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PEARLS OF LABORATORY MEDICINE

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TITLE: Thyroid Testing

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Slide 1: Hello, my name is Rob Nerenz and I am an assistant professor of pathology and laboratory medicine at Dartmouth-Hitchcock Medical Center. Welcome to this pearl of laboratory medicine on thyroid function testing.

Slide 2: Let's start with a historical perspective showing the evolution of thyroid function testing. As thyroid hormones are principally responsible for regulating the basal metabolic rate, thyroid function was assessed in the first half of the 20th century by measuring oxygen consumption while at rest. This approach left much to be desired as it didn't directly measure thyroid hormone concentration, thyroid hormones are not the only determinants of an individual's metabolic rate and the term "at rest" was difficult to define. Basal metabolic rate was replaced in the 1960s and 70s by the measurement of protein-bound iodine. In this approach, thyroid-binding proteins were precipitated and iodine was measured in the precipitated fraction. This technique was hampered by variable recovery and poor specificity caused by the presence of inorganic iodide in the form of iodine-containing drugs and cigarette smoke. The development of the total T4 competitive immunoassay in 1965 allowed the measurement of thyroid hormones for the first time and the introduction of the free hormone concept led to calculation of the free T4 index as an approximation of free T4 concentration from the 1970s through the late 1990s. Thyroid function testing as we know it began to take shape in the mid 1980s with the measurement of free T4 by equilibrium dialysis, followed shortly by the development of third generation TSH immunoassays with limits of detection capable of differentiating between euthyroid and hyperthyroid patients. Lastly, the development of one-step, automated free T4 immunoassays facilitated the routine measurement of free T4 in hospital laboratories.

Slide 3: In the initial workup of thyroid disease, TSH is the best screening test. If TSH is within the reference interval, thyroid disease is unlikely and no further testing is performed. As discussed in other Pearls dedicated to hypothyroidism and thyrotoxicosis, high concentrations of TSH indicate hypothyroidism while low concentrations indicate hyperthyroidism. If TSH is above or below the reference interval, free T4 is the most appropriate next test to confirm thyroid disease. In routine practice, it is relatively common for both TSH and free T4 to be measured in tandem during the initial evaluation of thyroid disease. In the vast majority of cases, free T4 is preferred over total T4 as free T4 is the thyroid hormone fraction available to thyroid-responsive tissues. In addition, total T4 increases or decreases in response to fluctuations in the concentrations of thyroid hormone binding proteins, while free T4 remains relatively constant in euthyroid individuals.

Slide 4: On most automated analyzers, TSH is measured using a two site sandwich immunoassay using a capture antibody directed at one TSH epitope and a labeled detector antibody directed at a second TSH epitope. Using this assay format, signal is directly proportional to TSH concentration: as TSH concentration increases, greater signal is generated. Currently available TSH assays are referred to as "third generation" assays with an analytical sensitivity defined by the lowest concentration at which a 20% coefficient of variation can be achieved. Third generation assays exhibit a 20% CV at 0.01 mIU/L and can adequately distinguish between hyperthyroid, euthyroid and hypothyroid patients. Previously available 1st and 2nd generation assays could identify hypothyroid patients but had difficulty discriminating between hyperthyroid and euthyroid patients.

Slide 5: Total T4 and T3 methods begin with an initial pretreatment step that displaces thyroid hormone from binding protein. ANS is most frequently used for this purpose. Following displacement of T4 and T3 from binding protein, displaced hormone is measured using a one-step competitive immunoassay format. Two options exist for this purpose: one in which a labeled T4 or T3 analog is used and one in which a labeled detector antibody targeting T4 or T3 is used. In the "labeled analog" format, labeled analog competes with thyroid hormone in the patient sample for a limited number of capture antibody binding sites. In the "labeled antibody" format, thyroid hormone in the patient sample competes with hormone bound to the solid phase for a limited number of labeled detector antibodies. In both cases, signal is inversely proportional to thyroid hormone concentration.

Slide 6: Measurement of free T4 in hospital laboratories is performed using automated instruments that do not physically separate free and protein-bound hormone. Automated free T4 methods typically fall into one of two categories – assays in which a T4 analog carries the detector label and assays in which an anti-T4 antibody carries the detector label. Regardless of the assay format, the assay reagent contains either exogenous analog or exogenous antibodies that disrupt the equilibrium between free and protein-bound hormone. Because of this, automated assays are more of an estimate of free T4 concentration but these assays typically correlate well with reference methods in patients whose T4 protein-binding capacity matches that of the assay calibrators. Inaccurate results are expected in patients with significantly altered T4 binding protein concentration or affinity. Both formats are one-step competitive immunoassays in which signal is inversely proportional to free T4 concentration: as free T4 concentration increases, signal decreases.

Slide 7: In one free T4 assay format, labeled anti-T4 detector antibody is added to patient sample and allowed to bind free T4. The key point to remember is that this detector antibody has relatively low affinity for T4, which allows most protein-bound T4 to remain protein-bound and minimizes disruption of the free:protein-bound equilibrium. Any labeled antibody not bound to free T4 in the patient sample binds to T4 attached to the solid phase and a subsequent wash step removes patient sample and any antibody not bound to the solid phase. Immobilized detector antibody signal is then measured and as this is a competitive immunoassay, signal is inversely proportional to free T4 concentration.

Slide 8: In another free T4 assay format, labeled T4 analog is added to patient sample. Similar to the previous assay format, the key point here is that this labeled analog has relatively low affinity for thyroid binding proteins, which allows most protein-bound T4 to remain protein-bound and minimizes disruption of the free:protein-bound equilibrium. Labeled analog then competes with free T4 in the

patient sample for a limited number of solid phase anti-T4 capture antibodies. After a wash step that removes patient sample and any analog not bound to the solid phase, signal is measured. Similar to the previous assay format, signal is inversely proportional to free T4 concentration.

Slide 9: As described earlier, measurement of free T4 is technically challenging because the vast majority of T4 is protein-bound and any attempt to measure the free fraction will inevitably disrupt the equilibrium between free and bound hormone. However, methods used to measure free T4 in reference labs disrupt this equilibrium only minimally and provide the most accurate approximation of free T4 concentration. In both equilibrium dialysis and ultrafiltration, free and protein-bound hormone are physically separated and the concentration of the separated free hormone is measured. In equilibrium dialysis, samples are placed in a dialysis membrane with a small molecular weight cutoff, allowing free hormone to escape but retaining protein-bound hormone within the membrane. The downside to this method is that dialysis requires ~20 hrs, and is manually intensive, making it impractical for most hospital laboratories. Ultrafiltration also uses a size-selection membrane filter to separate free and protein-bound hormone but applies centrifugation to reduce the time required for separation from 20 hrs to 1 hr. Results generated using ultrafiltration correlate well with equilibrium dialysis, but protein leakage into the filtrate can cause an overestimate of free T4 concentration.

Slide 10: Once a diagnosis of hyperthyroidism or hypothyroidism is established following TSH and free T4 measurement, autoantibody measurement is frequently performed to confirm an autoimmune cause of disease. Autoantibodies that stimulate the TSH receptor are frequently found in patients with Graves' Disease and can be detected using several different assay formats. In the bioassay format, patient sample is added to Chinese Hamster Ovary cells engineered to express the TSH receptor and a cAMP luciferase reporter. Following stimulation of the TSH receptor by Anti-TSHR antibodies, cAMP production increases inside the cell, stimulating the cAMP reporter to produce luciferase which generates light. In this assay format, signal is directly proportional to antibody concentration. This format is specific for activating antibodies but requires cell culture and is labor-intensive. In another format, the competitive immunoassay, anti-TSHR antibody in the patient sample competes with labeled anti-TSHR antibody in the assay reagent to bind to a limited number of TSH receptors. In this assay format, signal is inversely proportional to antibody concentration. This assay is automated and easily implemented in a hospital laboratory but it is not specific for activating antibodies as inhibitory immunoglobulins that block the TSH receptor will also be detected. In the third assay format, the bridge immunoassay, anti-TSHR antibody forms a bridge between an immobilized portion of the TSH receptor and a second, labeled portion of the TSH receptor. The labeled TSH receptor fragment is only immobilized when anti-TSHR antibody is present. In this assay format, signal is directly proportional to antibody concentration. Also, this assay is automated and specific for activating antibodies as the TSH receptor fragment included in the assay is targeted by activating but not inhibitory antibodies.

Slide 11: Anti-thyroperoxidase antibodies (anti-TPO) and anti-thyroglobulin antibodies (anti-Tg) are associated with cell-mediated autoimmune destruction of the thyroid gland, a condition known as Hashimoto thyroiditis. These antibodies are most frequently measured using a noncompetitive immunoassay in which anti-TPO antibody in the patient sample binds to immobilized TPO. Non-specific

antibodies are washed away and anti-human detector antibody is added. In this assay format, signal is directly proportional to antibody concentration.

Slide 12: Thyroglobulin is most frequently measured as a tumor marker in patients with differentiated thyroid cancer (DTC). These cancers arise from thyroid follicular cells and frequently produce thyroglobulin, which can be measured to assess disease progression or response to treatment. Most frequently, thyroglobulin is measured using a two-site immunometric sandwich assay but radioimmunoassays are available in some laboratories. It is important to remember that 15-35% of DTC patients produce anti-thyroglobulin antibodies that interfere with immunoassay-based thyroglobulin measurement. Anti-thyroglobulin antibodies cause falsely low thyroglobulin values if a noncompetitive immunometric assay is used and falsely high thyroglobulin values if a radioimmunoassay is used. For this reason, thyroglobulin and thyroglobulin antibodies should be measured in tandem in patients with DTC and thyroglobulin results should be interpreted with caution if thyroglobulin antibodies are present. Recently developed mass spectrometry-based methods are not susceptible to anti-thyroglobulin antibody interference and provide an alternative testing option in patients with anti-thyroglobulin antibodies.

Slide 13: In summary, TSH is the preferred initial test in the assessment of thyroid disease. If TSH is abnormal, free T4 should be measured to confirm disease and identify the underlying mechanism. Autoantibody measurement is a useful tertiary test to confirm or rule out an autoimmune etiology.

Slide 14: Here is a list of the references used in this pearl.

Slide 15: None

Slide 16: None

Slide 17: Thank you for joining me for this pearl of laboratory medicine on thyroid function testing.