobulin autoantibodies (TgAb). Clinical and methodological relevance of the interaction between Tg and TgAb in vivo and in vitro. *J Endocrinol Invest* 1985;8:571-6.

276. Spencer CA, Wang C, Fatemi S, Guttler RB, Takeuchi M and Kazarosyan M. Serum Thyroglobulin Autoantibodies: Prevalence, influence on serum thyroglobulin measurement and prognostic significance in patients with differentiated thyroid carcinoma. *J Clin Endocrinol Metab* 1998;83:1121-7.
277. Pacini F, Mariotti S, Formica N and Elisei R. Thyroid autoantibodies in thyroid cancer: Incidence and relationship with tumor outcome. *Acta Endocrinol* 1988;119:373-80.
278. Rubello D, Casara D, Girelli ME, Piccolo M and Busnardo B. Clinical meaning of circulating antithyroglobulin antibodies in differentiated thyroid cancer: a prospective study. *J Nucl Med* 1992;33:1478-80.
279. Nordyke RA, Gilbert FI, Miyamoto LA and Fleury KA. The superiority of antimicrosomal over antithyroglobulin antibodies for detecting Hashimoto's thyroiditis. *Arch Intern Med* 1993;153:862-5.
280. Di Cerbo A, Di Paola R, Menzaghi C, De Filippis V, Tahara K, Corda D et al. Graves' immunoglobulins activate phospholipase A2 by recognizing specific epitopes on the thyrotropin receptor. *J Clin Endocrinol Metab* 1999;84:3283-92.
281. Kung AWC, Lau KS and Kohn LD. Epitope mapping of TSH Receptor-blocking antibodies in Graves' disease that appear during pregnancy. *J Clin Endocrinol Metab* 2001;86:3647-53.
282. Ueta Y, Fukui H, Murakami M, Yamanouchi Y, Yamamoto R, Murao A et al. Development of primary hypothyroidism with the appearance of blocking-type antibody to thyrotropin receptor in Graves' disease in late pregnancy. *Thyroid* 1999;9:179-82.
283. Gupta MK. Thyrotropin-receptor antibodies in thyroid diseases: advances in detection techniques and clinical application. *Clin Chem Acta* 2000;293:1-29.
284. Kung AW, Lau KS and Kohn LD. Characterization of thyroid-stimulating blocking antibodies that appeared during transient hypothyroidism after radioactive iodine therapy. *Thyroid* 2000;10:909-17.
285. Filetti S, Foti D, Costante G and Rapoport B. Recombinant human thyrotropin (TSH) receptor in a radioreceptor assay for the measurement of TSH receptor antibodies. *J Clin Endocrinol Metab* 1991;72:1096-101.
286. Adams DD and Purves HD. Abnormal responses in the assay of thyrotropin. *Proc Univ Otago Med Sch* 1956;34:11-12.
287. Morgenthaler NG. New assay systems for thyrotropin receptor antibodies. *Current Opinion Endocrinol Diabetes* 1998;6:251-60.
288. Kamijo K, Nagata A and Sato Y. Clinical significance of a sensitive assay for thyroid-stimulating antibodies in Graves' disease using polyethylene glycol at high concentration and porcine thyroid cells. *Endocrinol J* 1999;46:397-403.
289. Takasu N, Yamashiro K, Ochi Y, Sato Y, Nagata A, Komiya I et al. TSBAb (TSH-Stimulation Blocking Antibody) and TSAb (Thyroid Stimulating Antibody) in TSBAb-positive patients with hypothyroidism and Graves' patients with hyperthyroidism. *Horm Metab Res* 2001;33:232-7.
290. Costagliola S, Swillens S, Niccoli P, Dumont JE, Vassart G and Ludgate M. Binding assay for thyrotropin receptor autoantibodies using the recombinant receptor protein. *J Clin Endocrinol Metab* 1992;75:1540-44.
291. Morgenthaler NG, Hodak K, Seissler J, Steinbrenner H, Pampel I, Gupta M et al. Direct binding of thyrotropin receptor autoantibody to in vitro translated thyrotropin receptor: a comparison to radioreceptor assay and thyroid stimulating bioassay. *Thyroid* 1999;9:466-75.
292. Akamizu T, Inoue D, Kosugi S, Kohn LD and Mori T. Further studies of amino acids (268-304) in thyrotropin (TSH)-lutropin/chorionic gonadotropin (LH/CG) receptor chimeras: Cysteine-301 is important in TSH binding and receptor tertiary structure. *Thyroid* 1994;4:43-8.
293. Grasso YZ, Kim MR, Faiman C, Kohn LD, Tahara K and Gupta MK. Epitope heterogeneity of thyrotropin-blocking antibodies in Graves' patients as detected with wild-type versus chimeric thyrotropin receptors. *Thyroid* 1999;9:521-37.
294. Kim WB, Chung HK, Lee HK, Kohn LD, Tahara K and Cho BY. Changes in epitopes for thyroid stimulation antibodies in Graves' disease sera during treatment of hyperthyroidism: Therapeutic implications. *J Clin Endocrinol Metab* 1997;82:1953-9.
295. Shewring G and Smith BR. An improved radioreceptor assay for TSH receptor. *Methods Enzymol* 1982;17:409-17.

296. Costagliola S, Morganthaler NG, Hoermann R, Badenhop K, Struck J, Freitag D, Poertl S, Weglohner W, Hollidt JM, Quadbeck B, Dumont JE, Schumm-Draeger PM, Bergmann A, Mann K, Vassart G and Usadel KH. Second generation assay for thyrotropin receptor antibodies has superior diagnostic sensitivity for Graves' disease. *J Clin Endocrinol Metab* 1999;84:90-7.
297. Schott M, Feldkamp J, Bathan C, Fritzen R, Scherbaum WA and Seissler J. Detecting TSH-Receptor antibodies with the recombinant TBII assay: Technical and Clinical evaluation. 32 2000;:429-35.
298. Feldt-Rasmussen U. Analytical and clinical performance goals for testing autoantibodies to thyroperoxidase, thyroglobulin and thyrotropin receptor. *Clin Chem* 1996;42:160-3.
299. Giovanella L, Ceriani L and Garancini S. Clinical applications of the 2nd. generation assay for anti-TSH receptor antibodies in Graves' disease. Evaluation in patients with negative 1st. generation test. *Clin Chem Lab med* 2001;39:25-8.
300. Momotani N, Noh JY, Ishikawa N and Ito K. Effects of propylthiouracil and methimazole on fetal thyroid status in mothers with Graves' hyperthyroidism. *J Clin Endocrinol Metab* 1997;82:3633-6.
301. Brown RS, Bellisario RL, Botero D, Fournier L, Abrams CA, Cower ML et al. Incidence of transient congenital hypothyroidism due to maternal thyrotropin receptor-blocking antibodies in over one million babies. *J Clin Endocrinol Metab* 1996;81:1147-51.
302. Gerding MN, van der Meer Jolanda WC, Broenink M, Bakker O, W. WM and Prummel MF. Association of thyrotropin receptor antibodies with the clinical features of Graves' ophthalmopathy. *Clin Endocrinol* 2000;52:267-71.
303. Bartelena L, Marcocci C, Bogazzi F, Manetti L, Tanda ML, Dell'Unto E et al. Relation between therapy for hyperthyroidism and the course of Graves' disease. *N Engl J Med* 1998;338:73-8.
304. Bech K. Immunological aspects of Graves' disease and importance of thyroid stimulating immunoglobulins. *Acta Endocrinol (Copenh) Suppl* 1983;103:5-38.
305. Feldt-Rasmussen U. Serum thyroglobulin and thyroglobulin autoantibodies in thyroid diseases et al. *ergy* 1983;38:369-87.
306. Nygaard B, Metcalfe RA, Phipps J, Weetman AP and Hegedus L. Graves' disease and thyroid-associated ophthalmopathy triggered by ¹³¹I treatment of non-toxic goitre. *J Endocrinol Invest* 1999;22:481-5.
307. Ericsson UB, Tegler L, Lennquist S, Christensen SB, Stahl E and Thorell JI. Serum thyroglobulin in differentiated thyroid carcinoma. *Acta Chir Scand* 1984;150:367-75.
308. Haugen BR, Pacini F, Reiners C, Schlumberger M, Ladenson PW, Sherman SI, Cooper DS, Graham KE, Braverman LE, Skarulis MC, Davies TF, DeGroot LJ, Mazzaferri EL, Daniels GH, Ross DS, Luster M, Samuels MH, Becker DV, Maxon HR, Cavalieri RR, Spencer CA, McEllin K, Weintraub BD and Ridgway EC. A comparison of recombinant human thyrotropin and thyroid hormone withdrawal for the detection of thyroid remnant or cancer. *J Clin Endocrinol Metab* 1999;84:3877-85.
309. Spencer CA, LoPresti JS, Fatemi S and Nicoloff JT. Detection of residual and recurrent differentiated thyroid carcinoma by serum Thyroglobulin measurement. *Thyroid* 1999;9:435-41.
310. Schlumberger M, C. P., Fragu P, Lumbroso J, Parmentier C and Tubiana M. Circulating thyrotropin and thyroid hormones in patients with metastases of differentiated thyroid carcinoma: relationship to serum thyrotropin levels. *J Clin Endocrinol Metab* 1980;51:513-9.
311. Pacini F, Fugazzola L, Lippi F, Ceccarelli C, Centoni R, Miccoli P, Elisei R and Pinchera A. Detection of thyroglobulin in fine needle aspirates of nonthyroidal neck masses: a clue to the diagnosis of metastatic differentiated thyroid cancer. *J Clin Endocrinol Metab* 1992;74:1401-4.
312. Spencer CA, Takeuchi M and Kazarosyan M. Current Status and Performance Goals for Serum Thyroglobulin Assays. *Clin Chem* 1996;42:164-73.
313. Feldt-Rasmussen U and Schlumberger M. European interlaboratory comparison of serum thyroglobulin measurement. *J Endocrinol Invest* 1988;11:175-81.
314. Feldt-Rasmussen U, Profilis C, Colinet E, Black E, Bornet H, Bourdoux P et al. Human thyroglobulin reference material (CRM 457) 2nd part: Physicochemical characterization and certification. *Ann Biol Clin* 1996;54:343-348.
315. Schlumberger M J. Papillary and Follicular Thyroid Carcinoma. *NEJM* 1998;338:297-306.
316. Hjiyiannakis P, Mundy J and Harmer C. Thyroglobulin antibodies in differentiated thyroid cancer. *Clin Oncol* 1999;11:240-4.
317. Spencer CA. Recoveries cannot be used to authenticate thyroglobulin (Tg) measurements when sera contain Tg autoantibodies. *Clin Chem* 1996;42:661-3.

318. Massart C and Maugeudre D. Importance of the detection method for thyroglobulin antibodies for the validity of thyroglobulin measurements in sera from patients with Graves' disease. *Clin Chem* 2002;48:102-7.
319. Mariotti S, Barbesino G, Caturegli P, Marino M, Manetti L, Pacini F, Centoni R and Pinchera A. Assay of thyroglobulin in serum with thyroglobulin autoantibodies: an unobtainable goal? *J Clin Endocrinol Metab* 1995;80:468-72.
320. Black EG and Hoffenberg R. Should one measure serum thyroglobulin in the presence of anti-thyroglobulin antibodies? *Clin Endocrinol* 1983;19:597-601.
321. Schneider AB and Pervos R. Radioimmunoassay of human thyroglobulin: effect of antithyroglobulin autoantibodies. *J Clin Endocrinol Metab* 1978;47:126-37.
322. Spencer CA, Platler BW and Nicoloff JT. The effect of 125-I thyroglobulin tracer heterogeneity on serum Tg RIA measurement. *Clin Chem Acta* 1985;153:105-115.
323. Bugalho MJ, Domingues RS, Pinto AC, Garrao A, Catarino AL, Ferreira T, Limbert E and Sobrinho L. Detection of thyroglobulin mRNA transcripts in peripheral blood of individuals with and without thyroid glands: evidence for thyroglobulin expression by blood cells. *Eur J Endocrinol* 2001;145:409-13.
324. Bellantone R, Lombardi CP, Bossola M, Ferrante A, Princi P, Boscherini M et al. Validity of thyroglobulin mRNA assay in peripheral blood of postoperative thyroid carcinoma patients in predicting tumor recurrence varies according to the histologic type: results of a prospective study. *Cancer* 2001;92:2273-9.
325. Bojunga J, Roddiger S, Stanisch M, Kusterer K, Kurek R, Renneberg H, Adams S, Lindhorst E, Usadel KH and Schumm-Draeger PM. Molecular detection of thyroglobulin mRNA transcripts in peripheral blood of patients with thyroid disease by RT-PCR. *Br J Cancer* 2000;82:1650-5.
326. Smith B, Selby P, Southgate J, Pittman K, Bradley C and Blair GE. Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. *Lancet* 1991;338:1227-9.
327. Luppi M, Morselli M, Bandieri E, Federico M, Marasca R, Barozzi P, Ferrari MG, Savarino M, Frassoldati A and Torelli G. Sensitive detection of circulating breast cancer cells by reverse-transcriptase polymerase chain reaction of maspin gene. *Ann Oncol* 1996;7:619-24.
328. Ghossein RA and Bhattacharya S. Molecular detection and characterisation of circulating tumour cells and micrometastases in solid tumours. *Eur J Cancer* 2000;36:1681-94.
329. Ditkoff BA, Marvin MR, Yemul S, Shi YJ, Chabot J, Feind C et al. Detection of circulating thyroid cells in peripheral blood. *Surgery* 1996;120:959-65.
330. Arturi F, Russo D, Giuffrida D et al. Early diagnosis by genetic analysis of differentiated thyroid cancer metastases in small lymph nodes. *J Clin Endocrinol Metab* 1997;82:1638-41.
331. Ringel MD, Balducci-Silano PL, Anderson JS, Spencer CA, Silverman J, Sparling YH, Francis GL, Burman KD, Wartofsky L, Ladenson PW, Levine MA and Tuttle RM. Quantitative reverse transcription-polymerase chain reaction of circulating thyroglobulin messenger ribonucleic acid for monitoring patients with thyroid carcinoma. *J Clin Endocrinol Metab* 1998;84:4037-42.
332. Biscolla RP, Cerutti JM and Maciel RM. Detection of recurrent thyroid cancer by sensitive nested reverse transcription-polymerase chain reaction of thyroglobulin and sodium/iodide symporter messenger ribonucleic acid transcripts in peripheral blood. *J Clin Endocrinol Metab* 2000;85:3623-7.
333. Takano T, Miyauchi A, Yoshida H, Hasegawa Y, Kuma K and Amino N. Quantitative measurement of thyroglobulin mRNA in peripheral blood of patients after total thyroidectomy. *Br J Cancer* 2001;85:102-6.
334. Chelly J, Concordet JP, Kaplan JC and Kahn A. Illegitimate transcription: transcription of any gene in any cell type. *Proc Natl Acad Sci USA* 1989;86:2617-21.
335. Premawardhana LDKE, Phillips DW, Prentice LM and Smith BR. Variability of serum thyroglobulin levels is determined by a major gene. *Clin Endocrinol* 1994;41:725-9.
336. Bertelsen JB and Hegedus L. Cigarette smoking and the thyroid. *Thyroid* 1994;4:327-31.
337. Knudsen N, Bulow I, Jorgensen T, Perrild H, Oversen L and Laurberg P. Serum Tg - a sensitive marker of thyroid abnormalities and iodine deficiency in epidemiological studies. *J Clin Endocrinol Metab* 2001;86:3599-603.

338. Van den Briel T, West CE, Hautvast JG, Vulsma T, de Vijlder JJ and Ategbo EA. Serum thyroglobulin and urinary iodine concentration are the most appropriate indicators of iodine status and thyroid function under conditions of increasing iodine supply in schoolchildren in Benin. *J Nutr* 2001;131:2701-6.
339. Gardner DF, Rothman J and Utiger RD. Serum thyroglobulin in normal subjects and patients with hyperthyroidism due to Graves' disease: effects of T₃, iodide, ¹³¹I and antithyroid drugs. *Clin Endocrinol* 1979;11:585-94.
340. Feldt-Rasmussen U, Petersen PH, Date J and Madsen CM. Serum thyroglobulin in patients undergoing subtotal thyroidectomy for toxic and nontoxic goiter. *J Endocrinol Invest* 1982;5:161-4.
341. Hocesvar M, Auersperg M and Stanovnik L. The dynamics of serum thyroglobulin elimination from the body after thyroid surgery. 1997;23:208-10.
342. Cohen JH, Ingbar SH and Braverman LE. Thyrotoxicosis due to ingestion of excess thyroid hormone. *Endocrine Rev* 1989;10:113-24.
343. Mitchell ML and Hermos RJ. Measurement of thyroglobulin in newborn screening specimens from normal and hypothyroid infants. *Clin Endocrinol* 1995;42:523-7.
344. Smallridge RC, De Keyser FM, Van Herle AJ, Butkus NE and Wartofsky L. Thyroid iodine content and serum thyroglobulin: clues to the natural history of destruction-induced thyroiditis. *J Clin Endocrinol Metab* 1986;62:1213-9.
345. Pacini F, Molinaro E, Lippi F, Castagna MG, Agate L, Ceccarelli C, Taddei D, Elisei R, Capezzone M and Pinchera A. Prediction of disease status by recombinant human TSH-stimulated serum Tg in the postsurgical follow-up of differentiated thyroid carcinoma. *J Clin Endocrinol Metab* 2001;86:5686-90.
346. Cobin RH. 1992. Medullary carcinoma of the thyroid. *In* Malignant tumors of the thyroid: clinical concepts and controversies. S. D. Cobin RH, editor. Springer-Verlag, New York. 112-41.
347. Dunn JT. When is a thyroid nodule a sporadic medullary carcinoma? *J Clin Endocrinol Metab* 1994;78:824-5.
348. Pacini F, Fontanelli M, Fugazzola L and et. al. Routine measurement of serum calcitonin in nodular thyroid diseases allows the preoperative diagnosis of unsuspected sporadic medullary thyroid carcinoma. *J Clin Endocrinol Metab* 1994;78:826-9.
349. Mulligan LM, Kwok JB, Healey CS, Elsdon MJ, Eng C, Gardner E et al. Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* 1993;363:458-60.
350. Hofstra RM, Landvaster RM, Ceccherini I et al. A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature* 1994;367:375-6.
351. Heyningen van V. One gene-four syndromes. *Nature* 1994;367:319-20.
352. Becker KL, Nylen ES, Cohen R and Snider RH. Calcitonin: structure, molecular biology and actions. *In*: J.P. Belezianian, L.E. Raisz, G.A. Rodan eds. Principle of bone biology, Academic Press, San Diego 1996;:471-4.
353. Motte P, Vauzelle P, Gardet P, Ghillani P, Caillou B, Parmentier C et al. Construction and clinical validation of a sensitive and specific assay for mature calcitonin using monoclonal anti-peptide antibodies. *Clin Chim Acta* 1988;174:35-54.
354. Zink A, Blind E and Raue F. Determination of serum calcitonin by immunometric two-site assays in normal subjects and patients with medullary thyroid carcinoma. *Eur J Clin Chem Biochem* 1992;30:831-5.
355. Engelbach M, Gorges R, Forst T, Pflutzner A, Dawood R, Heerdt S, Kunt T, Bockisch A and Beyer J. Improved diagnostic methods in the follow-up of medullary thyroid carcinoma by highly specific calcitonin measurements. *J Clin Endocrinol Metab* 2000;85:1890-4.
356. Milhaud G, Tubiana M, Parmentier C and Coutiris G. Epithelioma de la thyroïde secretant de la thyrocalcitonine. *C.R. Acad. Sci (serie D)*, Paris 1968;266:608-10.
357. Guilloteau D, Perdrisot D, Calmettes C and et. al. Diagnosis of medullary carcinoma of the thyroid by calcitonin assay using monoclonal antibodies. *J Clin Endocrinol Metab* 1990;71:1064-7.
358. Niccoli P, Wion-Barbot N, Caron P and et.al. Interest of routine measurement of serum calcitonin (CT): study in a large series of thyroidectomized patients. *J Clin Endocrinol Metab* 1997;82:338-41.
359. Wells SA, Baylin SB, Linehan W, Farrell RE, Cox EB, Cooper CW. Provocative agents and the diagnosis of medullary carcinoma of the thyroid gland. *Ann Surg* 1978;188:139-41.
360. Gagel RF. The abnormal pentagastrin test. *Clin Endocrinol* 1996;44:221-2.

361. Wion-Barbot N, Schuffenecker I, Niccoli P et al. Results of the calcitonin stimulation test in normal volunteers compared with genetically unaffected members of MEN 2A and familial medullary thyroid carcinoma families. *Ann Endocrinol* 1997;58:302-8.
362. Barbot N, Calmettes C, Schuffenecker I et al. Pentagastrin stimulation test and early diagnosis of medullary carcinoma using an immunoradiometric assay of calcitonin: comparison with genetic screening in hereditary medullary thyroid carcinoma. *J Clin Endocrinol Metab* 1994;78:114-20.
363. Erdogan MF, Gullu S, Baskal N, Uysal AR, Kamel N, Erdogan G. Omeprazole: calcitonin stimulation test for the diagnosis follow-up and family screening in medullary carcinoma of the thyroid gland. *Ann Surg* 1997;188:139-41.
364. Vieira AEF, Mello MP, Elias LLK et al. Molecular and biochemical screening for the diagnosis and management of medullary thyroid carcinoma in multiple endocrine neoplasia Type 2A. *Horm Metab Res* 2002;34:202-6.
365. Wells SA, Chi DD, Toshima K, Dehner LP, Coffin CM, Dowton SB, Ivanovich JL, DeBenedetti MK, Dille WG and Moley JF. Predictive DNA testing and prophylactic thyroidectomy in patients at risk for multiple endocrine neoplasia type 2A. *Ann Surg* 1994;220:237-50.
366. Telander RL and Moir CR. Medullary thyroid carcinoma in children. *Semin Pediatr Surg* 1994;3:188-93.
367. Niccoli-Sire P, Murat A, Baudin E, Henry JF, Proye C, Bigorgne JC et al. Early or prophylactic thyroidectomy in MEN2/FMTC gene carriers: results in 71 thyroidectomized patients. *Eur J Endocrinol* 1999;141:468-74.
368. Niccoli-Sire P, Murat A, Rohmer V, Franc S, Chabrier G, Baldet L, Maes B, Savagner F, Giraud S, Bezieau S, Kottler ML, Morange S and Conte-Devolx B. Familial medullary thyroid carcinoma (FMTC) with non-cysteine RET mutations: phenotype-genotype relationship in large series of patients. *J Clin Endocrinol Metab* 2001;86:3756-53.
369. Body JJ, Chanoine JP, Dumon JC and Delange F. Circulating calcitonin levels in healthy children and subjects with congenital hypothyroidism from birth to adolescence. *J Clin Endocrinol Metab* 1993;77:565-7.
370. Gharib H, Kao PC and Heath H. Determination of silica-purified plasma calcitonin for the detection and management of medullary thyroid carcinoma: comparison of two provocative tests. *Mayo Clin Proc* 1987;62:373-8.
371. Telander R, Zimmerman D, Sizemore GW, van Heerden JA and Grant CS. Medullary carcinoma in children. Results of early detection and surgery. *Arch Surg* 1989;124:841-3.
372. Calmettes C, Ponder BA, Fisher JA and Raue F. Early diagnosis of multiple endocrine neoplasia type 2 syndrome: consensus statement. European community concerted action: medullary thyroid carcinoma. *Eur J Clin Invest* 1992;22:755-60.
373. Modigliani E, Cohen R, Campos JM, Conte-Devolx B, Maes B, Boneu A et al. Prognostic factors for survival and biochemical cure in medullary thyroid carcinoma: results in 899 patients. *Clin Endocrinol* 1998;48:265-73.
374. Machens A, Gimm O, Ukkat J et al. Improved prediction of calcitonin normalization in medullary thyroid carcinoma patients by quantitative lymph node analysis. *Cancer* 2000;88:1909-15.
375. Fugazzola L, Pinchera A, Lucchetti F et al. Disappearance rate of serum calcitonin after total thyroidectomy for medullary thyroid carcinoma. *Internat J Biolog Markers* 1994;9:21-4.
376. Vierhapper H, Raber W, Bieglmayer C and et.al. Routine measurement of plasma calcitonin in nodular thyroid diseases. *J Clin Endocrinol Metab* 1997;82:1589-93.
377. Ferreira-Valbuena H, Fernandez de Arguello E, Campos G, Ryder E and Avellaneda A. Serum concentration of calcium and calcitonin in hyperthyroidism caused by Graves' disease. *Invest Clin* 1991;32:109-14.
378. Lips CJM, Hoppener JWM and Thijssen JHH. Medullary thyroid carcinoma: role of genetic testing and calcitonin measurement. *Ann Clin Biochem* 2001;38:168-79.
379. Niccoli P, Brunet Ph, Roubicek C, Roux F, Baudin E, Lejeune PJ et al. Abnormal calcitonin basal levels and pentagastrin response in patients with chronic renal failure on maintenance hemodialysis. *Eur J Endocrinol* 1995;132:75-81.
380. Snider RH, Nylen ES and Becker KL. Procalcitonin and its component peptides in systemic inflammation: immunochemical characterization. *J Invest Med* 1997;47:552-60.

381. Russwurm S, Wiederhold M, Oberhoffer M et al. Molecular aspects and natural source of Procalcitonin. *Clin Chem Lab Med* 1999;37:789-97.
382. Niccoli P, Conte-Devolx B, Lejeune PJ, Carayon P, Henry JF, Roux F et al. Hypercalcitoninemia in conditions other than medullary cancers of the thyroid. *Ann Endocrinol* 1996;57:15-21.
383. Baudin E, Bidart JM, Rougier P et al. Screening for multiple endocrine neoplasia type 1 and hormonal production in apparently sporadic neuroendocrine tumors. *J Clin Endocrinol Metab* 1999;84:114-20.
384. DeLellis RA. C-Cell hyperplasia. In: Rosai J., Carangiu M.L., DeLellis R.A. eds: *Atlas of Tumor Pathology*, 3rd. series, Fasc 5: tumors of the thyroid gland. Washington DC, Armed Forces Institute of Pathology. 1992;:247-58.
385. Guyetant S, Wion-Barbot N and Rousselet MC. C-cell hyperplasia associated with chronic lymphocytic thyroiditis: a retrospective study of 112 cases. *Hum Pathol* 1994;25:514-21.
386. Albores-Saavedra J, Monforte H, Nadji M and Morales AR. C-Cell hyperplasia in thyroid tissue adjacent to follicular cell tumor. *Hum Pathol* 1988;19:795-9.
387. Mulligan LM, Marsh DJ, Robinson BG, Schuffenecker I, Zedenius J, Lips CJ et al. Genotype-phenotype correlation in multiple endocrine neoplasia type 2: report of the international RET mutation consortium. *J Intern Med* 1995;238:243-6.
388. Eng C, Clayton D, Schuffenecker I, Lenoir G, Cote G, Gagel RF et al. The relationship between specific RET proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International RET mutation consortium analysis. *JAMA* 1996;276:1575-9.
389. Ito S, Iwashita T, Asai N, Murakami H, Iwata Y, Sobue G et al. Biological properties of RET with cysteine mutations correlate with multiple endocrine neoplasia type 2A, familial medullary thyroid carcinoma and Hirschsprung's disease phenotype. *Cancer Res* 1997;57:2870-2.
390. Heshmati HM, Gharib H, Khosla S et al. Genetic testing in medullary thyroid carcinoma syndromes: mutation types and clinical significance. *Mayo Clin Proc* 1997;72:430-6.
391. Berndt I, Reuter M, Saller B et al. A new hot spot for mutations in the RET proto-oncogene causing familial medullary thyroid carcinoma and multiple endocrine neoplasia type 2A. *J Clin Endocrinol Metab* 1998;83:770-4.
392. Komminoth P, Roth J, Muletta-Feurer S, Saremaslani P, Seelentag WKF and Heitz PU. RET proto-oncogene point mutations in sporadic neuroendocrine tumors. *J Clin Endocrinol Metab* 1996;81:2041-6.
393. Conte-Devolx B, Schuffenecker I, Niccoli P, Maes B, Boneu A, Barbot N et al. Multiple Endocrine Neoplasia Type 2: Management of patients and subjects at risk. *Horm Res* 1997;47:221-6.
394. Smith DP, Houghton C and Ponder BA. Germline mutation of RET codon 883 in two cases of de novo MEN2B. *Oncogene* 1997;15:1213-7.
395. Carlson KM, Bracamontes J, Jackson CE, Clark R, Lacroix A, Wells SA Jr et al. Parent-of-origin effects in multiple endocrine neoplasia type 2B. *J Hum Genet* 1994;55:1076-82.
396. Moers AMJ, Landsvater RM, Schaap C, van Veen JM, de Valk IAJ, Blijham GH et al. Familial medullary thyroid carcinoma: not a distinct entity/ Genotype-phenotype correlation in a large family: familial medullary thyroid carcinoma revisited. *Am J Med* 1996;101:634-41.
397. Dunn JT. Iodine deficiency - the next target for elimination. *N Engl J Med* 1992;326:267-8.
398. Delange F. Correction of iodine deficiency: benefits and possible side effects. *Eur J Endocrinol* 1995;132:542-3.
399. Dunn JT. Whats happening to our iodine. *J Clin Endocrinol Metab* 1998;83:3398-3400.
400. Knudsen N, Christiansen E, Brandt-Christensen M, Nygaard B and Perrild H. Age- and sex-adjusted iodine/creatinine ratio. A new standard in epidemiological surveys? Evaluation of three different estimates of iodine excretion based on casual urine samples and comparison to 24 h values. *Eur J Clin Nutr* 2000;54:361-3.
401. Aumont G and Tressol JC. Improved routine method for the determination of total iodine in urine and milk. *Analyst* 1986;111:841-3.
402. Unak P, Darcan S, Yurt F, Biber Z and Coker M. Determination of iodine amounts in urine and water by isotope dilution analysis. *Biol Trace Elem Res* Winter 1999;71-2:463-70.
403. Kilbane MT, Ajja RA, Weetman AP, Dwyer R, McDermott EWM, O'Higfins NJ and Smyth PPA. Tissue Iodine content and serum mediated 125I uptake blocking activity in breast cancer. *J Clin Endocrinol Metab* 2000;85:1245-50.

404. Liberman CS, Pino SC, Fang SL, Braverman LE and Emerson CH. Circulating iodine concentrations during and after pregnancy. *J Clin Endocrinol Metab* 1998;83:3545-9.
405. Vought RL, London WT, Lutwak L and Dublin TD. Reliability of estimates of serum inorganic iodine and daily faecal and urinary iodine excretion from single casual specimens. *J Clin Endocrinol Metab* 1963;23:1218-28.
406. Smyth PPA, Darke C, Parkes AB, Smith DF, Hetherington AM and Lazarus JH. Assessment of goitre in an area of endemic iodine deficiency. *Thyroid* 1999;9:895-901.
407. Thomson CD, Smith TE, Butler KA and Packer MA. An evaluation of urinary measures of iodine and selenium status. *J Trace Elem Med and Biol* 1996;10:214-22.
408. Als C, Helbling A, Peter K, Haldimann M, Zimmerli B and Gerber H. Urinary iodine concentration follows a circadian rhythm: A study with 3023 spot urine samples in adults and children. *J Clin Endocrinol Metab* 2000;85:1367-9.
409. Lightowler H and Davis JG. Iodine intake and iodine deficiency in vegans as assessed by the duplicate-portion technique and urinary iodine excretion. *Br. J Nutr* 1999;80:529-35.
410. Utiger RD. Maternal hypothyroidism and fetal development. *N Engl J Med* 1999;341:601-2.
411. Aboul-Khair S, Crooks J, Turnbull AC and Hytten FE. The physiological changes in thyroid function during pregnancy. *Clin Sci* 1964;27:195-207.
412. Smyth PPA, Smith DF, Radcliff M and O'Herlihy C. Maternal iodine status and thyroid volume during pregnancy: correlation with neonatal intake. *J Clin Endocrinol Metab* 1997;82:2840-3.
413. Gunton JE, Hams GH, Fiegert M and McElduff A. Iodine deficiency in ambulatory participants at a Sydney teaching hospital: Is Australia truly iodine replete? *Med J Aust* 1999;171:467-70.
414. Smyth PPA. Variation in iodine handling during normal pregnancy. *Thyroid* 1999;9:637-42.
415. Institute of Medicine. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium and Zinc. National Academic Press 2001
416. Koutras DA, Papadopoulos SN, Sfontouris JG and Rigopoulos GA. Comparison of methods for measuring the plasma inorganic iodine and the absolute iodine uptake by the thyroid gland. *J Clin Endocrinol Metab* 1968;28:757-60.
417. Mizukami Y, Michigishi T, Nonomura A, Hashimoto T, Tonami N, Matsubara F et al. Iodine-induced hypothyroidism: a clinical and histological study of 28 patients. *J Clin Endocrinol Metab* 1993;76:466-71.
418. Heymann WR. Potassium iodide and the Wolff-Chaikoff effect: relevance for the dermatologist. *J Am Acad Dermatol* 2000;42:490-2.
419. Stanbury JB, Ermans AE, Bourdoux P, Todd C, Oken E, Tonglet R, Bidor G, Braverman LE and Medeiros-Neto G. Iodine-induced hyperthyroidism: occurrence and epidemiology. *Thyroid* 1998;8:83-100.
420. Roti E and Uberti ED. Iodine excess and hyperthyroidism. *Thyroid* 2001;5:493-500.
421. Baltisberger BL, Minder CE and Burgi H. Decrease of incidence of toxic nodular goitre in a region of Switzerland after full correction of mild iodine deficiency. *Eur J Endocrinol* 1995;132:546-9.
422. Bacher-Stier RG, Totsch M, Kemmler G, Oberaigner W and Moncayo R. Incidence and clinical characteristics of thyroid carcinoma after iodine prophylaxis in an endemic goiter country. *Thyroid* 1997;7:733-41.
423. Barakat MCD, Hetherington AM, Smyth PPA and Leslie H. Hypothyroidism secondary to topical iodine treatment in infants with spina bifida. *Acta Paediatr* 1994;83:741-3.
424. Martino E, Safran M, Aghino-Lombardi F, Rajatanavin R, Lenziardi M, Fay M et al. Environmental iodine intake and thyroid dysfunction during chronic amiodarone therapy. *Ann Intern Med* 1984;101:28-34.
425. Rose NR, Rasooly L, Saboori AM and Burek CL. Linking iodine with autoimmune thyroiditis. *Environmental Health Perspectives* 1999;107:749-52.
426. Premawardhana LDKEPA, Smyth PPA, Wijeyaratne C, Jayasinghe A, De Silva H and Lazarus JH. Increased prevalence of thyroglobulin antibodies in Sri Lankan schoolgirls - is iodine the cause? *Eur J Endocrinol* 2000;143:185-8.
427. Costa A, Testori OB, Cenderelli C, Giribone G and Migliardi M. Iodine content of human tissues after administration of iodine containing drugs or contrast media. *J Endocrinol Invest* 1978;1:221-5.

428. May W, Wu D, Eastman C, Bourdoux P and Maberly G. Evaluation of automated urinary iodine methods: problems of interfering substances identified. *Clin Chem* 1990;35:865-9.
429. Lauber K. Iodine determination in biological material. Kinetic measurement of the catalytic activity of iodine. *Analyt Chem* 1975;47:769-71.
430. Mantel M. Improved method for the determination of iodine in urine. *Clin Chim Acta* 1971;33:39-44.
431. Dunn JT, Crutchfield HE, Gutenkunst R and Dunn AD. Two simple methods for measuring iodine in urine. *Thyroid* 1993;3:119-23.
432. May SL, May WA, Bourdoux PP, Pino S, Sullivan KM and Maberly GF. Validation of a simple, manual urinary iodine method for estimating the prevalence of iodine-deficiency disorders and interlaboratory comparison with other methods. *J Clin Nutr* 1997;65:1441-5.
433. Ohashi T, Yamaki M, Pandav SC, Karmarkar GM and Irie M. Simple microplate method for determination of urinary iodine. *Clin Chem* 2000;46:529-36.
434. Rendl J, Seybold S and Borner W. Urinary iodine determined by paired-ion reverse-phase HPLC with electrochemical detection. *Clin Chem* 1994;40:908-13.
435. Tsuda K, Namba H, Nomura T, Yokoyama N, Yamashita S, Izumi M and Nagataki S. Automated Measurement of urinary iodine with use of ultraviolet radiation. *Clin Chem* 1995;41:581-5.
436. Haldimann M, Zimmerli B, Als C and Gerber H. Direct determination of urinary iodine by inductively coupled plasma mass spectrometry using isotope dilution with iodine-129. *Clin Chem* 1998;44:817-24.
437. Mura P, Piriou A, Guillard O, Sudre Y and Reiss D. Dosage des iodures urinaires par electrode specifique: son interet au cours des dysthyroides. *Ann Biol Clin* 1985;44:123-6.
438. Allain P, Berre S, Krari N, Laine-Cessac P, Le Bouil A, Barbot N, Rohmer V and Bigorgne JC. Use of plasma iodine assays for diagnosing thyroid disorders. *J Clin Pathol* 1993;46:453-5.
439. Vander JB, Gaston EA and Dawber TR. The significance of nontoxic thyroid nodules: Final report of a 15-year study of the incidence of thyroid malignancy. *Ann Intern Med* 1968;69:537-40.
440. Rojeski MT and Gharib H. Nodular thyroid disease: Evaluation and management. *N Engl J Med* 1985;313:428-36.
441. Mazzaferri EL. Management of a solitary thyroid nodule. *N Engl J Med* 1993;328:553-9.
442. Kirkland RT and Kirkland JL. Solitary thyroid nodules in 30 children and report of a child with thyroid abscess. *Pediatrics* 1973;51:85-90.
443. Rallison ML, Dobyns EM, Keating FR, Rall J and Tyler E. Thyroid nodularity in children. *JAMA* 1975;233:1069-72.
444. Khurana KK, Labrador E, Izquierdo R, Mesonero CE and Pisharodi LR. The role of fine-needle aspiration biopsy in the management of thyroid nodules in children, adolescents and young adults: A multi-institutional study. *Thyroid* 1999;4:383-6.
445. Aghini-Lombardi F, Antonangeli L, Martino E, Vitti P, Maccherini D, Leoli F, Rago T, Grasso L, Valeriano R, Balestrieri A and Pinchera A. The spectrum of thyroid disorders in an iodine-deficient community: the Pescopagano Survey. *J Clin Endocrinol Metab* 1999;84:561-6.
446. Hamburger JL, Husain M, Nishiyama R, Nunez C and Solomon D. Increasing the accuracy of fine-needle biopsy for thyroid nodules. *Arch Pathol Lab Med* 1989;113:1035-41.
447. Hundahl SA, Cady B, Cunningham MP, Mazzaferri E, McKee RF, Rosai J, Shah JP, Fremgen AM, Stewart AK and Holzer S. Initial results from a prospective cohort study of 5583 cases of thyroid carcinoma treated in the United States during 1996. *Cancer (Cytopathol)* 2000;89:202-17.
448. Leenhardt L, Hejblum G, Franc B, Du Pasqueir Fediaevsky L, Delbot T, De Guillouzie D, Menegaux F, Guillausseau C, Hoang C, Turpin G and Aurengo A. Indications and limits of ultrasound-guided cytology in the management of nonpalpable thyroid nodules. *J Clin Endocrinol Metab* 1999;84:24-8.
449. Braga M, Cavalcanti TC, Collaco LM and Graf H. Efficacy of ultrasound-guided fine-needle aspiration biopsy in the diagnosis of complex thyroid nodules. *J Clin Endocrinol Metab* 2001;86:4089-91.
450. Cochand-Priollet B, Guillausseau P, Chagnon S, Hoang C, Guillausseau-Scholer C, Chanson P, Dahan H, Warnet A, Tran Ba Huy PT and Valleur P. The diagnostic value of fine-needle aspiration biopsy under ultrasonography in nonfunctional thyroid nodules: a prospective study comparing cytologic and histologic findings. *Am J Med* 1994;97:152-7.
451. Takashima S, Fukuda H and Kobayashi T. Thyroid nodules: Clinical effect of ultrasound-guided fine needle aspiration biopsy. *J Clin Ultrasound* 1994;22:535-42.
452. Gharib H. Fine-needle aspiration biopsy of thyroid nodules: Advantages, limitations and effect. *Mayo Clin Proc* 1994;69:44-9.

453. Hamberger B, Gharib H, Melton LF III, Goellner JR and zinsmeister AR. Fine-needle aspiration biopsy of thyroid nodules. Impact on thyroid practice and cost of care. *Am J Med* 1982;73:381-4.
454. Grant CS, Hay ID, Gough IR, McCarthy PM and Goellner JR. Long-term follow-up of patients with benign thyroid fine-needle aspiration cytologic diagnoses. *Surgery* 1989;106:980-6.
455. Liel Y and Barchana M. Long-term follow-up of patients with initially benign fine-needle aspirations. *Thyroid* 2001;11:775-8.
456. Belfiore A, La Rosa G, La Porta GA, Giuffrida D, Milazzo G, Lupo L, Regalbutto C and V. R. Cancer Risk in patients with cold thyroid nodules: Relevance of iodine intake, sex, age and multinodularity. *J Amer Med* 1992;93:363-9.
457. Tuttle RM, Lemar H and Burch HB. Clinical features associated with an increased risk of thyroid malignancy in patients with follicular neoplasia by fine-needle aspiration. *Thyroid* 1998;8:377-83.
458. Kumar H, Daykin J, Holder R, Watkinson JC, Sheppard M and Franklyn JA. Gender, clinical findings and serum thyrotropin measurements in the prediction of thyroid neoplasia in 1005 patients presenting with thyroid enlargement and investigated by fine-needle aspiration cytology. *Thyroid* 1999;11:1105-9.
459. Moosa M and Mazzaferri EL. Outcome of differentiated thyroid cancer diagnosed in pregnant women. *J Clin Endocrinol Metab* 1997;82:2862-6.
460. Oertel YC. A pathologist trying to help endocrinologists to interpret cytology reports from thyroid aspirates. *J Clin Endocrinol Metab* 2002;87:1459-61.
461. De Micco, Zoro P, Garcia S, Skoog L, Tani EM, C. PK and Henry JF. Thyroid peroxidase immunodetection as a tool to assist diagnosis of thyroid nodules on fine-needle aspiration biopsy. *Eur J Endocrinol* 1994;131:474-9.
462. Faroux MJ, Theobald S, Pluot M, Patey M and Menzies D. Evaluation of the monoclonal antithyroperoxidase MoAb47 in the diagnostic decision of cold thyroid nodules by fine-needle aspiration. *Pathol Res Pract* 1997;193:705-12.
463. Inohara H, Honjo Y, Yoshii T, Akahani S, Yoshida J, Hattori K, Okamoto S, Sawada T, Raz A and Kubo T. Expression of galectin-3 in fine-needle aspirates as a diagnostic marker differentiating benign from malignant thyroid neoplasms. *Cancer* 1999;85:2475-84.
464. Medeiros-Neto G, Nascimento MC, Bisi H, Alves VA, Longatto-Filho A and Kanamura CT. Differential reactivity for Galectin-3 in Hurthle Cell Adenomas and Carcinomas. *Endocr Pathol* 2001;12:275-9.
465. Saggiorato E, Cappia S, De Guili P, Mussa A, Pancani G, Caraci P, Angeli A and Orlandi F. Galectin - 3 as a presurgical immunocyto-diagnostic marker of minimally invasive follicular carcinoma. *J Clin Endocrinol Metab* 2001;86:5152-8.
466. Bartolazzi A, Gasbarri A, Papotti M, Bussolati G, Lucante T, Khan A, Inohara H, Marandino F, Orkandi F, Nardi F, Vacchione A, Tecce R and Larsson O. Application of an immunodiagnostic method for improving preoperative diagnosis of nodular thyroid lesions. *Lancet* 2001;357:1644-50.
467. Goellner JR. Problems and pitfalls in thyroid cytology. *Monogr Pathol* 1997;39:75-93.
468. Oertel YC, O. J. Diagnosis of benign thyroid lesions: fine-needle aspiration and histopathologic correlation. *Ann Diagn Pathol* 1998;2:250-63.
469. Baldet L, Manderscheid JC, Glinoe D, Jaffiol C, Coste-Seignovet B and Percheron C. The management of differentiated thyroid cancer in Europe in 1988. Results of an international survey. *Acta Endocrinol (Copenh)* 1989;120:547-58.
470. Baloch ZW, Fleisher S, LiVolsi VA and Gupta PK. Diagnosis of "follicular neoplasm": a gray zone in thyroid fine-needle aspiration cytology. *Diagn Cytopathol* 2002;26:41-4.
471. Herrmann ME, LiVolsi VA, Pasha TL, Roberts SA, Wojcik EM and Baloch ZW. Immunohistochemical expression of Galectin-3 in benign and malignant thyroid lesions. *Arch Pathol Lab Med* 2002;126:710-13.
472. Leteurtre E, Leroy Z, Pattou F, Wacrenier A, Carnaille B, Proye C and Lecomte-Houcke M. Why do frozen sections have limited value in encapsulated or minimally invasive follicular carcinoma of the thyroid? *Amer J Clin Path* 2001;115:370-4.
473. Stojadinovic A, Ghossein RA, Hoos A, Urist MJ, Spiro RH, Shah JP, Brennan MF, Shaha AR and Singh B. Hurthle cell carcinoma: a critical histopathologic appraisal. *J Clin Oncol* 2001;19:2616-25.
474. Carmeci C, Jeffrey RB, McDougall IR, Nowels KW and Weigel RJ. Ultrasound-guided fine-needle aspiration biopsy of thyroid masses. *Thyroid* 1998;8:283-9.

475. Yang GCH, Liebeskind D and Messina AV. Ultrasound-guided fine-needle aspiration of the thyroid assessed by ultrafast papanicolaou stain: Data from 1135 biopsies with a two- six-year follow-up. *Thyroid* 2001;6:581-9.
476. Fisher DA, Dussault JH, Foley TP, Klein AH, LaFranchi S, Larsen PR, Mitchell NL, Murphey WH and Walfish PG. Screening for congenital hypothyroidism: results of screening one million North American infants. *J Pediatr* 1979;94:700.
477. Brown AL, Fernhoff PM, Milner J, McEwen C and Elsas LS. Racial differences in the incidence of congenital hypothyroidism. *J Pediatr* 1981;99:934-.
478. LaFranchi SH, Dussault JH, Fisher DA, Foley TP and Mitchell ML. Newborn screening for congenital hypothyroidism: Recommended guidelines. *Pediatrics* 1993;91:1203-9.
479. Gruters A, Delange F, Giovanelli G, Klett M, Richiccioli P, Torresani T et al. Guidelines for neonatal screening programmes for congenital hypothyroidism. *Pediatr* 1993;152:974-5.
480. Toublanc JE. Guidelines for neonatal screening programs for congenital hypothyroidism. *Acta Paediatr* 1999;88 Suppl 432:13-4.
481. Vulsma T, Gons MH and de Vijlder JJ. Maternal-fetal transfer of thyroxine in congenital hypothyroidism due to a total organification defect or thyroid agenesis. *N Engl J Med* 1989;321:13-6.
482. Gruneiro-Papendieck L, Prieto L, Chiesa A, Bengolea S, Bossi G and Bergada C. Usefulness of thyroxine and free thyroxine filter paper measurements in neonatal screening for congenital hypothyroidism of preterm babies. *J Med Screen* 2000;7:78-81.
483. Hanna DE, Krainz PL, Skeels MR, Miyahira RS, Sesser DE and LaFranchi SH. Detection of congenital hypopituitary hypothyroidism: Ten year experience in the Northwest Regional Screening Program. *J Pediatr* 1986;109:959-64.
484. Fisher DA. Hypothyroxinemia in premature infants: is thyroxine treatment necessary? *Thyroid* 1999;9:715-20.
485. Wang ST, Pizzalato S and Demshar HP. Diagnostic effectiveness of TSH screening and of T4 with secondary TSH screening for newborn congenital hypothyroidism. *Clin Chim Acta* 1998;274:151-8.
486. Delange F. Screening for congenital hypothyroidism used as an indicator of the degree of IDD and its control. *Thyroid* 1998;8:1185-92.
487. Law WY, Bradley DM, Lazarus JH, John R and Gregory JW. Congenital hypothyroidism in Wales (1982-93): demographic features, clinical presentation and effects on early neurodevelopment. *Clin Endocrinol* 1998;48:201-7.
488. Mei JV, Alexander JR, Adam BW and Hannon WH. Use of filter paper for the collection and analysis of human whole blood specimens. *J Nutr* 2001;131:1631S-6S.
489. LaFranchi SH, Hanna CE, Krainz PL, Skeels MR, Miyahira RS and Sesser DE. Screening for congenital hypothyroidism with specimen collection at two time periods: Results of the Northwest Regional Screening Program. *J Pediatr* 1985;76:734-40.
490. Zakarija M, McKenzie JM and Eidson MS. Transient neonatal hypothyroidism: Characterization of maternal antibodies to the Thyrotropin Receptor. *J Clin Endocrinol Metab* 1990;70:1239-46.
491. Matsuura N, Yamada Y, Nohara Y, Konishi J, Kasagi K, Endo K, Kojima H and Wataya K. Familial neonatal transient hypothyroidism due to maternal TSH-binding inhibitor immunoglobulins. *N Engl J Med* 1980;303:738-41.
492. McKenzie JM and Zakaria M. Fetal and neonatal hyperthyroidism and hypothyroidism due to maternal TSH receptor antibodies. *Thyroid* 1992;2:155-9.
493. Vogiatzi MG and Kirkland JL. Frequency and necessity of thyroid function tests in neonates and infants with congenital hypothyroidism. *Pediatr* 1997;100.
494. Pohlenz J, Rosenthal IM, Weiss RE, Jhiang SM, Burant C and Refetoff S. Congenital hypothyroidism due to mutations in the sodium/iodide symporter. Identification of a nonsense mutation producing a downstream cryptic 3' splice site. *J Clin Invest* 1998;101:1028-35.
495. Nordyke RA, Reppun TS, Mandanay LD, Wood JC, Goldstein AP and Miyamoto LA. Alternative sequences of thyrotropin and free thyroxine assays for routine thyroid function testing. Quality and cost. *Arch Intern Med* 1998;158:266-72.