

Review Article

Assessment of Thyroid Function

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Thyroid disorders are among the most common endocrine disorders and accounts for a significant amount of Thyroid Function Tests (TFT) ordered. To interpret TFT effectively a firm appreciation of thyroid pathophysiology is required. Almost all the thyroid hormones are bound by thyroid binding proteins with small quantities of free hormones that are the active moieties. Levels of thyroid hormones are affected by alterations in their binding protein concentrations and thus their use has been surpassed by the availability of free hormone tests. Small changes in thyroid hormones exert a much larger inverse change in Thyroid Stimulating Hormone (TSH) levels. Thus TSH will better reflect thyroid dysfunction compared to thyroid hormones except when the pituitary-thyroid axis is deranged or when thyroid function is unstable (e.g. immediately after commencing/altering therapy and in critical inter-current illness). Thyroid testing in sick individuals is to be avoided since TFT may be abnormal without intrinsic thyroid disease. TSH is the best initial screening test. For diagnosis free thyroxine (fT4) is required for confirmation, identifying subclinical dysfunction and quantifying disease severity. Thyroid antibodies aid in differential diagnosis - anti-thyroid peroxidase for autoimmune thyroiditis and anti-TSH receptor for Graves' disease. Thyroglobulin (Tg) serves as a tumor marker in the follow-up of differentiated thyroid cancer. As anti-thyroglobulin (Tg-Ab) is quite prevalent in thyroid cancer patients, the presence of Tg-Ab will falsely lower Tg readings. Thus concomitant Tg-TgAb measurements are needed; for Tg-Ab positive patients Tg-Ab may serve as a surrogate tumor marker. In pregnancy the normal adult reference intervals must be replaced by trimester-specific ranges for fT4 and TSH as well as the use of thyroid antibody testing where appropriate. When thyroid tests are at variance with the clinical picture rarer entities (e.g. thyroid hormone resistance, pituitary TSH-secreting adenoma) as well as assay artifacts and antibody interferences have to be considered.

Keywords: Thyroid Function; TSH; Free T4; Thyroid Antibodies**Abbreviations**

TFT: Thyroid Function Tests; TRH: Thyrotropin Releasing Hormone; TSH: Thyroid Stimulating Hormone; T4: Thyroxine; T3: Tri-Iodo Thyronine; FT4: Free T4; FT3: Free T3; TT4: Total T4; TT3: Total T3; Tg: Thyroglobulin; Tg-Ab: Anti-Thyroglobulin; TPO-Ab: Anti-Thyroid Peroxidase; TSI: Thyroid Stimulating Immunoglobulin; TRAb: TSH Receptor Antibodies; HCG: Human Chorionic Gonadotropin; GD: Graves' Disease; NTI: Non-Thyroidal Illness; MNG: Multi-Nodular Goiter; TSHoma: TSH-Secreting Pituitary Adenoma; RTH: Thyroid Hormone Resistance

Introduction

Thyroid disorders are the fifth most common endocrine disorder in adults in the United States (US) after diabetes, obesity and dyslipidemia, osteoporosis, and erectile dysfunction [1]. Consequently, thyroid function tests are very commonly ordered [2]. In fact the annual volume of Thyroid Stimulating Hormone (TSH) tests ordered is 59 million in the US [3] and another 10 million in the United Kingdom [4].

This review provides an update on laboratory testing of thyroid dysfunction (hypothyroidism and hyperthyroidism). Structural disorders such as goiter, nodules, and cancer (which are more

appropriately investigated with imaging) will only be covered briefly where relevant. The availability of the new Thyroid Stimulating Immunoglobulin (TSI) assay, recent acknowledgement of high dose biotin interference on thyroid tests, and increase in gestational TSH thresholds will be considered. Each common thyroid test will be reviewed followed by their clinical use in the areas of screening, diagnosis, and monitoring therapy.

Brief Pathophysiology

Interpretation of thyroid function tests requires a firm understanding of thyroid physiology [5]. Thyrotropin Releasing Hormone (TRH) from the hypothalamus stimulates the release of TSH from the anterior pituitary gland. Thereafter, TSH regulates the production of thyroid hormone, thyroxine (T4) and triiodothyronine (T3), by the thyroid gland. The bulk of thyroid hormones (approximately 85%) is secreted as T4, while a smaller fraction (15%) is secreted as T3. The thyroid hormones are highly protein bound, mainly by thyroid binding globulin (TBG) and to a lesser extent transthyretin and serum albumin. It is the non-protein-bound or free T4 (fT4) and free T3 (fT3) that are the biologically active forms of thyroid hormone. While T3 is the active moiety much of its serum concentration derives from the peripheral conversion of T4 to T3 by deiodinases. FT3 and fT4 exert negative feedback on the hypothalamus

and pituitary. Their relationship to TSH is inversely log-linear such that minor changes in the pituitary-thyroid axis produce a large change in TSH concentrations [6-8]. Each individual has a unique TSH-fT4 set point [9]. In early thyroid dysfunction fT4 remains normal while TSH may be decreased (subclinical hyperthyroidism) or increased (subclinical hypothyroidism) [10].

Analytes

Despite progress in immunoassay technology all laboratory tests, including thyroid function, are still plagued by reports of false positive or negative results [11]. Sources of interferences include serum proteins (e.g. rheumatoid factor, binding proteins), heterophile (anti-animal) antibodies, drugs and their metabolites [12], and other cross-reacting substances (autoantibodies) with assay components [13]. Only the clinician can suspect such interferences and alert the laboratory to investigate the discordance between test results and the patient's condition. Close rapport and communication between the clinic and laboratory is vital for an efficient and seamless thyroid testing service.

TSH

TSH is a large 28 to 30-kDa dimeric glycopeptide hormone that shares a common 92 amino acid alpha subunit with human chorionic gonadotrophin (hCG), follicle-stimulating hormone and luteinizing hormone, but it has a unique 118 amino acid beta chain. With the introduction of highly sensitive TSH immunometric assays capable of detecting TSH <0.01mIU/L in the 90s [6,14-15], TSH is now the most widely ordered thyroid function test and is recommended for first line screening [16]. This development has also led to the decline in the measurement of TSH release following thyrotropin-releasing hormone stimulation [17]. While all analytical platforms will not produce exactly equivalent TSH values, the reference range for TSH in healthy iodine-replete subjects is generally quite similar at between 0.4-4.0 mIU/L even in different geographic and ethnic groups. However, TSH alone is not sufficient for assessing subclinical disease and states with deranged pituitary-thyroid axis such as central hypothyroidism, hospitalized patients or in the early phases of therapy (antithyroid drugs or thyroid hormone replacement) [18]. In these circumstances concomitant fT4 is required [19]. In comparison to other thyroid function parameters TSH is least susceptible to autoantibody interferences [20]. However, metformin (a commonly used drug in type 2 diabetes) can depress TSH levels [21].

Free T3 and T4

As the biologically active forms of thyroid hormones, fT4 and fT3 are considered to be more sensitive and meaningful indicators of thyroid disease [18] than total T3 (tT3) and total T4 (tT4). Since fT3 and fT4 exist in minute concentrations (pico-molar) compared to tT3 and tT4 (nano-molar), free hormones are harder to measure accurately [3]. Traditionally fT3 and fT4 were measured directly by equilibrium dialysis and ultrafiltration, but their tedium and expense have confined them to the realms of research. The widespread use of free thyroid hormones began with automation and advent of non-isotopic assays [22,23]. The current available free hormone tests are indirect assays as they use a specific high-affinity antibody to extract free thyroid hormones from serum [24,25]. Thereafter, the antibody-containing bound thyroid hormone is separated from serum prior to incubation with a labeled probe. The free hormone

concentration is inversely proportional to the unoccupied antibody-binding sites. Unlike TSH, the reference interval for fT4 shows a wider spread of between 9.5-25.0 pmol/L on different assay systems. One must be cognizant of some causes of misleading fT4 results especially autoantibody interferences [26]. Although rare, familial dysalbuminemic hyperthyroxinemia is an autosomal dominant condition with an albumin possessing greatly enhanced affinity for T4 (and occasionally T3) resulting in spuriously high free (and total) thyroid hormones [27].

Total T4 and total T3

Radioimmunoassays for tT4 and tT3, widely used in the 70s, have been largely replaced by automated non-isotopic immunoassays [13]. With the progress and wide availability of fT3 and fT4 assays, tT3 and tT4 now play a secondary role and are largely used to confirm doubtful free thyroid hormone results [18]. As the concentration of fT4 is 10-fold higher than tT3, its measurement is more reliable. TT3 and tT4 concentrations are also impacted by variations in thyroid binding proteins. In pregnancy, thyroid binding globulin increases up to 2.5 fold that of antenatal value and stays constant till term [28] while albumin concentration decreases by 25% due to increase in plasma volume. In critically ill patients, decreases in thyroid binding proteins also contribute to a fall in tT4 [29]. In theory, the diagnostic accuracy of total hormone measurements would be equivalent to free hormone tests if patients have similar binding protein concentrations. This free-total thyroid hormone discrepancy is particularly noticeable at the high and low ends of hormone concentrations and also in hospitalized patients [30].

Anti-TPO (TPO-Ab)

Anti-TPO is a common autoantibody against Thyroid Peroxidase (TPO). Historically, anti TPO were detected as thyroid microsomal antibodies using agglutination or immuno-fluorescence methods. It was only in 1985 that TPO was recognized as the target antigen of thyroid autoantibodies [31]. Thereafter, radioimmunoassay [32] and chemiluminescent assays [33] for TPO-Ab became available. Eighty percent of Graves' disease patients have high levels of anti-TPO antibodies [34], while positive anti-TPO Ab is detected in over 90% of patients with autoimmune thyroid disease [35]. In the community, the prevalence of TPO-Ab is 11-12 % euthyroid subjects [32,33].

Anti-TSH receptor (Anti-TSHR)

Anti-TSHR or TSH Receptor antibodies (TRAbs) are antibodies that bind to the TSH Receptor. TRAbs may be stimulatory or inhibitory; distinguishing between them is unnecessary as it is evident from their clinical presentation. In Graves' disease (GD) stimulatory TRAbs bind to and activate the TSH receptor [36] causing production of excess thyroid hormones. The fully automated Roche Elecsys TRAb, a 27 minute assay, became available in 2008 [37-38]. Sensitivity and specificity of TRAbs in the differential diagnosis of GD is excellent, with sensitivity and specificity above 90% [39]. In subclinical disease, the more specific thyroid stimulating immunoglobulins (TSI) might improve diagnosis. Algorithms incorporating TSI have shown faster diagnosis for GD and cost savings [40]. However, TSI bioassays are cumbersome, time-consuming and unsuitable for routine use in clinical laboratories. This changed in 2016 when FDA approved the fully automated TSI on the Siemens Immulite 2000 [41]. As stimulatory antibodies account for the majority of TRAbs,

concordance between TRABs and TSI should be similar. A recent study indeed demonstrated equivalent performance between the Roche TRAb and Siemens TSI in patients with untreated GD [42]. Hence, the new TSI may add limited value to the already widely available Roche TRABs besides the TSI involves a longer assay time (60 mins). The clinical utility of TRABs [39] include predicting short-term relapse of GD after antithyroid drug therapy but are less effective in predicting long-term relapse or remission. In pregnant women with GD, a negative TRAb are unlikely to result in neonatal thyrotoxicosis while high titers of TRAb need close monitoring. GD patients with ophthalmopathy have high TRAb levels; positive TRAb in unilateral proptosis favors a diagnosis of GD, but TRABs are unable to predict the course of ophthalmopathy or its response to treatment.

Anti-Thyroglobulin (Tg-Ab)

Tg-Ab on its own adds limited value to the diagnosis of autoimmune thyroid disease with anti-TPO more sensitive and specific for Hashimoto thyroiditis [43], and TRABs more useful for GD. The clinical utility of Tg-Ab is to ensure the reliability of Tg results in the setting of DTC. In fact for Tg-Ab positive DTC, Tg-Ab may serve as a surrogate tumor marker instead of Tg [44,45].

Thyroglobulin (Tg)

Tg, a dimeric protein produced by the thyroid gland, is undetectable in patients with differentiated thyroid cancer (DTC) after thyroidectomy or radioiodine ablation. With improved assay sensitivity Tg is a valuable tumor marker for the follow-up of DTC [46]. However, the concomitant presence of thyroglobulin antibodies (Tg-Ab) in patient sera will result in the under-estimation of the true Tg result [47]. As the prevalence of Tg-Ab is 25% in DTC patients [48] and 10% in the general population [43], it would be prudent for the lab to provide the Tg-Ab titers together with Tg for a meaningful interpretation of all Tg results [49].

The Biotin Effect

Many automated immunoassays make use of biotin-labelled antigen or antibody [50]. Biotin's natural affinity with streptavidin enables the biotinylated antigen-antibody complex to bind to the streptavidin with very high avidity and specificity. By coating streptavidin onto a solid phase (coated tube or micro particles) it can capture and bind the biotinylated antibody (e.g. TRAb) or biotinylated antigen (e.g. TSH, fT4). In one popular automated system (Roche) TSH is a non-competitive immunoassay. A monoclonal biotinylated TSH-specific antibody and a monoclonal ruthenium-labelled TSH-specific antibody reacts with TSH in the patient's serum to form a sandwich complex. Following addition of streptavidin-coated microparticles, the sandwich complex is bound onto this solid phase through the interaction of biotin and streptavidin. The TSH-antibody-microparticle complex is separated, washed and bound to a magnetic electrode. When a voltage is applied a chemiluminescent signal is generated and this signal is proportional to the sample TSH concentration. In the Roche system, fT4 and TRAb are competitive immunoassays. A monoclonal ruthenium-labelled T4-specific antibody extracts fT4 in the patient's serum. Thereafter biotinylated-T4 competes for unoccupied binding sites on the ruthenium-T4-Ab. The biotin-T4-ruthenium-T4-Ab sandwich complex binds onto the streptavidin-coated micro particle solid phase through the interaction of biotin and streptavidin. This entire antibody-hapten complex is

magnetically captured and a chemiluminescent signal generated; luminescence is inversely proportional to fT4 in the sample. High levels of exogenous biotin in the patient sample will saturate the streptavidin reagent and inhibit the proper formation of the antibody complex, resulting in a low signal. In non-competitive immunoassays low signal translates into spuriously low analyte concentration, while in competitive immunoassays low signal will result in falsely high concentrations of analyte [51].

The combination of low TSH, high fT4 and high TRAB results engendered by biotin will be translated as biochemical hyperthyroidism. However, this biotin effect on TFT is seen in patients prescribed high doses of biotin. Patients with inborn error of metabolism such as Biotin-Thiamine-responsive Basal Ganglia Disease (BTBGD) or biotin cycle defects (biotinidase deficiency and multiple carboxylase deficiency) are prescribed megadoses of 10-15mg/kg biotin per day [51]. Factitious Graves' disease was also reported in a patient with multiple sclerosis prescribed 300 mg biotin daily [52]. It is thus important, for the ordering doctor and the scientist to be aware about their assay methodology [53]. The Roche package insert clearly states that biotin interference is only encountered in patients consuming more than 5 mg biotin per day. Although there exist dedicated biotin supplement available for purchase over the counter or internet, most adult multivitamin supplements on the market contain less than 1mg of biotin per tablet and so will not cause any assay interference. Hence in the usual clinical practice, the issue of biotin induced assay interference is unlikely to be encountered. When clinically discordant thyroid results are found, a detailed history (including supplements) will rule out biotin interference. This interference can be confirmed by re-testing samples on biotin-free automated immunoassays. Another method of overcoming such interference is biotin neutralization. A simple method of sample pretreatment using streptavidin reagents to neutralize biotin has been recently described [54].

Clinical Evaluation

Screening

The purpose of screening is early detection of asymptomatic disease and to improve clinical outcomes. The US National Health Screening program, NHANES 2007-2012, shows a prevalence of 7.1% for thyroid dysfunction - subclinical hyperthyroidism 3.1%, clinical hyperthyroidism 0.3%, subclinical hypothyroidism 3.5%, and clinical hypothyroidism 0.2% respectively [55]. Despite the substantial burden of thyroid disorders, screening asymptomatic adults remains controversial. The American Thyroid Association, American Academy of Clinical Endocrinology, American Academy of Family Physicians and American College of Physicians recommend screening while the Royal College of Physicians London and the United States Preventive Services Task Force are against it [56]. For screening asymptomatic ambulatory subjects, TSH alone is sufficient. When TSH exceeds the reference range, the laboratory may provide reflex testing instead of repeat testing at another occasion. In view of significant clustering of thyroid disease in families [57], women over 50 and the elderly [58], screening may also be done on these at-risk populations. If abnormal, thyroid antibodies (TPO-Ab) may be used to assess risk for future thyroid dysfunction. However, cognizance must be given to the extraneous effects of various conditions, especially medications, on thyroid test results [12,56].

Diagnosis of thyroid conditions

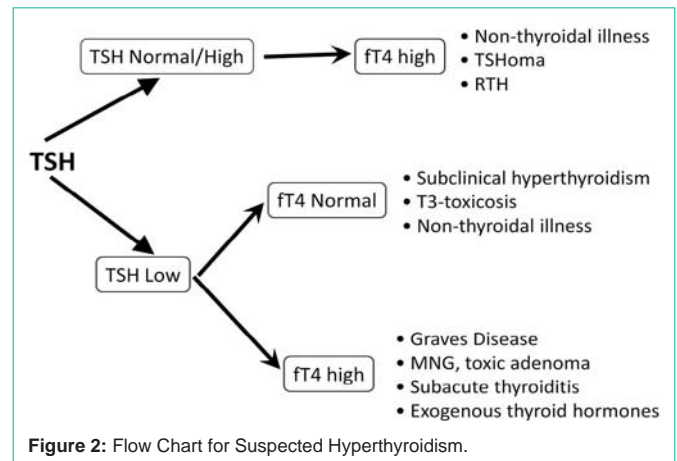
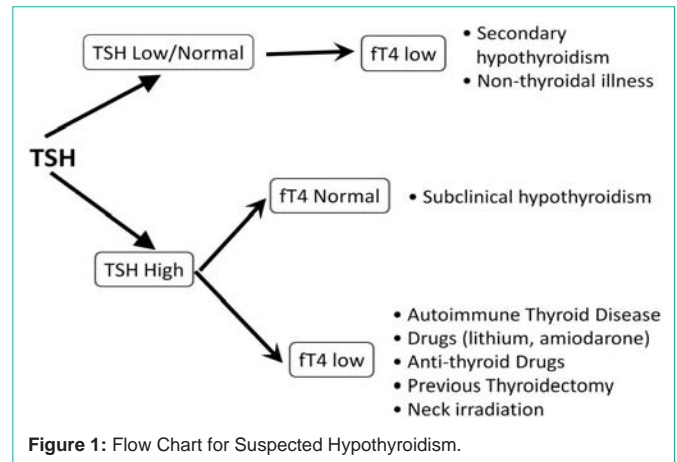
In symptomatic or patients suspected of thyroid dysfunction, the concurrent use of fT4 and TSH is preferred [59]. Thereafter, further testing (thyroid antibodies or radionuclide uptake studies) may be undertaken.

Suspected hypothyroidism: A first line thyroid panel comprising of fT4 and TSH should be done see Figure 1. TSH in overt primary hypothyroidism is often greater than 15.0mIU/L with corresponding low fT4 levels. In subclinical hypothyroidism TSH levels are typically between 4.0-10.0 mIU/L with normal fT4 levels. The most frequent cause of hypothyroidism is Hashimoto’s thyroiditis. A proper history is necessary to elicit other causes such as previous treatment (thyroidectomy, radioiodine ablation, and neck irradiation) and current medications (e.g. lithium, amiodarone and interferon-α) [12,56]. FT3 or tT3 has no clinical utility in the evaluation of hypothyroidism as their levels remain in the normal range until quite late due to hyper-stimulation of the remaining functioning thyroid tissue by TSH and up-regulation of type 2 iodothyronine deiodinases [60]. Deranged thyroid function tests, notably a low T3 or fT3, is frequently encountered in hospitalized patients with non-thyroidal inter-current illnesses or non-thyroidal illness (NTI) [61,62]. The NTI syndrome is characterized by a combination of abnormalities in the pituitary-thyroid axis, thyroid hormone binding proteins, and thyroid hormone action without any intrinsic pituitary or thyroid disease. Hence a low fT3 is neither specific nor sensitive for hypothyroidism, and adds little value in the diagnosis of hypothyroidism. Thyroid function tests should not be ordered in sick hospital inpatients unless absolutely necessary.

Thyroid antibodies can be used to assess the risk of developing overt hypothyroidism in females with TSH >6mIU/L - 4.3 % per year in those with elevated TPO-Ab versus 2.1% in those with normal TPO-Ab [63]. In addition, women with normal TSH (2.5-4.0 mIU/L) and TPO-Ab positivity also progressed to subclinical and overt hypothyroidism [64]. When fT4 is decreased in the face of low or normal TSH, consider central or secondary hypothyroidism [56]. Such patients may have a history of post-partum hemorrhage, head injury, neurosurgery, short stature, amenorrhea, and infertility. A full pituitary panel (growth hormone, prolactin, follicle-stimulating hormone, luteinizing hormone, testosterone, adrenocorticotropic hormone) will be necessary to assess for concomitant hypopituitarism from pituitary or hypothalamic tumors; imaging (CT or MRI) will be required for their delineation (Figure 1).

Suspected hyperthyroidism: Hyperthyroidism refers to the inappropriately high synthesis and secretion of thyroid hormones by the thyroid gland, while the term “thyrotoxicosis” refers to a clinical state that results from inappropriately high thyroid hormone action in tissues [59]. Symptoms and signs include heat intolerance, nervousness, hyperactivity, tremor, weakness, diarrhea and increased appetite. Elderly subjects may display a more sedate, apathetic presentation with weight loss and cardiac symptoms such as heart failure [65,66].

For suspected hyperthyroidism, a thyroid panel comprising TSH and fT4 is sufficient (see Figure 2). In overt hyperthyroidism TSH is often <0.01mIU/L while fT4 is elevated. The most frequent cause of hyperthyroidism is GD followed by toxic multinodular



goiter (MNG), whilst autonomously functioning thyroid adenoma or thyroiditis are less common [67]. Eye signs and smooth goiters are suggestive of GD. TRAbs are useful in the diagnosis of GD when the presentation is atypical. Radionuclide scanning may be done if the diagnosis is still uncertain. There will be a diffuse uptake in GD, while in toxic MNG the thyroid gland uptake will be focal. Subacute thyroiditis may present with pain (localized or radiating to the jaw) and elevated C-reactive protein and erythrocyte sedimentation rate, while drug-induced thyroiditis are usually painless [68]. In subacute or drug-induced thyroiditis the radionuclide uptake will be reduced or absent.

For subclinical hyperthyroidism, fT4 is normal but TSH is suppressed. In a subset of patients with T3-toxicosis, only fT3 is elevated while fT4 is normal and TSH suppressed (<0.01mIU/L) or undetectable. These changes may represent the earliest stages of disease or that caused by an autonomously functioning thyroid nodule [46]. Unless T3-toxicosis is suspected there is no need to order fT3 or tT3.

In patients with elevated fT4, but normal or raised TSH, the possibility of rare disorders such as TSH-secreting pituitary adenoma (TSHoma) or thyroid hormone resistance (RTH) must be considered. The prevalence of TSHoma has been reported to be 2.8 per million [69]. Some TSHoma are polymorphous and may co-secrete growth hormone and prolactin. In TSHoma, patients exhibit more classical

symptoms of thyrotoxicosis. Biochemically, the TSH α -subunit and Sex-Hormone Binding Globulin (SHBG) are increased with blunted TSH response to TRH stimulation. Resistance to thyroid hormone (RTH) is caused by a mutation in the thyroid hormone receptor β gene [70]. Three quarters of RTH are autosomal dominant. In RTH, the TSH response to TRH stimulation is normal or exaggerated while the TSH α -subunit and SHBG are normal (Figure 2).

Monitoring

Patients on treatment with thyroxine or anti-thyroid drugs should be monitored with TSH. During the initial weeks after commencing therapy fT4 must be used due to the lag in pituitary homeostatic response [56,59]. The aim is to keep TSH within the normal reference range. Excessive suppression increases the risk of osteoporosis and atrial fibrillation while hypothyroidism will occur with over treatment with anti-thyroid drugs. A minimum re-testing interval of 1-3 months is recommended for TSH to attain a steady state. In secondary hypothyroidism, pituitary TSH release is impaired and so fT4 should be used to monitor thyroxine replacement. The aim is to keeping fT4 in the upper third of the reference range. For patients who are on T3 replacement, fT3 may then be used for monitoring.

Pregnancy and Postpartum

In normal pregnancy thyroid hormone binding proteins are elevated, thyroid hormone production is increased, and hCG exerts a stimulatory effect on the thyroid gland. These factors in turn impact thyroid function tests in normal pregnancy and they are quite different from healthy nonpregnant women [71]. The reference ranges for TSH and fT4 differ and vary significantly in different populations and on different assay systems. TSH levels decrease in early pregnancy before gradually recovering towards non-pregnant levels by the third trimester. FT4 increases early in the first trimester from the effects of hCG before declining for the rest of the trimesters. Thus laboratories have to provide trimester- and instrument- specific reference intervals.

The stimulatory effects of hCG in the first trimester may result in transient gestational hyperthyroidism. This condition may be accompanied by hyperemesis gravidarum and often resolves without treatment. Equally, subclinical hyperthyroidism needs no therapy as it is not associated with adverse outcomes. Overt hyperthyroidism is uncommon and may be due to pre-existing or new GD. While overt hypothyroidism is associated with adverse pregnancy outcomes for mother and fetus, the impact of gestational subclinical hypothyroidism and its treatment is unclear. For optimum obstetric care of thyroid disease in pregnancy close monitoring (every 3-6 weeks) of thyroid function is required.

Up to 10% of women have thyroiditis (autoimmune) or exacerbation of GD in the postpartum period. Thyroid function tests may be variable and proceed to permanent hyper or hypothyroidism. Close follow-up is required.

Conclusion

Thyroid function tests are integral in today's clinical practice. Utilization of tests needs to be rational in support of clinical suspicion. Care has to be exercised when results are discordant with the clinical picture [72]. A thorough history including medication, previous

therapies (radioiodine ablation, thyroidectomy) and consideration of non-thyroidal illness, will usually uncover the underlying cause. Reassessment of thyroid function and exclusion of assay artifacts should be undertaken next. Lastly, consider the possibility of rare disorders. With good knowledge of laboratory science and appreciation of medical context, users will be able to interpret thyroid function tests successfully.

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