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FTIR-ATR SPECTROSCOPY AS A DIAGNOSTIC TOOL IN HYPOTHYROIDISM INDUCED BY CARBIMAZOLE – A STUDY IN ANIMAL MODEL

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ABSTRACT
Hypothyroidism known as underactive thyroid, is a condition wherein the thyroid gland doesn't produce enough thyroid hormone. It is metabolically inter-related and affect/damage multiple organs leading to various complications and early screening is advised to check the disorder through proper medications. Carbimozole (20 mgs/Kg body weight) was given to induce hypothyroidism in male wistar rats for 21 days. The hypothyroid status has been assessed by measuring T3,T4 and TSH in serum by ELISA method as well as by FTIR-ATR spectral analysis. In addition to this other biochemical components were also analyzed to correlate the hypothyroid status. Current study focused on concept of economic and timely screening of the hypothyroid disease condition adopting reliable method. FTIR spectroscopy is used as a diagnostic tool in detecting disease condition by analyzing the functional groups of the biomarker(s) of concerned diseases. The spectral bands (4000 - 450 $^{cm\ -1}$) obtained from serum was characterized for different biomarkers to assess the diseases status which aid in complete screening to control and management the disease. The chemical bonding stretches reveals the nature of bio molecule levels in serum. The results obtained on control and experimental animals were showed statistically more significant. The spectral study gives an additional information about the molecular basis which help in the detection and severity of disease condition. The results obtained were also correlated with other standard methods for comparison.

KEYWORDS: Hypothyroidism, Biomarkers, FTIR-ATR Spectroscopy, male wistar rats.

INTRODUCTION
Thyroid diseases are common in general population nowadays and their prevalence increases with age and affect females more than males. Hypothyroidism defined as a deficiency of thyroid activity (Farwell et al., 1996) and is one of the most frequent thyroid disorders. Primary hypothyroidism is the most common thyroid disorder next to hyperthyroidism. Hypothyroidism caused by inflammation of the thyroid gland, results in thyroid damaged (or dead) and incapable of producing sufficient hormone. The most common cause of thyroid gland failure is called Hashimoto's thyroiditis (also called autoimmune thyroiditis), a form of thyroid inflammation caused by the patient's own immune system. Another cause is surgical removal of a portion or all of the thyroid gland where the patient will develop hypothyroidism. The rare causes of hypothyroidism, thyroid gland not making enough hormone due to problem in the pituitary gland, which do not produce sufficient TSH. Thyroid gland is specialized for production, storage and release of the thyroid hormones thyroxine (T4) and tri iodothyronine (T3). T3 and T4 are known as cell metabolism regulators (Silva JE, 1995) and they are required for normal cell growth and development (Cavalieri MM, 1997). Hypothyroidism results from reduced secretion of both T_4 and T_3. (Seely EW and Williams GH 2001). Biochemically decrease in T_4 and T_3 concentrations lead to hyper secretion of pituitary thyroid stimulating hormone (TSH) and an amplified increase in serum TSH levels. This is a key laboratory finding, particularly in the early detection of thyroid failure.

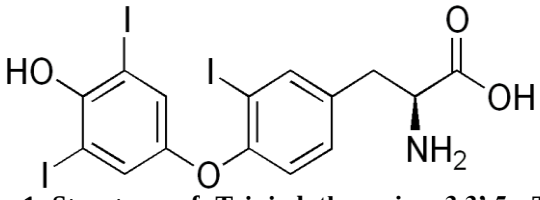

Fig. 1 Structure of Tri iodothyronine 3,3',5- Tri-iodothyronine (T3)

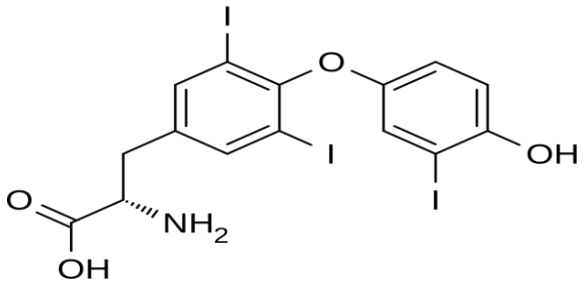

Fig. 2 Tetra iodothyronine Thyroxine (T4)

Experimental studies associated with thyroid dysfunctions and changes in the metabolism and body development are based on the suppression of hormone production (McCardle et al., 1998; Ferreira et al., 2003, Khotimche YS, Serguschchenko IS 2003 and Silva CM et al., 2004). Hypercholesterolemia is favored due to the hormone deficit and to the decreased activity of the lipoprotein lipase (Galesanu C etal., 2004). Development of atherosclerosis in cholesterol fed animals is enhanced by the presence of hypothyroidism and reduced when thyroid hormone is administered **(**Vela BS 2001**)**. Thyroid dysfunction can have an important effect on lipid profile (Liberopoulos EN and Elisaf MS, 2002). Biochemical screening for thyroid dysfunction is critical in all dyslipidemic patients, as well as in all patients with unexpected improvement or worsening of their lipid profile. Thyroid function regulates a wide array of metabolic parameters. Thyroid function significantly affects lipoprotein metabolism as well as some cardiovascular disease (CVD) risk factors, thus influencing overall CVD risk (Duntas LH, 2002 and Canaris GJ et al., 2000). Indeed, even within the normal range of thyroid-stimulating hormone (TSH) values, a linear increase in total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TGs) and a linear decrease in high-density lipoprotein cholesterol (HDL-C) levels has been observed with increasing TSH (Asvold BO et al., 2007). Apart from general metabolic disturbance, impairment of thyroid hormone production causes serious intellectual and behavioral abnormalities that may affect patient's daily functioning and result in additional stress and depression. Therefore, the research on the thyroid disorders has not only significant medical but also social implication.

Based on literature (Hardisty JF and Boorman GA 1999) carbimazole induction of hypothyroidism in male wistar rat is chosen as a model to elucidate mechanisms underlying hypothyroidism and to contribute to resolve accompanied problems. In the present study we artificially induced hypothyroidism in otherwise healthy and metabolically stable male wistar rats in order to elucidate the existing controversies related to effects of experimentally induced hypothyroidism in male wistar rats, As well as an attempt to give acceptable explanation for discrepancies which exist in that regard between human and animal models of hypothyroidism. Hypothyroidism whereas provoked by potent anti thyroid drug carbimazole that is frequently and preferentially used in the treatment of human hyperthyroidism (Zaidi TM et al., 2004).

Diagnostic tests are an important part of medical care and tests performed on samples taken on and from the body and used in a broad range of applications. Test results can be used to aid the patient, physician, and caregiver in reaching decisions. FTIR-ATR spectroscopic instruments is low cost with easy handling without skilled persons and reagents and results obtained could extend that the level of different bond stretching in bio molecules. FTIR-ATR spectroscopic imaging has significant advantages composed to many other imaging methods for the characterization of bio molecule molecules because it relies on the characteristic absorbance of corresponding molecular vibration in the sample functional group of chemical compounds such as carbohydrates, cholesterol, Triglycerides, albumin, proteins, as well as inter atom chemical bonds. The concept of using FTIR-ATR in the analysis of biological samples like Different organs in addition to blood serum dates back over half a centaury (Blout ER et al., 1949, Kraft C and Sergo V, 2006). Nowadays this technique has become an independent modality, especially in its imaging system, for searching spectral biomarker of a disease, Since infra red spectrum provides information about biochemical components in a sample such as proteins, lipids, carbohydrates and nucleic Acid. Further, current study involves in FTIR-ATR spectroscopic method where the spectrum obtained due different bond stretching and functional groups exist in serum sample might be the additional information to the earlier methods.

MATERIALS AND METHODS

Induction of Hypothyroidism

Male wistar rats were housed in the animal house of Research and Development, Saveetha Medical College and Hospital, Thandalam Chennai, India. All experiments were carried out according to the guidelines for care and use of experimental animals and are approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Study proposal was approved by the Institutional Animal Ethical Committee. Three-Four male wistar rats per cage were housed in polypropylene cages (32.5 ×21×14) cm lined with raw husk which was renewed every 48 hours. The animal house was maintained at an average temperature (24.0^0C± 2^0C) and 30-70% RH, with 12 hr. light-dark cycle (lights on from 8.00 a.m. to 8.00 p.m.). Animals received human care and were fed with commercial pellet diet and the animals were acclimatized for one week before the start of the experiment.

The male wistar rats were divided into two groups: animals from the first group were made hypothyroid by drinking 0.02% Carbimazole (Sigma St. Louis, USA) solution in drinking water for three weeks to develop

hypothyroid condition following standard procedure (Zaidi TM et al., 2004) and animals in the other group were untreated control. On day 22nd of the experiment animals were weighed and sacrificed by decapitation.

Preparations for Biochemical studies

At the end of experiment (21 days) the male wistar rats were fasted overnight. Blood samples of the male wistar rats were withdrawn from the heart. under mild anaesthesia before scarifying and collected in plain and EDTA tubes for further analysis. Plasma and serum were separated by centrifugation at 3000 rpm for 15 minutes. The blood serum properly stored for estimation of biochemical parameters including Albumin, Glucose, LDL concentration by CLIA and Total serum T_4,T_3 and TSH concentration were determined by ELISA (detection kits provided by Transasia, Zemun, SCG) in a reputed clinical laboratory in Chennai. FTIR-ATR Spectral Analysis the serum samples were properly preserved in ice bags and immediately transported to the wet lab for spectral studies.

FTIR-ATR spectral Measurements

The FTIR-ATR spectroscopy is based on the phenomenon known as Total Internal Reflection (TIR) (Katon JE and Micron 1996 and Baulsir CF and Simler RJ, 1996). This radiation strikes the interface between the IRE and the serum sample composed of a lower refractive index. This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal into the serum sample held in contact with the crystal. It can be easier to think of this evanescent wave as a bubble of infrared that sits on the surface of the crystal. This evanescent wave protrudes only a few microns (0.5µ-5µ) beyond the crystal surface and into the sample. The depth of penetration of infrared radiation from denser IRE into the test material depends on refractive indices of the materials to be investigated and the wave number of the infrared radiation. As the sample absorbs IR radiation at certain frequencies, the resultant totally reflected radiation (or) evanescent wave will be attenuated (altered) in regions of the infrared spectrum where the sample absorbs energy Katon JE and Micron 1996, Baulsir CF and Simler RJ 1996). This attenuated IR radiation of evanescent wave is passed back to the IR beam, which then exits the opposite end of the crystal and it is detected by the detector in IR spectrometer. The system generates an infrared spectrum.

FTIR-ATR spectral measurements of serum samples of male wistar rat induced hypothyroidism were carried out at Sophisticated Analytical Instrumentation facility (SAIF-SPU), St. Peter's University, Avadi and Chennai-600 054, using PerkinElmer Spectrum-Two FTIR Spectrophotometer with attenuated Total Reflectance accessory having highly reliable and single bounce diamond as its Internal Reflectance Element (IRE). Experimental serum samples were analyzed immediately for spectral recordings in the Mid IR region of 4000-450cm^{-1}. As water is a good absorbent of infrared radiation, it affects the actual spectral response of the test material and dominated in the FTIR spectrum of serum sample and therefore it was placed on the IRE crystal and water content on the serum sample is removed by air drier. FTIR spectral measurements were carried at room temperature and each measurement was repeated to ensure the reproducibility of the spectra. These spectra were subtracted against the background of air spectrum. After every scan, the crystal is cleaned with isopropyl alcohol or methanol soaked tissue and a background of new reference air was taken to ensure the crystal cleanliness.

Statistical Analysis

All statistical analysis were performed using Statistical Package for Social Science (SPSS, version 17) for Microsoft windows. The data were not normally distributed. And therefore Non - parametric tests were performed. Descriptive statistics were presented as numbers and percentages. The data were expressed as Mean and SD.

A one way analysis of variance (ANOVA) /Kruskal-Wallis test with a post hoc Tukey HSD was used. Independent sample student t test / Mann-Whitney test were used to compare continuous variables between two groups. A two sided p value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

A. Quantification Biochemical Composition by CLIA

Oral dosage of carbimazole in drinking water is a conventionally used method for establishing hypothyroidism in experimental animals (Chehade J et al., 1999, Cooper DS et al., 1984 and Petrovi N et al., 2001). Carbimazole acts as false substrate for thyroid peroxidase, this blocking the iodination of thyroid residues within thyroglobulin and the coupling of iodothyrosines into iodothyronines (Farwell AP et al., 1996 and Cooper DS et al., 1999). Since the release of hormone not affected, the onset of effects is somewhat delayed and slowed, until the depletion of thyroid gland hormone stores. In our study the significant decrease in the total T4 and T3 serum levels and increased TSH levels pointed out that administered dose and duration of the treatment were sufficient to induce hypothyroidism in the experimental group of wistar male rat (Table 1) which support the study of Maja Aki Milo et al., 2004. Although at the beginning of the experiment the biochemical composition of the both control and experimental group of animal was analytically equal, but at end of induction the male wistar rat from experimental group were significantly different. Wiseman SA et al., 1993 and Mya MM and Arnow WS,2002 were also found that developing atherosclerosis with increased level of total cholesterol, Low LDL cholesterol and Apolipoprotein B (ApoB) which also Support this study. Additionally similar observations

were also recorded in patient with hypothyroidism have an increased risk of developing atherosclerosis and the sub clinical stage is also considered a risk factor for this disease (Donnimi D etal., 2003). Further, LDL cholesterol receptor gene contains a thyroid hormone responsive element (TRE) that could allow T3 hormones to modulate gene expression of LDL cholesterol receptor resulting in LDL cholesterol receptor synthesis. (Adriana S 2012, Taddei S et al., Lee WY et al., 2004 and Iqbal A, 2006). The results obtained between TSH and total cholesterol and LDL and HDL fraction as well as T3 and T4 hormones showing positive correlation with Triglycerides are similar to earlier literature but few studies not showed significant difference (Geul KW and Al-Tonsi AA. 1993**.**

This study also suggest association between TSH levels and lipid levels in addition to protein and carbohydrates. It is also evident that insulin resistance bears an indispensable role in Type 2 Diabetes Mellitus and hypothyroidism. Thyroid hormone directly control insulin secretion. In hyper thyroidism, there is reduction in glucose induced insulin secretion by bête cells and the response of beta cells to glucose or catecholamine in increased hyper thyroidism due to increased beta cell mass. (Donkier JE, 2003 and Stanicka S et al., 2005) which support the present study. Increased lipolysis is observed in thyroidism resulting increased free fatty acids that stimulate hepatic gluconeogenesis and similar findings were observed with increased Free Fatty acids (Vaughan M, 1967).

Table 1. Changes in biochemical composition levels in Blood serum before and after Carbimazole treatment in male wistar rat

Biochemical Composition	Control	Carbimazole treated	Statistics
T3(ng/dl)	161±5.87	212+8.19**	P<0.01
T4(µ/dl)	5.9±3.20	15.9+3.99***	P<0.001
TSH (mIU/dl)	4.8±2.33	12.8±3.11**	P<0.01
Plasma Glucose (mgs/dl)	112±5.42	149±4.48*	P<0.05
Total Cholesterol (mgs/dl)	167±3.10	219±3.82**	P<0.01
Triglyceride(mgs/dl)	120±3.89	135±4.07*	P<0.05
HDL Cholesterol (mgs/dl)	47±3.87	48±3.90	NS
Urea (mgs/dl)	30±7.10	43±7.20*	P<0.05
Creatinine (mgs/dl)	0.7±0.4	0.76±0.5	NS
Uric acid (mgs/dl)	4.8 ±1.1	4.9±1.3	NS
Calcium (mgs/dl)	7.9±1.4	8.0±1.7	NS
Total Protein (gms/dl)	7.1±2.89	7.6±2.96*	P<0.05
Albumin (gms/dl)	5.1±2.11	5.3±2.02	NS
Globulin (gms/dl)	2.0±1.0	2.3±1.09	NS
SGOT (IU/l)	55±5.1	58±4.8	NS
SGPT(IU/l)	61±7.5	60+11.4	NS
SAP(IU/l)	121±12	133±15.1	NS

B. FTIR-ATR Spectral Analysis

The FTIR-ATR spectrum obtained reveals the nature of the bio molecules based on its structural and functional group configurations. The spectrum obtained on spectroscopic studies is an reflection of infra red colour pattern characteristic of the sample. The current study focus on qualitative and quantitative changes in biochemical composition in control and carbimazole induced Hypothyroid male wistar rat. The spectral pattern for control male wistar rat and experimental carbimazole induced hypothyroid male wistar rat are given in Fig.3.

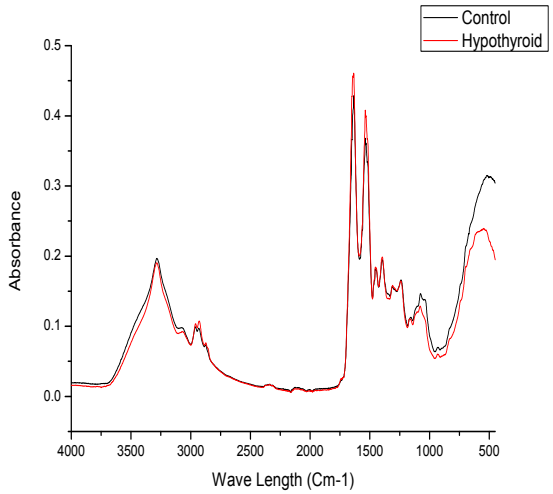

Fig3. FTIR-ATR spectral pattern of control and experimental carbimazole induced hypothyroid in Wistar rat

Quantification of bio molecule composition each constituents is studied with unique absorption pattern to over all spectrum governed. Spectra obtained were overlaid in order to distinguish the spectral signatures of control and hypothyroid induced male wistar rat serum.

C.FTIR Band Assignment of control and Carbimazole induced hypothyroid male wistar rat

The spectral bands of proteins, lipids, glycogen bands due to IR absorption of hypothyroid serum indicates that these are the key markers in the investigation of hypothyroid in male wistar rat. FTIR vibration band assignment of blood serum of control and Hypothyroid induced male wistar rat were shown in Table 2. The FTIR-ATR spectroscopic technique adopted for certain bio molecules such as proteins, lipids and glycogen to represent the hypothyroidism in male wistar rat. Generally the band assignment done with the idea of the group frequencies of the various components in the sample. The prominent absorption peak 3283 cm^{-1} is due to the N-H stretching of mode (Amide) of protein in the present study is similar to that of recent studies on serum immunoglobulin (Sankari et al., 2010 and Kamatchi S et al., 2016). The observed absorptions are the aliphatic C-H stretch of both saturated and unsaturated long chain fatty acids, alcohols and esters. In the present study the absorption band at 2931 cm^{-1} and 2961 cm^{-1} are due to asymmetric /symmetric stretch vibrations of methyl and methylene C-H lipids and protein molecules which support the study of Akthar et al., 1997. Further Chen Y J et al., 2006 reported that absorption at 1540 cm^{-1} is similar to that of present study in which Aryl substituted C=C Amide I and Amide II of band mainly due to C=O, C=N and N-H stretching peaks observed at 1634 cm^{-1} and 1538 cm^{-1}. The absorption peak at 1453 $cm^{-1}$and 1395cm^{-1} is due to vibrations of proximate compositions like protein, lipid, carbohydrate and nucleic acid and similar was reported by Bantignies L et al., 1998. Moreover stretching of glucose are observed at 1076 cm^{-1} and polysulfide S-S stretch in cystic acid at 532 cm^{-1} in this study is slightly different to that of earlier work where stretching of glycogen was observed at 1118 cm^{-1} and Polysulfidic S-S stretch in cystic acid at 516 cm^{-1} recently in hair samples of hypothyroid patients (Kamatchi S et al., 2016).

Table 2. FTIR Vibration Band assignment of blood serum of control and Hypothyroid induced male wistar rat

S.No	Wave Number (cm^{-1})	Vibration Band assignment
1	3283	N-H stretch due to protein and Urea
2	3071	Amide B band due to overtone of Amide I band and olefinic group C-H stretch Lipids of Unsaturated fatty acid
3	2961	C-O-C Asymmetric / Symmetric stretch vibrations of Methyl group of Protein and C-H Lipids (Fatty acids and TGL)
4	2931	Asymmetric stretching vibrations of Methylene group of protein and lipids
5	2879	Symmetric stretching vibrations of Methylene group of protein and lipids
6	1742	C=O group of cholesterol ester (HDL)
7	1634	Aryl substituted C=C Amide I band mainly due to C=O ,C=N and N-H stretching
8	1538	Amide II band due to NH vibrations stretching coupled with C-N stretching vibrations in protein.
9	1453	Asymmetric bending vibrations of lipids, proteins of CH3 groups.
10	1395	Free Amino Acid and Fatty Acids
11	1313	Amide III erythrocyte
12	1240	Amide III and Asymmetric PO2 stretching vibration mode of Nucleic acid
13	1165	Ring vibrational mode of C-O-H and C-O-C bonds (CO-O-C) asymmetric Cholesterol ester, Phosphoric acid
14	1115	Stretching vibration of glycogen
15	1076	C-O chacterization stretching of glucose
16	1040	Primary alcohol C-O stretch glucose-Muco Poly saccharide
17	934	Ribose , Phospholipids
18	532	Polysulfidic S-S stretch in cystic acid

Internal ratio parameter is calculation

These spectra were used in Internal ratio Parameter calculation and analysis requires spectra with change in sensitive peaks and no change in sensitive peaks for control and experimental. Internal ratio parameter is calculated to fortify the results obtained from the FTIR intensity of absorptions. Internal ratio Parameter ignores the difference in the amount of sample analyzed, it nullifies the contradiction in the quantity of the sample and gives measured out exact deviations in the male wistar ratio ($I_{2961}/_{1240}$, $I_{2879}/_{1453}$, $I_{2931}/_{1395}$ and $I_{1538}/_{1313}$). The internal ratio parameter of protein, lipid and glycogen of control and hypothyroid experimental animals given in the Table 3. The results shows that peak ratio for lipids and protein (Lipoprotein) to Amide III and PO2 of nucleic acid (Nucleoprotein) is increased in male wistar rat induced with hypothyroid than control. The small changes in the absorption is also appreciable in the FTIR-ATR spectra as it depends on the short existing, effective evanescent wave with 0.5 µ-5 µ depth

of penetrations. The absorption ratio of symmetric Nucleoprotein (Symmetric Methylene group of protein and lipids) to asymmetric nucleoprotein (Asymmetric Methylene group of protein and lipids) among control and male wistar rat induced thyroid found to be slightly high. Further the internal peak absorption ratio of asymmetric lipoprotein (Asymmetric methylene group of protein and lipids) to amino acid and Fatty acid (Free amino and fatty acid) and Amide II (NH vibrational stretch coupled with C-N stretch vibrations in protein) to Amide III were significantly elevated in hypothyroid male wistar rat than in control. The trends observed on absorptions of internal peak male wistar ratio of experimental animal was more than control which support earlier studies on different diseases like thyroid, Renal, atherosclerosis, cancer, Hepatitis (Kamatchi S et al., 2016, Renugadevi TSR et al., 2009, Haas SL et al., 2010, Dimitrova N 2009, Mackanos, MA et al., 2009 Gunasekaran, S. et al 2008, 2010, Sankari G etal., 2010).

Table 3 Internal Standard ratio Parameters calculation of Lipids, Proteins and Glucose between control and Hypothyroid blood serum of male wistar rat

Peak ratio	Wave Number (cm^{-1})	Absorbance	
		Control	Hypothyroid
$(Lipoprotein)_{asym}$ /Nucleoprotein	$I_{2961/1240}$	0.5975	0.6257
$(Lipoprotein)_{sym}$ /$(Lipoprotein)_{asym\ bending}$ -LDL	$I_{2879/1453}$	0.3995	0.4050
$(Lipoprotein)_{asym}$ /AA and FA	$I_{2931/1395}$	0.4982	0.5402
Amide II/ Amide III	$I_{1538/1313}$	2.3497	2.6200

CONCLUSION

This study allowed elaborating an experimental protocol of thyroid dysfunctions in adult male wistar rats and study the biochemical variations FTIR –ATR spectroscopic technique in addition to ELISA and other existing methods, which has not been previously described in the literature. Biochemical and spectral analysis confirmed carbimazole action in the induction of hypothyroidism in male wistar rats. In addition, the administration of drugs in the drinking water was an effective and practical procedure and with little animal handling, important factors when using animals as experimental models. There have been studies suggesting that screening for thyroid dysfunction is cost effective. This study is the economic, less time consuming and reliable technique to study the biochemical variations qualitatively and quantitatively in the medical and its allied field of research.

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