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Frequency of Abnormal Thyroid Function Tests in Type 1 and Type 2 Diabetic Patients

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Abstract:

Background: *It is known that thyroid hormones are insulin antagonists, both insulin and thyroid hormones are involved in cellular metabolism. Excess and deficiency of anyone can result in functional derangement of the other.*

Aim: *To check the frequency of abnormal thyroid function tests in diabetic patients.*

Method: *Cross-sectional study of thyroid hormones alterations in which data from the Diabetic Clinic of Jinnah Hospital LHR, from 1 August 2014 to 31 January 2015 were reviewed. Patients and their thyroid profile results are present in simple average and percentages.*

Results: *One hundred and five patients were selected from Diabetic clinic of Jinnah hospital Lahore. Among them 46 were with type 1(19 males,27 females). 54.5% having normal level, 41.3% having sub-clinical hypothyroid, 2.1% having sub-clinical hyper thyroid and 2.1% having hypothyroid functioning and 59 were with type 2 (28 males, 31 females) 66% having normal level, 20% having sub clinical hypo thyroid,10% having sub clinical hyperthyroid and 4% primary hypo thyroid function.*

Conclusion*: We conclude that screening for thyroid disease among patients with diabetes mellitus should be routinely performed considering the prevalence of new cases diagnosed. Failure to recognize the presence of abnormal thyroid hormone level may be one of the reasons of poor outcome of diabetes management.*

Keywords: abnormal thyroid function tests, Type 1 and Type 2 diabetic patients

INTRODUCTION and LITERATURE REVIEW

Diabetes mellitus is a growing problem in our country. It has been long recognized that thyroid hormones have marked effects on glucose homeostasis. Diabetes mellitus and thyroid dysfunction are the two most common endocrine disorders in clinical practice. The unrecognized thyroid dysfunction may adversely affect the metabolic control and add more risk to already predisposing scenario for cardiovascular diseases. Glucose intolerance is associated with hyperthyroidism and most recently it was shown that hypothyroidism is characterized by insulin resistance. Since type 1 diabetes also has autoimmunity it is not unsual to find patients with concomitant diabetes and thyroid dysfunction. Some genetic factors might contribute to the co-occurrence of autoimmune thyroid disease and type 1diabetes (Pearce SH and Merriman TR, 2009). The 2 main thyroid hormones are t3,(triidothyronine) and t4 (thyroxine). T3 and t4 regulate body's temperature metabolism and heart rate. The amount of thyroid hormones secreted is controlled by another hormone, called thyroid stimulating hormone (TSH) which is released from the pituitary gland in our brain.

Hyperthyroidism is a condition in which thyroid gland produces too much of the hormone thyroxine. Following are the symptoms of Hyperthyroidism: Anxiety, Irritability or moodiness, Nervousness, hyperactivity, Sweating or sensitivity to higher temperatures, Hand trembling, Hair loss, Missed or less menstrual periods.

Following are the symptoms of **Hypothyroidism:** Trouble sleeping**,** Tiredness and fatigue**,**Difficulty in concentrating**,** Dry skin and hair**,** Depression**,** Sensitivity to cold temperature.

Diabetes mellitus has become one of the greatest threats for 21st centuary. (Soumya D and Srilatha B. 2011). The global rise in diabetes has led to increase in health care expenditure significantly.

In developing countries the prevalence of diabetes is rising rapidly,and it is estimated that among adults aged ≥ 20 years , the global number of diabetes is to reach 366 millions in 2030 (Li M.-Z,

Su LY, et al. 2013). Both type 1 and type 2 diabetes are one of the powerful and independent risk factors for coronary artery diseases (CAD), stroke , and peripheral arterial diseases (Aronson D. 2008)

The pathological features of T2DM include increased intestinal glucose absorption, reduced insulin secretion, and change in the β-cell mass. (*Clark A et al. 2001.)* Further, symptoms also include increased insulin degradation, increased glucagon secretion, increased hepatic glucose production, enhanced catecholamines, and insulin resistance. These factors have been investigated to be an integral part of hyperthyroidism as well. Hence, an intersection of pathological basis occurs which gives us cue to an array of physiological aberrations which are common in hyperthyroidism and T2DM. Among the above-mentioned symptomatology, insulin resistance has been the most important facet connecting thyroid dysfunction and T2DM. Insulin resistance is a condition which occurs in both hypothyroidism and hyperthyroidism. (*Brenta G et al. 2009).*Insulin resistance in the muscles and liver is a characteristic feature of T2DM. An undisturbed glucose homeostasis and intact insulin secretary response and unperturbed sensitivity of the tissues to insulin are essential to maintain normal blood glucose levels.

Glucose disposal is mediated by the conjoint effect of insulin and hyperglycemia to modulate three basic phenomenon. Firstly, diminution of endogeneous (hepatic) glucose production. Secondarily, enhanced uptake of glucose (hepatic and splanchnic). Thirdly, upregulation of glucose by peripheral tissues (skeletal muscles). Glucose uptake into muscles is modulated by glycolysis and glycogen synthesis. Hepatic insulin resistance is characterized by glucose overproduction inspite of fasting hyperinsulinemia, and enhanced rate of hepatic glucose output was the pivotal modulator of increased fasting plasma glucose (FPG) concentration inT2DM subjects. (*Med Clin North Am. 2004).* In insulin resistance in the postabsorptive state, muscle glucose is upregulated but the efficiency of uptake is reduced. In the wake of such conditions, reduced glucose uptake into the muscles and enhanced hepatic glucose output lead to worsening of glucose metabolism.

Thyroid hormones directly influence insulin secretion. There is a reduction in glucose-induced insulin secretion by beta cells, In hypothyroidism, and the response of beta cells to glucose or catecholamine is increased in hyperthyroidism due to increased in

beta cell mass. Moreover, in thyrotoxicosis, insulin clearance is increased.(Stanická S *et al.* 2005) (. Mitrou P et al. 2010)

There is a deep underlying relation between thyroid dysfunction and diabetes mellitus. (Wang C. 2013). Studies have found that thyroid dysfunction is much common in diabetic population compared to nondiabetic population, both diabetes and thyroid disorders have been shown to mutually influence each other. (Al-Geffari et al., 2013) **(**Hage et al., 2011)

Most often type 2 diabetes is mainly due to insulin resistance,whereas type 1 diabetes is due to an autoimmune condition (Helfand M and Crapo L. M. 1990).

Hence it seems that diabetes particularly type 1, has potential link with thyroid dysfunction and vice versa. In subclinical or overt hypothyroidism, insulin resistance leads to glucose-stimulated insulin secretion. Low thyroid hormones level, besides the effects due to high blood glucose in diabetics, independently increase the risk of cardiovascular diasease in both diabetic and non-diabetic patients. **(** Van Tienhoven-Wind L. J. N and Dullaart R. P. F.. 2015)

A plethora of studies have evidenced an arrangement of complex biochemical, genetic and hormonal malfunctions showing this pathophysiological association. It has been shown that 5′ adenosine monophosphate–activated protein kinasa (AMPK) is a central target for modulation of insulin sensitivity and feedback of thyroid hormones associated with appetite and energy expenditure. (Brenta G et al. 2007) **(** Goglia F et al . 1999)

Reduced glucose absorption from gastrointestinal tract accompanied by prolonged peripheral glucose accumulation, gluconeogenesis, low hepatic glucose output and reducuction in the disposal of glucose are hallmarks of hypothyroidism .In subclinical hypothyroidism, reduce rate of insulin stimulated glucose transport rate caused by perturbed expression of glucose transporter type 2 gene (GLUT 2) translocation may contribute to insulin resistance. Moreover, due to reduced renal clearance of insulin in hypothyroid conditions, physiological requirements of insulin were reduced. Anorectic conditions in hypothyroidism may also contribute to reduced insulin in this condition.An increased dose of insulin is required to ameliorate hypothyroidism, (Duntas LH et al. 2011).

Moreover the association between type 1 diabetes and AITD is considered one of the variants of the autoimmune polyglandular

syndrome. The MHC locus on chromosome 6p21 is one of the susceptibility loci for both of diseases. Although autoimmune thyroid disease is more prevalent in type 1 diabetes as a result of their common origin, in patients with type 2 diabetes, the prevalence of hypothyroidism and hyperthyroidism is similar to that of general population autoimmunity has been implicated to be the major cause of abnormal thyroid functions associated with diabetes mellitus. However, in type 2 diabetic patients, the presence of the highly frequent sub-clinical forms of hyperthyroidism and hypothyroidism should be ruled out since they may be associated with higher cardiovascular risk. One study done in Spain by (Díe*z JJ et al. 2011).* who found an overall prevalence of thyroid dysfunction in 32.4% in type 2 diabetics patients. A study by (*Perros P et al. 1995).* showed a prevalence of thyroid dysfunction 13.4% in diabetics, though the prevalence was more in female type 1 diabetics. A study in Jordan by (*Radaideh AR et al. 2004).* Thyroid dysfunction in patients with type 2 found the overall prevalence of thyroid disease in type 2 DM to be 12.5% and 6.6% in the control group and the most common was Subclinical Hypothyroidism. In another study by (*Akbar DH et al. 2006).* in Saudi Arabia the association between thyroid autoimmunity in diabetics was 10% and in control it was 5%, while thyroid dysfunction was found in 16% diabetics and 7% control. In a study done in Greece the prevalence of thyroid dysfunction in type 2 DM was found to be 12.3% with a higher prevalence in females (Papazafiropoulou A et al. 2010).

In view of the relatively high prevalence of both endocrinopathies, it is important to investigate all diabetic patients for thyroid disorders. However, screening has been recommended only in children and adolescents with type 1 diabetes (WiericingW,1995).

In patients with type 1 diabetes the most prevalent immunological diseases are autoimmune thyroid function disorders (*Perros P et al.* 1995)

Prevalence of Hypothyroidism in patients with type 1 diabetes 12-24% of female and 6% of maleand 3-6% of patients with type 2 diabetes have been reported in cross-sectional studies.

It was found that Hyperthyroidsm occurs in 1-2% of patients suffering from diabetes It has been reportedin that the prevalence of positive thyroid peroxidase (TPO) antibodies is ~80% type 1 diabetic patients and elevated TSH levels and is 10-20% in those diabetics

individuals having normal TSH levels.Development of diabetes usually precedes the diagnosis of hypothyroidism, in most patients having subclinical diseases.(Nabarro JD et al. 1979) ***(****Radetti G et al.*1995)

Regular screening of TSH is recommended in patients with type 1 diabetes due to the increased prevalence of thyroid dysfunction in these subjects. However, the natural history of thyroid disorders and long term prospective trials to evaluatethe incidence in patients with type 1 diabetes are lacking. Therefore, there must be evaluation of thyroid status and the presence of TPO antibodies in all the patients having diabetes and followed them prospectively over the past 18 years. Thyroid dysfunctions are highly prevalent in general population. (*Wang C,Crapo,1997)*Cross-sectional studies have reported that 2.8% men and 7.5% of women of all ages in whickham,U.K, had abnormal serum thyroid –stimulating hormone levels.In the 20 years follow up study of the wickham survey cohort,the mean incidence of spontaneous hypothyroidism in women who had positive thyroid antibodies was increased from 4-27%. (*Tunbridge WM, et al. 1997).* The Colorado Thyroid Disease Prevalence Study reported that 11.7% of subjects among 25,682 subjects who were attending a state wide health fair had abnormal serum TSH concentration. Hyperthyroidism was detected in 2.1% and Primary Hypothyroidism was detected in 9.5% of subjects, most of whom were asymptomatic.

Recently, from a sample of 17,353 people aged≥12 years representing the geographic and ethnic distribution of u.s population,by the third national health and nutrition survey ,reported a prevalence of hypothyroidism in 4.6% of which 0.3% clinical and 4.3% subclinical and Hyperthyroidism in 1.3% of which 0.5% clinical and 0.7% subclinical *(Hollowell JG et al.* 2002)

In diabetes the role of hypothyroidism was investigated in 1927, by Coller and Huggins proving the association of worsening of diabetes and hyperthyroidism. It was shown that there is ameliorative effect on restoration of glucose tolerance by the surgical removal of parts of thyroid gland in hyperthyroid patients suffering from coexisting diabetes. (Coller FA, Huggins CB. 1927) Hashimoto's thyroiditis (hypothyroidism) or Graves's disease (thyroid over activity) has been investigated to be associated with diabetes mellitus. From meta-analysis it is reported a frequency of 11% in thyroid dysfunction

in patients of diabetes mellitus (Kadiyala R, Peter R, Okosieme. 2010). There is a deep underlying relation between diabetes mellitus and thyroid dysfunction (*Brenta G et al. 2007)* plethora of studies have evidenced an array of complex intertwining biochemical, genetic, and hormonal malfunctions mirroring this pathophysiological association (*Goglia F et al. 1999)* 5′ adenosine monophosphate-activated protein kinase (AMPK) is a central target for modulation of insulin sensitivity and feedback of thyroid hormones associated with appetite and energy expenditure Hypothyroidism (Hashimoto's thyroiditis) or thyroid over activity (Graves' disease) has been investigated to be associated with diabetes mellitus. A meta-analysis reported a frequency of 11% in thyroid dysfunction in the patients of diabetes mellitus (*Kadiyala R et al. 2010).*

Autoimmunity has been implicated to be the major cause of thyroid-dysfunction associated diabetes mellitus (*Kordonouri O et al. 2009)* Unmanaged pro diabetes, both type 1 and type 2, may induce a "low T3 state" characterized by low serum total and free T3 levels, increase in reverse T3 (rT3) but near normal serum T4 and TSH concentrations . (Donckier JE JC, Williams G, 2003) The relation between T2DM and thyroid dysfunction has been a less explored arena which may behold answers to various facts of metabolic syndrome including atherosclerosis, hypertension, and related cardiovascular disorders. T2DM owes its pathological origin to inappropriate secretion of insulin, due to defective islet cell function or beta cell mass. Continuous consumption of calories-rich meals, junk food and sedentary lifestyle have culminated into an epidemic of diabetes projected to afflict around 300 million people across the globe by 2020 (*Baxter JD et al, 2001)* Defective insulin secretion leads to various metabolic aberrations in T2DM, spanning from hyperglycemia due to defective insulin-stimulated glucose uptake and upregulated hepatic glucose production, along with dyslipidaemia, which includes impaired homeostasis of fatty acids, triglycerides, and lipoproteins *(Baxter JD and Webb P Nat Rev Drug Discov. 2009).*

Cappelli et al. evaluated the thyroid hormone profile by studying the interaction between metformin and circulating thyroid function parameters in patients who were started on metformin. (*Cappelli C et al ,2009)*. A pilot study on diabetic hypothyroid patient revealed baseline reduction of TSH level after 6 months; similarly a large cohort study on diabetic patients showed significant fall of TSH

level in euthyroid patients on L-T4 substitution and subclinical hypothyroid patients who did not receive LT4 treatment, except in euthyroid patients after 1 year on metformin. This study concluded that TSH lowering effect of metformin only seen in untreated hypothyroid patient and with L-T4 replacement therapy irrespective of thyroid function test. Similar findings were reported by Vigersky et al., (*Vigersky RA et al. 2006).*

In vitro studies support the use of metformin in other thyroid diseases other than hypothyroidism. Metformin has inhibited the cell proliferation and growth-stimulatory effect of insulin on thyroid carcinoma cell lines.

OBJECTIVES:

- To identify thyroid dysfunction in the management of diabetes mellitus.

MATERIALS AND METHODS

Study Design:
Cross-sectional study/ Purposive
Duration of Study:
6-8 months.
Study Population:
Patients visiting Diabetic Clinic of Jinnah hospital, Lahore and full filling the inclusion and exclusion criteria.
Sample Size:
A total of 105 samples were collected from Diabetic Clinic of Jinnah hospital, Lahore.
Sampling Technique:
Non probability/ Purposive

METHODOLOGY

Detailed clinical history and examination were carried out and recorded in a preformed Performa. 5cc blood were drawn from anti cubital vein of fore-arm and dispensed in a vial without any anticoagulant. The vial and request form were labeled properly. The blood sample were allowed to clot and then serum was separated out in a serum cup. Serum was then subjected to determination of TSH,

T3 and T4 by direct and sandwich ELISA technique. Serum random GLUCOSE level was performed using microlab300.

Sample Selection

Inclusion criteria:

- Patients having diabetes mellitus (type I & type II).
- Age: All age groups included.
- Gender: Both male and female.

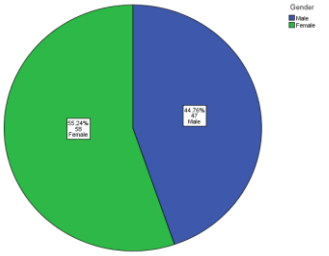
Exclusion criteria:

- Hemolysed sample.
- Patients on drugs affecting thyroid profile.

DATA ANALYSIS

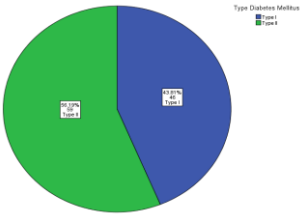
Age

N	Valid	105
	Missing	0
Mean		46.7333
Std. Deviation		13.03418
Minimum		13.00
Maximum		75.00



Statistics

		Blood Sugar Random mg /dl	Free T3 pg/dl	Free T4 pg/dl	TSH miu/l
N	Valid	105	105	105	105
	Missing	0	0	0	0
Mean		319.7714	2.1143	1.2581	5.7547
Std. Deviation		101.49330	.90622	.70884	7.23945
Minimum		200.00	.60	.10	.04
Maximum		749.00	6.50	5.20	33.70



Group Statistics

	Type Diabetes Mellitus	N	Mean	Std. Deviation	T test	P value
Free T3 pg/dl	Type I	46	1.9065	.73103	-2.108	.037
	Type II	59	2.2763	.99851		
Free T4 pg/dl	Type I	46	1.1391	.39411	-1.528	.130
	Type II	59	1.3508	.87205		
TSH miu/l	Type I	46	6.7400	7.58837	1.235	.220
	Type II	59	4.9864	6.92306		

Crosstab

			Type Diabetes Mellitus		
			Type I	Type II	Total
Free T3 ng/dl	Normal (2.3-6.19 ng/dl)	Count	46	58	104
		% within Type Diabetes Mellitus	100.0%	98.3%	99.0%
	High (> 6.19 ng/dl)	Count	0	1	1
		% within Type Diabetes Mellitus	.0%	1.7%	1.0%
Total		Count	46	59	105
		% within Type Diabetes Mellitus	100.0%	100.0%	100.0%

Chi-Square Tests					
	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.787[a]	1	.375		

Crosstab

			Type Diabetes Mellitus		
			Type I	Type II	Total
Free T4 ng/dl	Normal (0.7-1.9 ng/dl)	Count	46	52	98
		% within Type Diabetes Mellitus	100.0%	88.1%	93.3%
	High (> 1.9 ng/dl)	Count	0	7	7
		% within Type Diabetes Mellitus	.0%	11.9%	6.7%
Total		Count	46	59	105
		% within Type Diabetes Mellitus	100.0%	100.0%	100.0%

Chi-Square Tests					
	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5.847[a]	1	.016		

Crosstab

			Type Diabetes Mellitus		
			Type I	Type II	Total
TSH miu/l	Low (< 0.5 mIU/ml)	Count	4	8	12
		% within Type Diabetes Mellitus	8.7%	13.6%	11.4%
	Normal (0.5-6 uU/ml)	Count	25	39	64
		% within Type Diabetes Mellitus	54.3%	66.1%	61.0%
	High (> 6 mIU/ml)	Count	17	12	29
		% within Type Diabetes Mellitus	37.0%	20.3%	27.6%
Total		Count	46	59	105
		% within Type Diabetes Mellitus	100.0%	100.0%	100.0%

Chi-Square Tests			
	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.705[a]	2	.157

RESULTS AND DISCUSSION:

One hundred and five patients were selected from Diabetic clinic of Jinnah hospital Lahore. Among them 46 were with type 1(19 males,27 females). 54.5% having normal level,41.3% having sub-clinical hypothyroid,2.1% having sub-clinical hyper thyroid and 2.1% having hypothyroid functioning and 59 were with type 2 (28 males,31 females) 66% having normal level , 20% having sub clinical hypo thyroid,10% having sub clinical hyperthyroid and 4% primary hypo thyroid function.

In internal medicine, it is repeatedly proven that the association between thyroid dysfunction and diabetes mellitus is evident. Thyroid dysfunction chiefly comprises hypothyroidism and hyperthyroidism although the entity belongs to the same organ but with vast difference in pathophysiology as well as clinical picture. The interface between thyroid malfunction owing to diabetes is a matter of investigation. The literature suggests that polyendocrinal multidysfunction leads to stimulation of a cascade of reactions which are actually antihomeostatic in nature. For instance, hypoadrenalism as well as hypopituitarism exhibits strong linkage with hypothyroidism and consequently diabetes mellitus.

Recent findings have evidenced the intricate bond between subclinical hypothyroidism and diabetes mellitus that deceptively contribute to the major complications such as retinopathy and neuropathy. Cardiovascular events and micro- or macro-angiopathies are the counterreflection of resurgence of heavily disturbed lipid metabolism due to thyroid dyscrasias. It is also evident from the existing literature that insulin resistance bears an indispensable role in connecting T2DM and thyroid dysfunction. Novel molecules have shown the path for the development of suitable thyroid hormone receptor analogues to treat metabolic diseases. It is important to diagnose thyroid dysfunction in T2DM patients, and this practice should be inculcated in clinical settings with immediate effect to nourish further understanding of thyroid dysfunction and T2DM.

The “American Thyroid Association” guidelines for T2DM patients require frequent testing for thyroid dysfunction. They recommend testing from 35 years of age, and every 5 years thereafter in adults. High-risk patients may require more frequent testing. The American Association of Clinical Endocrinologists, Thyroid Disease

Clinical Practice Guidelines (2002) recommends thyroid palpation and TSH in diagnosis, especially if goitre or other autoimmune disease presents in association with T2DM. Regular screening for thyroid abnormalities in all diabetic patients will allow early treatment of subclinical thyroid dysfunction. A sensitive serum TSH assay is the screening test of choice. It has also been proposed that in T2DM patients, a TSH assay should be performed at diagnosis and then repeated at least every 5 years.

Population screening for thyroid dysfunction may prevent the development of overt thyroid dysfunction and may allow early treatment of hyperlipidemia .prevention of associated cardiovascular complications and metabolic bone disorders. The American College of Physicians recently published guidelines on screening for thyroid disease with a sensitive TSH test in the primary care setting .These guidelines state that screening in women <50 years of age and in men is not warranted because of the low frequency of thyroid dysfunction. Our results and previous studies indicate that these recommendations do not apply to patients with type 1 diabetes, since, compared with the general population, diabetic subjects develop thyroid dysfunction at an earlier age. In addition, our results indicate that long-term follow-up is necessary because the onset of diabetes usually precedes the diagnosis of thyroid dysfunction by approximately one decade.

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APPENDIX:

An enzyme-linked immunosorbent assay, also called ELISA or EIA, is a test that detects and measures antibodies in your blood. This test can be used to determine if you have antibodies related to certain infectious conditions.

APPENDIX A

TSH is measured by sandwich ELISA method by using anti TSH antibodies (Ab against ß subunit of TSH) as primary and HRP enzyme conjugated secondary antibody, here TSH is sandwiched between these two antibodies and color is produced when chromogenic substrate like TMB (Tetramethyl Benzidine) is added. Substrate A containing TMB and substrate B containing H2O2 is mixed and added. HRP will release [O] by hydrolysis of H2O2 which will oxidize TMB producing color.

APPENDIX B

The plasma concentration of free thyroid hormones are extremely small and as most of them are protein bound and especially in NTI or under medication, there is alteration in protein level or hormone itself making their measurement less informative. So, free hormone estimate is used for quantification. The binding of T4 to TBP is overcome by using barbital buffer which will selectively inhibit the binding. Similarly Anilino naphthalene sulfonic acid (ANS) is also used for this purpose. These agents displace T4 from TBG. This is in case of measurement of total T3 and T4.

Equilibrium dialysis and ultrafiltration methods are reference methods for measuring free thyroid hormones. But immunometric methods are used for routine purpose.

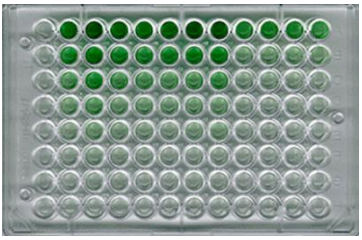

Fig1. ELISA plate (Color development)

Initially total T4 and T3 (free and protein bound) were measured, but there are various condition where results may be misleading. In pregnancy and oestrogen medication there is increased TBG concentration and thus increases in total T3 and T4. Use of contraceptives also increases TBG. Whereas Androgens, glucocorticoid, malnutrition, malabsorption, illness, liver disease, Cushing's disease, genetic variant of TBG, transthyretin, albumin with low affinity for T3, T4, etc. there is low TBG which will give false low results. NTI will produce modified TBG with diminished affinity for thyroid hormone.

Nowadays free T3 and T4 are measured by competitive ELISA method. There exists a competitive reaction between native Ag and enzyme-Ag conjugate for a limited number of immobilized binding sites on Ab coated on microwell. After the Ag-Ab reaction has taken place, the fraction of Ag in the conjugate or native Ag from sample

which does not bind to Ab coated in well is washed away (during this washing the unbound free and protein bound will be decanted off). The enzymatic activity in the Ab bound fraction, which is inversely proportional to the native Ag concentration, is measured by addition of the substrate. By utilizing calibrators of known concentration of conjugated Ag a dose response curve is generated from which the Ag concentration in a sample can be found out.

Here HRP enzyme is conjugated and TMB and H2O2 are used as substrates. Detergent like tween 20 is used in wash buffer.