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Thyroid Hormones and Interference in Thyroid Function Tests: A Review

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ABSTRACT: The objective of this paper was to provide a critical and concise review on thyroid hormones and what constitutes interference in thyroid function tests using appropriate Online resources.Thethyroid function tests, play a valuable and significant role in diagnosing and managing thyroid disease. Discordance can exist between test results and symptoms, leading to false laboratory reports, misdiagnosis, and mismanagement of thyroid disease. Interference in thyroid function tests can be caused by heterophile antibodies, macro TSH, biotin interference, anti-streptavidin and anti-ruthenium antibodies, thyroid hormone antibodies, and the high dose hook effect. This review paper also discusses the importance of studying interference, the mechanical perspectives of interference, and protocols to limit or eliminate interference in thyroid function assays. Additionally, it highlights quality control and laboratory safety aspects, as well as advancements in healthcare team deliveries related to thyroid function tests.

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Thyroid hormones are processed by the thyroid gland in association with the anterior pituitary and the hypothalamus within a self-regulatory axis defined as the hypothalamic-pituitary-thyroid (HPT) axis. These hormones are responsible for metabolism, growth, and various other systemic functions, and also impact reproductive health (Silva *et al*., 2018). The thyroid gland synthesizes two significant hormones, thyroxine or tetrahydrothyronine (T4) and triiodothyronine (T3), which circulate in the bloodstream bound to well-characterized carrier proteins, the thyroid hormone binding proteins (THBP), mainly of hepatocellular origin (Omon and Ajayi, 2023). Essentially, the thyroid-releasing hormone (TRH) originating from the hypothalamus, along with thyroid-stimulating hormone (TSH) from the anterior pituitary, and T4 exhibit harmony for appropriate physiological feedback and homeostasis (Bogazzi *et al*., 2010). Low and high levels of circulating thyroid hormones are identified as hypothyroidism and hyperthyroidism, respectively. Hypothyroidism presents as weight gain, fatigue, bradycardia, intolerance to cold, and constipation, and is caused by a weakened thyroid gland, while hyperthyroidism presents as weight loss, intolerance to heat, diarrhea, fine tremor, and muscle weakness (Shahid *et al*., 2023).Consequently, the objective of this paper is to provide a critical and concise review on thyroid hormones and what

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constitutes interference in thyroid function tests. The synthesis of thyroid hormones requires iodine, an essential trace element that is rapidly absorbed in the small intestine and is relevant for normal thyroid function. Iodine is an intrinsic constituent of T3 and T4. Sources of iodine include natural foods such as seafood and fish, seaweed, vegetables, and iodized table salt. A reduced quantity of iodide intake is responsible for iodine deficiency and reduced synthesis of thyroid hormones (Knudsen *et al*., 2002). Iodine (as iodide) is largely but not equally distributed in the earth's environment. Substantial iodine is present in the oceans (approximately 50µg/L), and iodide ions in seawater undergo oxidation to elemental iodine and volatilize into the atmosphere, returning to the soil through rainfall, completing the cycle. In many regions of the world, iodine cycling is decelerated and imperfect. Therefore, soils and groundwater begin to be deficient in iodine. Food plants grown in this deficient soil have low iodine content, and humans and animals ingesting foods grown in these soils will be iodine deficient. Iodine deficit in the body has a number of adverse effects, including thyroid disorders, especially hypothyroidism, goiter, cretinism, miscarriage, stillbirth, myxedema coma, neurocognitive impairment, and coma. The World Health Organization (WHO) has advocated universal iodination of salt (USI) for both human and animal consumption to enhance the iodine dietary status of people globally (WHO, 2014). Potential risk factors that may lead to iodine deficiency include reduced dietary iodine, selenium deficiency, pregnancy, radiation exposure, elevated intake of plasma levels of goitrogens such as calcium, gender (higher occurrence in women), tobacco smoking, alcohol, oral contraceptives, perchlorates, thiocyanates, age (for various types of iodine deficiency at different ages) (Panth*et al.*, 2019).

The thyroid gland of a normal adult produces about 90µg of thyroxine and less than 10µg of triiodothyronine (liothyronine) per day. Several tissues, especially the liver, convert less than 50% of T4 to T3. The majority of the daily secretion of T3 results from peripheral 5'-deiodination of T4 and not direct glandular secretion. Thyroid hormones are firmly bound to plasma proteins, especially T4 compared to T3. This accounts for the lengthy half-lives, eight days for T4 and just about a day for T3. Nuclear receptor activity for thyroid hormones favors T3 binding more efficiently than T4, which reasonably explains the rapid onset of action and excellent biological strength of T3 (Schalliol and Pitman, 2021). The cellular level activity of thyroid hormones begins with the regulation of thyroid hormones starting at the hypothalamus. Thyrotropin-releasing hormone (TRH) is released by the hypothalamus into the hypothalamic-hypophyseal portal system to the anterior pituitary gland. TRH activates the thyrotropin cells in the anterior pituitary to release thyroid-stimulating hormone (TSH). TRH is a peptide hormone and also a tropic hormone (indirectly impacting cells by stimulating other endocrine glands initially (Braun and Schweizer, 2018). It binds to TRH receptors on the anterior pituitary gland, which brings about a signaling response modulated by a G-protein-coupled receptor. Related effects following G-protein activation have been described (Braun and Schweizer, 2018). TSH in circulation binds to the thyroid-releasing hormone (TSH-R) on the basolateral aspect of the thyroid follicular cells. The TSH-R is a G-protein-coupled receptor, and following its activation, adenylcyclase and intracellular levels of cAMP are turned on (Shahid *et al.*, 2023). The elevated cAMP activates protein kinase A (PKA), which modulates the phosphorylation of distinct individual proteins to alter their activity (Braun and Schweizer, 2018). The five steps of thyroid hormone synthesis are properly elucidated and summarized as the synthesis of thyroglobulin, iodide uptake, iodination of thyroglobulin, storage, and release into circulation (Shahid*et al.*, 2023). However, the unutilized iodotyrosines are deiodinated, and the iodide is recycled.

Physiological Functions and Mechanism of Action of Thyroid Hormones: The physiological functions of thyroid hormones are significant and diverse. These include elevation of the basal metabolic rate, induction of lipolysis or lipid synthesis depending on the metabolic status, involvement in carbohydrate metabolism, permissive biochemical effects on catecholamines, anabolism of proteins, and catabolism if protein levels are high, synergistic activity with growth hormones to stimulate bone growth in children, brain maturation and impact on the central nervous system, and effects on fertility, ovulation, and menstruation in women (Shahid*et al*., 2023). Other functions include stimulation of the respiratory centers and increased development of type II fibers responsible for fast and powerful contractions. Various organ systems and metabolism are involved in all of these functions. The interaction of thyroid hormones with intranuclear receptors activates genes for an accelerated metabolic rate and thermogenesis. This leads to increased oxygen and energy consumption (Omon and Ajayi, 2023).

Thyroid hormones are characterized as lipophilic and circulate in the blood bound to transport proteins, with only about 0.2% of the hormone (Free T4) being unbound and active. Identified transporter proteins

include thyroxine-binding globulin (TBG), transthyretin, and albumin. Two-thirds of T4 is transported by TBG, while transthyretin transports thyroxine and retinol. When thyroid hormones reach a specific target site in the body, T3 and T4 dissociate from their binding proteins to enter cells either through diffusion or carrier-mediated transport (Shahid*et al*., 2023). Thyroid hormone action is modulated by a complex of thyroid hormone receptor isoforms derived from two separate genes. These receptors are part of a nuclear receptor superfamily that also includes receptors for other small lipophilic hormones. The function of thyroid hormone receptors is to bind to unique thyroid hormone-responsive elements in promoters of target genes and control transcription. Other associated mechanisms of action of thyroid hormones with their receptors are also described. Thyroid hormones are metabolized via sulfation and glucuronidation and excreted in the bile (Brent, 2012).

*Physiology of Thyroid Gland:*The physiology of the thyroid gland results in hyperthyroidism and hypothyroidism. Thyroid-stimulating hormone (TSH), produced by thyrotroph cells located in the anterior pituitary gland, modulates thyroid gland function, hormone formation, and release. The secretion of both TSH and thyrotropin-releasing hormone (TRH) is controlled by negative feedback from thyroid hormone, mostly T3 within the circulation, and/or T3 that is produced locally from intracellular conversion of T4 to T3. An elevation of circulating thyroid hormone levels leads to the synthesis and secretion of serum TSH being blunted. In contrast, when T4 and T3 levels in circulation are low, serum TSH is increased in a compensatory fashion. The mean level of serum TSH in apparently healthy individuals is approximately 1.5 µU/ml, as stated in the NHANES III study and by Hollowell*et al*., 2002. A healthy hypothalamic-pituitary function results in a marked suppression of TSH levels (<0.05 µU/ml) in patients with hyperthyroidism and elevated serum thyroxine levels in the blood, while a significant elevation in TSH (>5 µU/ml) presents in patients with hypothyroidism and reduced blood levels of T4.

Hyperthyroidism: Disorders associated with the thyroid gland can lead to an overproduction of T3 and T4 along with a compensatory decrease in TSH. Additionally, thyrotroph adenoma can result in unregulated TSH production, leading to elevated T3 and T4 levels. Ectopic production of thyroid hormones exists, producing elevated thyroid hormone levels with a compensatory decrease in TSH.

Hypothyroidism: This condition is varied and has been identified as primary, secondary, and tertiary. In primary hypothyroidism, reduced production of thyroid hormones by the thyroid gland results in a compensatory increase in TSH. In secondary hypothyroidism, pituitary disorders are responsible, leading to reduced TSH release and decreased T3 and T4 levels, while hypothalamic disorders are responsible for tertiary hypothyroidism, resulting in reduced TRH levels, reduced TSH, and T3/T4 levels. Graves disease and Hashimoto thyroiditis are frequently caused by hyperthyroidism and hypothyroidism, respectively, and have been well elucidated (Shahid*et al.*, 2023).

*Importance of Studying Thyroid Hormones and Interferences:*Thyroid function test is one of the commonly requested test globally given its role in metabolism and reproduction. For results to be reliable, relevant and reproducible, there is the need to investigate and identify possible interferences and those with possible threats of interference. Therefore, the importance of studying thyroid hormones and its interference in thyroid function tests immunoassays will assist facilitate detection algorithms, clinical impact of interference in immunoassays, and to avoid misdiagnosis. Others are to mitigate inappropriate request and prescription of unnecessary treatment, inappropriate suppression or the modification of a current therapy or the use of an unnecessary complimentary procedure such radio isotopic thyroid imaging science. Also, appropriate deployment of suitable machines could be enhanced given the problems associated with automated machines configurations for thyroid function test with manufactures of machines adequately and better informed.There is the benefit of knowing what to do in specific cases of interferences.Beyond these, there would be adequacy of knowledge to enhance interpretation of result and equally develop a nexus of communication between the laboratory and clinical staff and between the clinical staff and patient for better clinical outcome.

Generally, the diagnosis of thyroid disease by clinicians is based on blood levels of the thyroid hormones (TH), (L-3,3’5- triiodothyronine (T3) and (L -3,3’,5,5’-Tetraiodothyronine (thyroxine(T4) including the measurement of serum TSH , a loss of inverse relationship between TH and TSH concentration or a markedly different levels of blood T4 and T3 (such as high T3 and low T4 in a TH transporter defect); a broad differential diagnosis should be considered and correct diagnosis can be made from clinical observations and confirmed by appropriate genetic testing (Weiss *et al*., 2010). Initially, the tests of choice to investigate thyroid abnormalities are TSH and free T4 tests. These tests

determine whether the abnormality arises centrally from the thyroid gland (Primary abnormality), peripherally from the pituitary (Secondary abnormality) or hypothalamus (Tertiary) (Shahid*et al*., 2023). Free T4 can serve as a proxy for serum T3 levels. Frequently, thyroxine levels are the last abnormality to appear in thyroid disorders as upstream processes produce TSH and T4, maintaining available T3 at their own expense (Wang *et al*., 2019).

*Interferences with Clinical Analysis in the Laboratory:*Interferences in clinical analysis by endogenous and exogenous substances is a frequent and usual problem in medical laboratory testing and practice. In a review document on interferences with clinical laboratory analysis by Kroll and Elim, 1994, interferences had been defined as the alteration of the correct value of a result by the effect of a substance available in a sample. Two common definitions have been alluded to by the authors: 1). Analytical interference is the systematic error of estimation determined by a sample component which does not produce a signal by itself in the measuring system. 2). The effect of a substance on any step in the estimation of the concentration or catalytic activity of the analyte of interest. Some of the endogenous molecule that frequently interfere with medical laboratory reports are haemoglobin, lipids, bilirubin and paraproteins and the exogenous interfering molecule are prescription drugs for the patient (Kroll and Elim, 1994).

The interference of endogenous and exogenous agents in the estimation of analytes is significant. This obstacle is further compounded by the variety of reagents and instruments used in different procedures. The ability to anticipate and address potential interference is essential. This requires a method of understanding it. One effective way to comprehend interference in clinical chemistry is to clearly explain the sequence of reactions involved. These sequences are known as mechanisms. Mechanisms can be specific, global (generic), analytical, or chemical. Global mechanisms involve overall sequences affected by multiple interferents and procedures. Specific mechanisms pertain to the sequences for individual substances or categories of substances with a specified type of reaction. Analytical mechanisms demonstrate how interfering substances impact performance. Chemical mechanisms focus on the chemical reactivity, utilizing concepts and theories of organic, inorganic, physical, and biochemical composition, structure, properties, and changes (Kroll and Elim, 1994).

Global mechanisms of interference are generally analytical mechanisms that are based on whether the interference sequence involves the analyte, its products, or whether it prevents the analyte or its products from being detected. This is known as analyte-dependent interference (Sturgeon and Viljoen, 2011). Distinguishing between analyte-dependent and analyte-independent interference involves determining analyte levels at four or more concentrations of both analyte and interferent, followed by a multivariate regression analysis. A positive interference caused by an interferent indicates a lack of specificity in the method. Understanding the entire mechanism is crucial to avoid underestimating interference in clinically relevant concentrations of analyte and interferent (Kroll and Elim, 1994).

The impact of this interferent have been properly elucidated (Kroll and Elim, 1994). Unique subsets of macromolecular binding interactions have been identified in immunoassays. At least one antibody type is included in immunoassays. Cross reactivity between the analyte and other elemental substance in the specimen typifies or presents the major recognized form of interference in immunoassays. Antigen – antibody reactions entail fragile intramolecular strength of which hydrogen bonds and van der Waal associations are examples. This differentiates it from chemical reactivity which requires a change in covalent bonds. Antigen –antibody associations are based on the shape or structure of the antigen and not on the chemical reactivity. Numerous and valuable shapes obliged by antibody variable region leads to the vast assemblage of antibody capable of interacting with various biological components while sustaining its specificity. The ligand antibody interaction is static in majority of cases because of the non-covalent nature. The size of the variable regions on antibodies and its recognition patterns on ligands have been identified and represents a weakness of immunoassay due to the difference between the analyte and other chemical components being minute (Kroll and Elim, 1994).

*Laboratory Interferences of Thyroid Function Test:*Worldwide studies for thyroid hormones and thyroid stimulating hormones are well recognized and utilized. Results from these tests have important effects on how therapeutic and diagnostic process decisions are made and executed. Discordance between symptoms and laboratory reports or between reports of different test should be evaluated to eliminate misdiagnosis, unwarranted treatment and unrequired and repeat ordering. Some of the identified causes of inconsistences in hormone investigations are disease, physiological alterations in hormone equilibrium, drug consumption, laboratory auto-

antibodies, anti-rheumatism antibodies and streptavidin (Paczkowska*et al.*, 2020).

Investigating thyroid function is mostly based on measuring free thyroid hormones and thyrotropinlevels as thyroid function tests. Immunochemical methods or immunoassays are methods of choice for determination by antigen-antibody interactions. The bonding or combination activity between the antigen binding site of the antibody Fab fragment and specific determinant of the antigen molecule gives rise to an antigen –antibody complex (Ag-Ab) (Paczkowska*et al*., 2020). Immunochemical investigative methods fall into two (2) groupsdetermined by the principle of the test. These groups are competitive and non-competitive and have been well described (Haddad *et al.*, 2019).Estimation of free T4 and free T3 are done using a competitive assay in the medical laboratory while TSH estimation is done using non-completive assay. Immunochemical methods utilize tracers for the generation of signal. With this, rapid response and detection of low levels of measured parameters are possible. Labels are used in immunochemical methods with an initial isotope that facilitate radioactivity count (RIA-Radioimmunoassay) which can be identified. At the present time, this is infrequently utilized in routine studies as a result of non-availability of automated procedure. Various other labels are enzymatic, immunofluorometric types. Labels that give off light is called chemilumnescent assay (CLIA). Other assay type utilizeselectrochemiluminescence procedures (ECLIA) In this regard, chemiluminescence is facilitated by electrochemical reactions (Kohl and Ascoli, 2017).

Competitive procedures for the estimation of the routine low molecular weight analytes such as FT3 and FT4 and also the non-competitive procedures for the estimation of high molecular weight antigens necessitates the separation of the resulting complexes from reaction mix or combinations. These separations proceed in distinct ways. An example is the biotin – streptavidin system depending on the makers of the test kits. Steptavidin is a glycoprotein with high combination tendency for biotin. Biotin is a low molecular weight water soluble vitamin of 244.31Da and it is vital for enzymatic interactions needed for various metabolism in the human body (Hawker, 2012). Some of the unparalled or sole characteristic of the streptavidin and biotin association or interactivity makes it suitable for its wide use in immunochemical test. These interactivity or association are distinct, distinguished by a strong combination and resistance to different exploitations during estimations such as pH, temperature gradient washing and utilizations of denaturing substances. Also, an advantage of this reaction mix is that biotin does not alter the characteristics of the molecule that it binds to (Cinquanta*et al*., 2017).

Furtherance to this, is also the fact that it is little to be utilized in the estimations of small molecules such as thyroid hormones due to non-alteration of their biological effects such as the competence to bind the antibody. It should be noted that the biotin – streptavidin/-avidin system is a high combination isolation procedure that elevates the sensitivity of the test procedure (Paczkowska*et al*., 2020). The advent of immunochemical techniques, the application of distinct labels and the continuous upgrade of testmethods have heightened the sensitivity of the analytical determination of thyrotropin. Various detection limits have been identified. For first generation test, usually, RIA the detection limit is 1-2µIU/ml, the second-generation test commonly the enzyme linked immunosorbent assay (ELISA) took the detection limit further to 0.1–0.2µIU/ml with the third-generation tests attaining a functional sensitivity of 0.01 – 0.02µIU/ml. In routine medical laboratory studies, the third-generation assays are extensively used. As at 2007, many manufacturing companies had begun providing forth generation test with estimation sensitivity of a maximum of 0.001µIU/ml (Sarkar, 2014).

Laboratory Interferences in Thyroid Hormone Immunoassays: Two types of laboratory interference have been identified which are positive interference and negative interference. Positive interference give rise to a falsely increased test value and a negative interference is linked with false decreased test value (Vandendriessschee*t al*., 2020). All stages of measurement are affected in immunoassay by varying concerns and the major ones are: Heterophile antibodies, Macro TSH, biotin, antistreptavidin, anti-ruthenism antibodies and thyroid hormone auto antibodies.Aberant diagnosis as a result of laboratory interference is a significant concern. It becomes imperative laboratory reports should be linked with symptoms and signs prior interpretation. A considerable number of patients seeking advisories from specialist officers due to minimal and subjective complaints arising from varying aetiologies presents a challenging concern. The implication therefore is results of hormone test must be evaluated and interpreted with caution when hormonal parameters are considered.

In a well-documented report that analyzed the clinical consequence in not less than 150 cases of laboratory interference spanning about 40 years, not more than

8% of cases had absent clinical effect and 42% of cases was undetermined. The negative clinical outcome was noticed in about 50% of patients which involves inappropriate L-thyroxine or antithyroid drug treatment, radioactive scans, extra hormone assay and decelerated diagnosis and treatment (Paczkowska*et al*., 2020). When an interference is suspected by the clinician involving a test result, a request can be made that the laboratory executes an interference evaluation. This is due to the fact that the hallmark of interference is having a discordance between the test report and clinical manifestationof the patient. The impact of the inability to recognize interference can lead to adverse clinical consequences (Favresse*et al*., 2018).

Laboratory evaluations for interference involves disparity between various manufacturers procedures and protocols, addition of blocking agents and subsequently remeasuring the analyte and executing linearity studies inclusive of recoveries or precipitating immunoglobin with polyethylene glycol (PEG). Once analyte concentration is altered in response to any of these, it is suggestive of interference. However, a lack of effect does not rule out interference. The identification of interference as being non analyte specific or analyte specific has been described (Sturgeon and Villagoen, 2011). The non analyte specific interferences are protein interferences, congenital TBG excess or deficiency, pregnancy, familial dysalbuminaemic and transthyretin associated hyperthyroxinemias, heterophile antibodies (HAbs0, and anti-reagent antibodies while analyte specific interference are due to T4 and T3 auto-antibodies (Braunstein, 2022).

Endogenous Concerns Interfering with Hormone Immunoassay Measurements

1). Excessive normal blood or serum components.
a). Hyperlipidemia. b). Hyperbilirubinemia. c). Haemolysis. d). Human binding protein alterations or abnormalities.
2). Cross reacting substances.
a). Exogenous hormone or medicines. b). Hormone isoforms and subunits. c). Complex hormone protein interactions.
3). High dose hook effect.
4). Interference with assay components.
a). Auto antibodies. b). Heterophile antibodies. c). Human anti animal antibodies. d). Anti-rutherium antibodies. e). anti-streptavidin antibody. f). Biotin (Braunstein, 2022).

*Interference with Thyroid Hormone Assay Components***:** *Heterophile Antibodies:* Antibodies present in numerous patients' blood interfering with an immunoassay is misunderstood and remains unresolved. The reactivity of these antibodies with the analyte of concern or with the antibodies employed in the procedure gives rise to untrue results. There is occurrence of negative interference and lower than expected result in reactions between an analyte e.g.hormone and antibodies in serum which give rise to barrier between the analyte molecules and detection antibodies. However, positive interference brings about higher than expected result between interfered antibodies reactivity with detection antibodies (Paczkowska*et al.,* 2020). Heterophile antibodies are human poly-specific antibodies directed against animal antigens, the most prevalent being anti-mouse antibodies (HAMA).Another possibility is that heterophile antibodies can aim at human antigens. An example of heterophile antibody is the rheumatoid factor (RF) which is an immune protein frequently linked auto immune conditions. It has been demonstrated that RF interfers with free and total thyroid hormones test as well as TSH and thyroglobulin (Ismail, 2007).

Statistical evaluations in respect of heterophile antibodies by a number of authors show a prevalence of 30-40 % with a potential to interfere with a wide range of procedures that utilize immunometric assays otherwise known as non-competitive immunoassay (type 1) which uses an excess of labelled specific antibody directed at the analyte of interest. Despite the use of immunoglobulin blocker agents added to tests by manufacturers, interference has decreased from 2% - 5% but interference is still present in about 1% patient with elevated levels of HAbs that overwhelms the blocking agent. Heterophile antibodies has the ability to interfere with both free and total thyroid hormone test and also THBR, TSH, Tg, TgAb and calcitonin. Heterophile antibodies or HAMA (Human anti mouse antibodies) normally produce untrue elevated results for most analytes. Conversely, untrue low results may be exhibited. It should be noted that different manufacturers of test may have varying effects of interference,if any. Therefore, the initial requirement of investigating interference is re-measurement of the analyte in a different manufacturer's method (Nakano *et al.,* 2016).

It has been identified that recent vaccinations, blood transfusions or monoclonal antibodies for treatment or scintigraphy as well as animal health specialist who come in contact with zoological creatures in the work environment are particularly susceptible to test interference broughtabout by induced HAb and HAMA (Sareen*et al.,* 2019).Unknown exposures involving heterophile antibodies are frequent in about 40% of the general population. Different types exist but have no effect on immunoassays. However,

problematic ones exist and there are the ones with high binding capacity to the Fc- region ofantimouseIgG antibodies (Paczkowska*et al.,* 2020). There is an urgent need to understand the difficulty and time consumption in identifying interference in immunoassay. Therefore, to identify interference with heterophile antibodies in about 90% of suspected serum, three independent tests are required; 1). A repeat analysis with an alternate method or a different test platform is required even though interference may be present in both analysis. 2). Carrying out a serial dilution. 3). Analysing the serum sampleafter incubating with a commercial blocking agents or antibodies. Therefore, to improve detection capabilities, all three methods can be used when discrepancies in results are suspected due to heterophile antibodies (Paczkowska*et al*., 2020).

Macro TSH: MacroTSH circulates largely in the form of TSH molecules and bound to anti human TSH autoantibodies. As the name implies, macro TSH is a large molecular weight substance of not less than 150KDa compared to TSH molecule of 28KDa (Favresse*et al.,* 2018). Currently,macro TSH is considered inactive and restricted to the intravascular component because of its large size and with the auto antibodies bound to TSH, may avert the trigger of TSH receptors due to steric impediment (Favresse*et al*., 2018). Two site immunometric assays for TSH testing does not in its entirety distinguish macro TSH from bioactive free TSH despite the availability of platforms such as Cobas analyzer, Roche diagnostics which are highly sensitive to its presence than others such as Architect analyzer, Abbot (Chicago, IL). This therefore can lead to high TSH results that are false making interpretation challenging and difficult for the clinician. An ideal immunoassay should identify the presence of bioactive TSH and there should not be a reactivity with macroTSH. Thyroid disease without symptoms in patients but having an elevated TSH levels and FT3 and FT4 within the reference values in laboratory test should be a case with a high suspicion of Macro TSH (Paczkowska*et al.,* 2020).

Available data from a large population reveals the prevalence of macro TSH among patients with subclinical hypothyroidism is at less than 2%. Associated clinical and diagnostic conundrum has been highlighted and elucidated (Hattori *et al*., 2016). The wide utilization of polyethylene glycol (PEG) precipitation in routine clinical practice for screening and diagnosing macro prolactinaemia in those with high blood prolactin levels also cuts across macro TSH detection. The presence of macro TSH in a serum specimen is highly suspicious when TSH recovery is low. A number of PEG precipitation procedure is available with a percent recovery procedure typically executed.Most commonly, the percent recovery mathematics, up to 25% is used but the PEG precipitation technique seems inconclusive despite its use as a screening tool for macroTSH. Also, the prospects of recovery calculation being appropriate in the clinical and diagnostic process for managing thyroid conditions in some patient is still of concern and properly described (Beda-Maluga*et al*., 2014). When the recovery calculation is reduced, macro TSH can be identified with gel filtration chromatography which is a preferred analytical procedure for the identification of Macro TSH (Hattori *et al.,* 2016). Out of 117 patients with reduced PEG recovery of TSH (< 25%), the presence of macro TSH was confirmed in only seven using gel filtration chromatography (GFC).

Biotin Interference: The biotin–streptavidin combination is very much utilized in immunoassay for the estimation of TSH, FT3, FT4 and anti- TSHR. Biotin is a vitamin (vitamin H B7 or B8 of low molecular weight (244.3Da) and a cofactor for carboxylases produced by gut bacteria and a bioavailability from food intake. For an adequate intake, an evaluation of 30-35µg/d in adults is satisfactory while in children, 5-25µg/d is appropriate with basic diet providing 35-70µg/d. Biotin readily has great affinity for hormones and antibodies mostly used in test procedures. With additional biotin consumption, higher values of this molecule is found in blood thereby interfering in assays with a tendency for false positive or false negative results. Biotin is used for the treatment of dermatological concerns ranging from hair loss (alopecia to frail nails, and for unusual metabolic disturbances such asmultiple sclerosis, mitochondrial disease biotinidase deficiencies and propionic acidemia at a concentration of 10-40mg/d by dermatologist recommendation. Others are thiamine transporter -2 deficiency or holocarboxylasesynthetase deficiency (Ostrowska*et al*., 2019). Significant laboratory interferences are not observed in food supplements containing biotin because of its reduced dosage. Concerns arise when patients receive biotin more than the recommended intake e.g. 10,000 times and such is found in treatment of multiple sclerosis when dosage can be from 100-300mg of biotin per day (Minkovsky*et al*., 2016).Biotin was discovered from a simple observation with the consumption of raw egg whites giving rise to toxic effects which could be prevented using an unknown substance in the egg yolk.

Test platforms for hormone levels evaluation is linked with test interference because biotin may influence TSH, FT4, FT3 or all of it. TSH levels may be falsely reduced with FT4, T3 and T4 concentrations falsely

increased. This presentation should be in mind before therapy begins especially when patient does not exhibit symptoms (Elston*et al*., 2016). In a review by Holmes and colleagues, on manufacturers technical manual for more than 350 procedures used by eight of the most popular immunoassays, about 60% was biotinylated. The extent of this concern is huge given the increased rate of testing for thyroid dysfunction despite the fact that prevalence of biotin interference is unknown (Holmes *et al*., 2017). With great interest, in TSH sandwich assays excess biotin disrupted biotinylated antibody antigen nexus from streptavidin – coated microparticles leading to falsely low TSH concentrations (assay signal is directly proportional to TSH concentration). In the contrary, for competitive assays of FT4 and FT3, excess biotin resulted in over production of both hormones (assay signal is inversely proportional to hormone levels) (Favresse*et al.,* 2018). Various testing platforms have been dominated by effect of biotin and should be carefully noted. For instance, Roche platforms, TSH, FT4, FT3 may be influenced by excess biotin. Ortho clinical diagnostic platforms (Rarihan, NJ), TSH is reduced since FT4 and FT3 do not utilize the biotin –streptavidin interaction. Conversely, FT4 and FT3 are increased in Beckman Coulter diagnostics platforms (Brea, CA) while TSH is not affected. Curiously, the centaur FT4 platform (Siemens Health care, Erlangen, Germany employs a preformed streptavidin –biotin complex insensitive to the availability of biotin. Similarly, the abbot and diasorin (Saluggia, Italy) immunoassays are not influenced by biotin since the biotin –streptavidin immobilization system is unemployed for TSH, FT4 and FT3 estimations. The implication therefore is that these last listed platforms become a suitable method of choice for indirectly identifying biotin interference (Holmes *et al.,* 2017; Favresse*et al*., 2018).

Biochemical results expressed in patients taking biotin may wrongly affect the determination of thyroid state in varied ways with different platforms. With this,endogenous and endogenous hyperthyroidism may be suspected when hormone measurements are done on the Roche and Siemens platforms and subclinical hyperthyroidism or any other cause of TSH becoming gradually less may be obtained on the orthoplatform and drug interference or resistance to TH (e.g.amiodarone, heparin) may be seen on the Beckman Coulter platform. Amiodarone should be given some consideration here. It inhibits type 1 deiodinases and its initial use commonly leads to a brief and mild increase of FT4, reduced Ft3 and increase of TSH. Patients who live in iodine sufficient regions may present with amiodarone induced hypothyroidism (AIH), particularly patients with positive thyroid antibodies or basic Hashimoto's thyroiditis. In iodine deficient regions, amiodarone induced thyrotoxicosis (AIT) is a common occurrence than AIH. Two types of AIT exist and both needs to be treated for. Type 1 AIT occurs due to a surplus quantity of iodine in amiodarone and this provides high substrate for thyroid hormone synthesis (Jod-Basedow phenomenon. Patient with latent Grave's disease or established multinodular goiter may experience this phenomenon. Type 2 AIT occurs in apparently normal thyroid tissues or small goiters. Amiodarone produces direct cellular toxicity on the thyroid follicles with preformed thyroid hormones released (Soh and Aw, 2019;Bogazzi*et al.,* 2010).

To identify interference, a useful approach will be to have a conversation with the patient on biotin use. The non-availability of information in this regard or a denial and there is still doubts about biotin interference, a dilution with manufacturers diluents or a juxtaposition with a different method without biotin-streptavidin interaction can be employed. The European medicines agency deem it a mandate that a warning message must be part of biotin supplements containing a dose greater than 150 µg in respect of risk associated with erroneous laboratory results (Balzer and Whitehurst, 2023). Also, wash out interactivity is advised to make it free of interference with wash interactivity of 8hrs, 16hrs, 25hrs, 2 days, 3 or more. However, this may cause a problem implementing the wash out operating steps. In mitigating the arguments associated with biotin wash out periods, the use of streptavidin beads is suggested as a possible plan. Succinctly, the sample with biotin content is incubated with the streptavidin beads and then investigated on the same platform. The presence of biotin brings about a significant change from the base line value as required. The procedure will eliminate interruption in biotin treatment and a second blood sample becomes unnecessary (Ghazal *et al.,* 2022).

If biotin interference is properly identified, unwanted/unnecessary repeated blood test, specialist referrals, delayed treatment, not essential imaging, stress for the patient, the starting of inappropriate treatment like methimazole will be circumvented. It should be noted that biotin does not only interfere with thyroid hormones, other molecules are also affected such as estradiol, troponin–I, parathyroid hormone (PTH), testosterone, dehydroepiandrosteronesulphate (DHEAS), LH, 25-hydroxyvitamin D, prostate specific antigen. Some tumor markers have also been identified to be affected in this regard such as cancer antigen 19.9 (Ca19.9), carcinoembryogenic antigen (CEA) and alpha fetoprotein (Paczkowska*et al.,* 2020; Li *et al.,* 2018; Favresse*et al.,* 2018; Lam and Kyle, 2017; Piketty*et al.,* 2017). As an addendum to biotin interference, the extensive utilization of biotin–

streptavidin system in immunoassay and ample consumption of dietary supplements or drugs with high dosage of biotin in patients with various pathologies is linked with regular occurrence of interferences in clinical practice. Possible ways to avoid this is to: a). Evaluate patient medical history properly and supplementary intake prior carrying out a medical test. b). Giving information to patient about abstinence from biotin for at least 24 hrs before medical test. c). If kidney disease is suspected or biotin is taken in high doses for neurological disease, the time interval between biotin use and laboratory test should be longer, may be, to seven days (Ostrowska*et al.*, 2019; Pikett*yet al.,* 2017).

Depletion protocols make it possible for biotin in the sample to be removed before testing. This is done by adding streptavidin coated agarose beads to the sample which is followed by incubation and centrifugation. If the test result shows a significant difference before and after the removal, biotin interference is determined. a great benefit of using streptavidin coated beads is the speed up of separation of the free biotin in the sample with well-established methods and commercial concerns. Dilution of the sample can also reduce the level of biotin to help eliminate interference (Balzer and Whitehurst, 2023; Luong and Vashist, 2020; Bowen *et al*., 2019).

Anti –streptavidin and Anti-ruthenium antibodies: Anti-streptavidin and anti-ruthenium (Anti-Ru) antibodies are uncommonly seen interferences in thyroid function tests and influences immunoassays based on different methods. Streptavidin is a tetrameric protein identified as a production of the microbe (antibiotic secreting *Streptomyces avidinii*). It has a molecular weight of 60,000 Da and binds biotin with very high specificity and affinity with an affinity constant of 10^{15} L/mol. Its homologs have been found in a diverse number of organisms such as fungi, bacteria, chickens and frogs (Balzer and Whitehurst, 2023; Favresse*et al*., 2018). The functional and structural relevance of avidin and streptavidin have been properly elucidated (Balzer and Whitehurst, 2023). Comparatively anti streptavidin antibodies interferences on thyroid function test (TFT) are not as reported as biotin but interference may be seen in other test and may lead to disease management. Presentations of interference with anti-streptavidin has similarities with that of biotin. Findings are that of increased free T4 and free T3 and decreased levels of TSH on platforms that employs biotin streptavidin complexes. It has been observed that positive interference in evaluation of antibodies against TSH receptor (anti-TSHR), thyroid peroxidase (Anti–TPO) and thyroglobulin (anti-TG) does occur. Sandwich immunoassay (estimating TSH) is less influenced than competitive assays (estimating FT4 and FT3) and wash periods becomes necessary since antistreptavidin interference is endogenous and sustained e.g. between 18 to 24 months. In identifying this interference, challenging the patient's serum with a different platform that does not employ the biotin- streptavidin mix e.g.diascorin, Abbot, is beneficial. Other indications are the PEG precipitation procedure and dilution test. Others are incubating patient serum with streptavidin –agarose mix (scarce though) and utilization of manufacturer's streptavidin beads (favourable in routine investigations. These methods can be deployed for the screening of anti-streptavidin antibodies despite being proven and acceptable for biotin interference. Based on cases that have been published, sending a fraction of the sample to the manufacturer have to produce a definite result in identifying the interference (Paczkowska*et al.,* 2020; Favresse*et al*., 2018; Tranbas*et al*., 2018; Lam and Kyle, 2017;Pikett*yet al.,* 2017; Harsch*et al.,* 2017).

Anti-streptavidin antibodies can produce an interference with anti –TSHR estimation with amisdiagnosis of Grave's disease. Documented reports in the literature of five case showed two patients received antithyroid therapy. The first subject was on treatment for three (3) months while the other presented with hypothyroidism. If symptoms of hypothyroidism are absent and observed, laboratory measurements only suggest the disorder, interference should be suspected (Paczkowska*et al*., 2020).Immunoassay interference with anti-streptavidin is becoming more prevalent with numerous new cases as documented in recent literature. An attempt to determine the rate of occurrence of anti- streptavidin in Norwegian population found discordant thyroid function test in 42 serum samples as analyzed. Interference profiles of 34 of the 42(81%) serum samples were caused by the availability of endogenous anti-streptavidin. studies investigating falsely increased results of anti CPP IgG antibodies, a marker used for diagnosing rheumatoid arthritis, out of 1375 patients, 8(0.6%) were found to have falsely raised results determined by anti-streptavidin interference. Other associated concerns have been described (Dahll*et al.,* 2021; Berth *et al.,* 2018).

Ruthenium (Ru) is a chemical element and rare transition metal of the platinum group of the periodic table and inert to other chemicals. It has wide application as catalyst in electrical contacts, thick film chip resistors and platinum alloys. It has also been identified in the food chain and clothing residues (Favresse*et al*., 2018). The prevalence of anti-Ru

interference has up to 0.24% from 0.1% (Ando *et al.,* 2007). It is used as labels in some laboratory methods based on electrochemiluminescence with Roche diagnostics deploying it widely in its chemiluminescence immunoassays (Paczkowska*et al.,* 2020; Favresse*et al*., 2018). The amount of light emitted during electrochemiluminescence has an inverse relationship to the FT4 or FT3 concentration in the sample in a competitive assay and a direct relationship to TSH level in a one-step sandwich assay based on manufacturer' s description. The chemiluminescence reaction on execution are well described (Favresse*et al.,* 2018). It is rare to find anti-Ruthenium antibodies affecting laboratory test. The antibodies majorly have effect on FT3 and FT4 estimations as described by various authors with varied results but case reports abound which have identified false results of TSH levels (Ando *et al*., 2007). As observed by Ando *et al*. (2007), elevated free T3 values were as a result of non-specific activity against the Rucrosslinkercomplex $[tri(bipyridyl)_3^{2+,l}$·Ruthenylated anti-T3 antibody and the Ru cross linker complex is suggestive to be targets of anti-Ru antibodies. PEG precipiatation was found satisfactory in reducing (or normalizing the signal in three euthyroid patients that were investigated which therefore points to the interfering agents be formed of immunoglobulins.

Alternative non-Ru method utilization produced lower FT3 results (Favresse*et al.,* 2018; Ando *et al.*, 2007). The advent of anti-Ru interference described in 2007 was followed by a next generation diagnostic procedure in 2008 which reduced the frequency of interference but not eliminated completely. Varied methods have been identified to be beneficial in detecting anti-Ru antibodies. Blood sample should be investigated with an immunoassay that does not employ ruthenium as a label. In various cases studied, the search for confirmation of anti-Ruinterference was executed by sending a fraction of sample to the company that utilize this method in its assay which is Roche diagnostics for a proper characterization of these interferences (Paczkowska*et al.,* 2020; Favresse*et al.,* 2018). The influence of anti-Ruantibodies on other analytes have been studied and their impact revealed in α-fetoprotein, troponin, progesterone assays and cholesterol (Favresse*etal.*, 2018; Ando *et al*., 2007). The investigation of anti-Ru interference is pivotal to curb unneeded additional tests, referral to endocrinology clinics and the prescription of unsatisfactory drugs and treatment (Faverssee*t al.,* 2018).

Thyroid Hormone Autoantibodies (THAAbs): Thyroid hormone auto antibodies have been implicated in interference in immunoassays majorly in antagonism to T4 and T3. It wasrecognized in the 20th century. Anti T4 and anti T3 THAAbs are exclusively and uniquely responsible for interference in thyroid function tests. The anti-TSHR, anti –TPO and anti Tgdo not (Paczkowska*et al.,* 2020; Faveresse*et al*., 2018). In the general population, 1.8% of THAAbs have been determined but not greater than 40% in autoimmune thyroid disease (Paczkowska*et al.,* 2020; Favresse*et al*., 2018; Sakata *et al.,* 1994). Fine needle aspiration biopsy has been found to elicit increases in THAAbs inclusive of other diseases that results from an anomalous response of the adaptive immune system such as diabetes mellitus (Type 1), vitiligo (Paczkowska*et al.,* 2020; Benvenga*et al*., 2015; Kemp, 2014). Investigating for THAAbs becomes necessary in those with an anomalous response of the adaptive immune system should there be a suspicion of interference. However, the presence of THAABs in serum can be transient even as its prevalence is underrated given that it is not investigated for routinely (Benvenga and Trimarchi, 2004; Favresse*et al*., 2018).

One step immunoassays(OSIs) have been identified to be affected by interference with anti T4 and anti T3 antibodies but the rate differs between other types of immunoassays with OSIs being affected much more (Paczkowska*et al*., 2020; Zouwail*et al*., 2008). Some of the automated immunoassay systems identified I THAAbs interference in OSIs protocol are immulite2000 and 2500 (Siemens Healthcare), Advia Centaur (Siemens Healthcare), Tosoh AIA 1800 (Tosoh, Tokyo, Japan). Comparing results from the OSIs machines against a two-step immunoassay machines such as Abbot AxSYM or Architect, Beckman DXI 800 or Access (Beckman Coulter), Immunotech radio-immunoassay, RIA-gnost (Cis Bio Bioassays, Codolet, France) may be a valuable proposition to identify interference. In any case, practically speaking, numerous OSIs are insensitive towards THAAbs even though two step immunoassays may be affected by their availability (Favresse*et al*., 2018).THAAbs gets bound to an analyte or a labeled tracer producing untrue results. The proportional relations are of inverse relationship between hormone levels measured and signal in the OSIs. In this regard, THAAbs brings about a signal reduction by getting bound to a labeled hormone analogue which give rise to total and free T4 as well as T3 levels being falsely increased. It should be appreciated and considered that TSH measurements do not get interfered by THAAbs. With this in mind, assessing thyroid function in patients thyroid hormone auto antibodies availability in their blood, TSH

becomes authentic and appropriate (Paczkowska*et al.*, 2020; Zouwail, 2008).

With variable results arising from confirmation of discrepancies by different authors, there is insufficiency in testing samples with adifferent assay and serial dilution even though these methods may be useful (Paczkowska*et al.*, 2020). However, radioimmuno-precipitation has been identified as a more specific method of identifying interference even though it is complicated. The procedural steps are that serum samples are incubated with a radiolabelled hormone or its analogue, then it is followed with a precipitation step with PEG. The precipitate (mostly immune complexes) radioactivity is estimated and matched against the the total number of radioactive labels added. The quotient of the tracer quantity bound to total tracer number and this is reported as a percentage value. The PEG precipitation without the utilization of radioactive substance could be deployed which is easier and accessible given the difficulty in performing the PEG precipitation procedure. Generally, a 5% radioactivity is established in human sera samples (Paczkowska*et al.*, 2020; Faverese*et al.*, 2018; Srichomkwun*et al*., 2017; Massart*et al*., 2009).

The Hook Effect (High Dose Hook Effect): The hook effect also known as antibody excess (Prozone phenomenon) or antigen excess also known as (Postzone phenomenon) interferes with thyroid immunoassays. Hooke effect is an immunologic occurrence which alters the efficiency of Ab to form immune complexes when levels of antibody or antigens are elevated. The formation of immune complexes are retarded with increasing levels and then decreases with extreme concentration which produces a hook like shape on the measurement graph. It produces a false negative result or inappropriately low results while actual analyte concentration is significantly higher (Jacobs *et al.,* 2015). The hook effect has been seen to occur in sandwich immunoassays at high levels of analyte for which assay signal is saturated and levels off (Vashist and Luong, 2018). For sandwich immunoassays, given the observation of hook effect, the allowable maximum concentration is the one that correlates to the commencement of the saturated signal (Vashistand Luong, 2018).

Hook effect occurrence is typical of progressive tumor pathologies in which the extent of variations between the pathological and physiological concentrations can be at the maximum. This can be seen in cases such as prolactinaemia, thyroid carcinoma and hydatiform moles. Delayed diagnosis and therapy for severe cases or misdiagnosis with grave consequences can be an outcome. Given that hook effect impacts only sandwich immunoassays, sample dilution will disclose the availability of excess analyte. Following serial dilutions, the level of the analyte will be elevated until paired reasonable sequential dilutions are secured (Ghazal *et al.,* 2022). However, manufacturers deploy the utilization of excess Abs or decrease the necessitated and requisite sample quantity to reduce Ab saturation and rate of occurrence of the hook effect (Ghazal *et al.,* 2022).

*Other Forms of Interference:*Other forms of interference have been identified and these includeTH transport variants, human serum albumin (HAS)(Minoto*et al.,* 2018), thyroid binding globulin (TBG), Transthyretin (TTR) mutation, drugs which affect the equilibrium between T4 and T3 and the binding proteins which alters the free TH levels, These agents which have the capacity to displace are acetylsalicylic acid (Aspirin), carbamazepine, Phenobarbital, non-steroidal anti-inflammatory drugs (NSAID), phenylbutazone, phenytoin and unfractionated and fractionated heparin (Favresse*et al*., 2018). Accessing medication history will prove valuable in matters of thyroid hormone interference. The other identified forms of interferences are TSH variants, gammopathies (Paraproteins) which affect antibody binding. These interferences have been properly expatiated and extensively elucidated (Favresse*et al.*, 2018; Ghazal *et al.,* 2022).

Persons with threat possibilities for the presence of interference include;

i). Those found to have had inconsistences in their laboratory report.

ii). Those who have been identified to have had interference in their laboratory investigations.

iii). Those who had been administered therapy with anti-animal antibodies previously.

iv). Thoseadministered with biotin supplement of high dosage.

v). Those persons with rheumatoid arthritis.

vi). Those with elevated concentrations of rheumatoid factor in their blood(Paczkowska*et al.,* 2020).

*Quality Control and Laboratory Safety:*Quality control and laboratory safety are integral part of the laboratory when tests are being carried out and are vital when running thyroid function tests. Good laboratory practice and the quality assurance system as adapted globally including the ISO 15189, ISO 9001 and ISO 17025 have many rules that are properly established for the installation and maintenance of quality systems in the laboratory (Premnath and Zubair, 2024). Reference materials,internal reference samples and participation in external quality assurance programmes make analytical accuracy assessable.

Appropriate standards have to be deployed while carrying out thyroid function test as provided by the manufacturer. Technical manuals should be assessed to note the various risks, safe handling instructions, properties of reagents, contact information of manufacturers as well as date the technical manual was prepared as to the occupational safety and health administration guidelines. Green horn and new health care professionals in the employ of the laboratory should be given satisfactory time to get acquainted to procedures and utility concerns. All blood samples under investigation should be handled with utmost care to avoid any form of contamination as it were and laboratory staff should be held accountable for using aseptic techniques to derive clean samples. Samples and reagents used in carrying out thyroid function test should be properly handled, efficiently and effectively using appropriate procedures.

To ensure healthcare team deliveries, an efficient and effective interdisciplinary, multidisciplinary communication and appropriate training for team members have to be established. All phases (pre-analytical, analytical and post analytical) of laboratory studies have to be properly taken care of especially the pre-analytical phase where most errors of laboratory investigations begin. The clinical staff must work with the laboratory staff and vice versa to obtain the right and appropriate sample, handle sample properly and ensure specimen wholeness. Efficient communication between laboratory and clinical staff should be mutual and uncompromised and there should be a clear understanding of the importance of carrying out thyroid function tests and what the effects of the interference could produce. Experts with adequate knowledge of thyroid function test should always be consulted where and when applicable to evaluate results, confirm diagnosis and give advisories on future or immediate directions. These aspects of healthcare team deliveries if properly fused, healthcare experts will be optimistic of the relevance of thyroid function tests in providing patient centered responsibility, superior output, safe persons and facility (Premnath and Zubair, 2024).

*Conclusion:*Interference in thyroid function tests has complicated immunoassays for thyroid function. Access to adequate information could help in mitigating interference in these tests by utilizing available remedial actions. Effective and efficient strategies to eliminate interference could involve education and technical expertise, which play a vital role in intervention. Efforts should focus on collaboration between laboratory and clinical staff to accurately identify potential interferences and match symptoms of thyroid issues with laboratory reports. Additionally, current diagnostic machines are heavily automated and less susceptible to external factors. It is important to ensure proper observation of quality control, laboratory safety, and the delivery of healthcare team services.

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