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Physiological, Biochemical, and Molecular Study on Patients with Thyroid Disorders

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Summary

This study focused on 215 patients possessing thyroid dysfunction and were distributed as follow: 100 patients with hypothyroidism, 100 patients with hyperthyroidism, 15 patients with thyroid cancer, and 20 healthy persons as control. Their ages ranged between (20-70) years. Blood samples of those subjects were collected at AL- Yarmok Teaching Hospital (Baghdad), AL-Kadhymia Teaching Hospital (Baghdad), St. Rafael Hospital (Baghdad), Marjan General Surgery Hospital (Babylon), AL-Zahra Hospital (Najaf), AL-Basra General Hospital (Basra), AI- Ramadi General Hospital (Ramadi), AL-Shifaa General Hospital (Mousel), and Karkuk General Hospital (Karkuk).

The first objective of this research was to study the relationship between gender and thyroid dysfunction, and age at which the illness appears. Results showed that hyperthyroidism is more frequent in male than female, whereas hypothyroidism is significantly higher ($p<0.05$) in female than in male. The disease symptoms were also age related since most patients with thyroidites were in age of 41 – 50 and 51 – 60 respectively in both genders. The second objective is to study the effect of thyroid dysfunction on fertility. Results showed that there was a significant effect of thyroid disorders on fertility in both genders. In male, seminal fluid analysis showed a significant decrease ($P<0.05$) in total count when compared with normal, whereas in

women progesterone decreased significantly ($P<0.05$) when compared with normal value. The third objective is to determine the histopathic disorder in thyroid tissue. Histopathological examination of thyroid disorders showed the presence of papillary adenomas in patients with goiter and most of benign tumors appeared in female patients and were less in male. Cancer study showed that cancer cases studied were mostly of papillary carcinoma, whereas medullary carcinoma was less frequent. The fourth objective is the molecular analysis of thyroid dysfunction using RT – PCR performed on blood samples collected from subjects under study. Results showed no gene alteration in patients with hypothyroidism and hyperthyroidism, and illness may be due to physiological changes. In the case of cancer, molecular analysis of papillary carcinoma indicated the presence of point or deletion mutation that altered the normal function of thyroid gene(s), while in the case of medullary carcinoma, the mutation could be of amplification or gene rearrangement in thyroid gland.

List of contents

No.	Title	Page No.
Chapter One **Introduction and Literature review**		
1.	Introduction and Literature review	**1**
1.1.	Introduction	**1**
1. 2.	Literature review	**7**
1.2.1.	The endocrine system	**7**
1. 2.2.	Role in disease	**8**
1.2. 3.	Thyroid gland	**17**
1.2.3.1.	Embryological development	**20**
1.2.3.2.	Histology of thyroid gland	**21**
1.2.3.3.	Physiology of thyroid gland	**22**
1.2.3.4.	T3 and T4 regulation	**23**

1.2.3.5.	Calcitonin	23
1.2.3.6.	Significance of iodine	24
1.2.4.	Thyroid hormones	25
1.2.4.1.	Production of the thyroid hormones	27
1.2.5.	Thyroid disorders symptoms	29
1.2.6.	Types of Thyroid Disorders	31
1.2.6.1.	Hashimoto’s thyroiditis	31
1.2.6.2.	Graves’ disease	32
1.2.6.3.	Thyroid Nodules/Thyroid Cancer	34
1.2.6.4	Mutations in thyroid cancer	36
1.2.7.	Medical use of thyroid hormones	42
1.2.8.	Thyroid and fertility	43
1.2.9.	Molecular aspects of thyroid hormones	47
1.2.9.1.	Thyroid hormones synthesis	49

1.2.9.2.	Multiple thyroid hormone receptor isoforms	**52**
1.2.9.3.	Thyroid hormone receptor functional domains	**55**
1.2.9.4.	DNA-Binding Domain	**56**
1.2.9.5.	Ligand-Binding Domain	**57**
1.2.10.	Diagnosis of thyroid diseases	**61**
1.2.10.1.	Thyroid hormones and TSH changes during illness	**62**
1.2.10.2.	TSH/T4 relation	**63**
1.2.10.3.	Free T4 assay	**64**
1.2.10.4.	TSH assay	**64**
Chapter two Materials and methods		
2.	Materials and methods	**69**

2.1.	Materials	**69**
2.1.1.	Apparatus	**69**
2.1.2.	Chemicals	**70**
2.1.3.	Kits	**71**
2.1.4.	Blood samples collection	**72**
2.1.4.1.	Collection of blood from hypothyroidism, hyperthyroidism, thyroid cancer patients, and control (healthy)	**72**
2.1.4.2.	Collection of tumor tissue samples	**73**
2.1.5.	Buffers and solutions	**73**
2.1.5.1.	Sterile DEPC- treated solution	**73**
2.1.5.2.	NaOH , EDTA washing solution	**73**
2.1.5.3.	Thyroid Histopathological Examination	**73**
2.1.5.3.1.	Sodium bicarbonate solution (NaHCO3)	**73**

2.1.5.3.2.	Acid alcohol	**74**
2.1.5.4.	Hormones kit	**74**
2.1.5.4.1.	Fertility hormones	**74**
2.1.5.4.1.1.	FSH	**74**
2.1.5.4.1.2.	LH	**74**
2.1.5.4.1.3.	Testosterone	**74**
2.1.5.4.1.4.	Progesterone	**74**
2.1.5.4.2.	Thyroid hormones	**75**
2.1.5.4.2.1.	T3	**75**
2.1.5.4.2.2.	T4	**75**
2.1.5.4.2.3.	TSH	**75**
2.1.5.5.	RNA isolation system kit	**75**

2.1.5.6.	Determination of RNA yield and purity	**75**
2.1.5.6.1.	Gel electrophoresis analysis of RNA	**75**
2.1.5.6.1.1.	10X MOPS electrophoresis buffer	**76**
2.1.5.6.1.2.	RNA sample buffer	**76**
2.1.5.6.1.3.	RNA loading buffer	**76**
2.1.5.6.1.4.	RNA Marker	**76**
2.1.5.7.	Standardization one step – RT – PCR reaction	**77**
2.1.5.7.1.	Hexanucleotide containing all possible deoxynucleotide	**77**
2.1.5.7.2.	Cyclin A primer set	**77**
2.1.5.7.3.	Reverse Transcriptase	**77**
2.1.5.7.3.1.	AMV Reverse Transcriptase	**78**
2.1.5.7.3.2.	M-MLV Reverse Transcriptase	**78**

2.1.5.7.4.	(10X) PCR buffer	**78**
2.1.5.7.5.	dNTPs mixture	**78**
2.1.5.7.6.	Taq polymerase	**78**
2.1.5.7.7.	Ribonuclease inhibitor	**78**
2.1.5.8.	DNA gel electrophoresis	**79**
2.1.5.8.1.	(0.5 M) EDTA (pH 8.0)	**79**
2.1.5.8.2.	(5X) TBE buffer	**79**
2.1.5.8.3.	Loading buffer	**79**
2.1.5.8.4.	Ethidium bromide stock solution	**79**
2.1.5.8.5.	DNA marker	**80**
2.1.5.8.5.1.	Lambda DNA EcoR I , Hind III digest	**80**
2.1.5.8.5.2.	DNA ladder (123 bp)	**80**

2.1.6.	Preparation of cell culture medium for transportation and washing of tumor tissue samples	**80**
2.2.	Methods	**81**
2.2.1.	Sterilization methods	**81**
2.2.2.	Histopathological Examination	**81**
2.2.2.1.	Preparation of histological section	**81**
2.2.2.2.	Hematoxyline – Eosin Method	**82**
2.2.3.	Determination of FSH, LH, Testosterone, and Progesterone levels	**82**
2.2.4.	Determination of T3, T4 and TSH levels	**83**
2.2.5.	RNA extraction	**84**
2.2.6.	Determination of RNA yield and purity	**85**
2.2.6.1.	Gel electrophoresis analysis RNA	**85**
2.2.6.2.	Determination of RNA yield and purity spectrophotometrically	**85**

2.2.7.	One step – RT – PCR reaction.	**86**
2.2.8.	DNA (RT – PCR) product gel electrophoresis	89
2.3.	Statistical analysis	90
Chapter Three **Results and discussion**		
3.	Results and discussion	91
3.1.	Patients categorizing and thyroid hormone concentrations measurements	91
3.1.1.	Statistical analysis of patients with hypothyroidism	95
3.1.2.	Statistical analysis of patients with hyperthyroidism	97
3.2.	Effect of thyroid dysfunction on fertility	100
3.3.	Histopathology of thyroid tumors	112
3.3.1.	Thyroid cancer	117
3.4.	Molecular analysis of thyroid mRNA from blood	122

Chapter four Conclusions and Recommendations		
4.	Conclusions and Recommendations	**130**
4.1.	Conclusions	**130**
4.2.	Recommendations	**132**
	References	**133**

List of Tables

No.	Title	Page No.
Table (1.1)	Hormones released by hypothalamus	**9**
Table (1. 2)	Hormones released by Pituitary gland	**10**
Table (1. 3)	Hormones released by Thyroid	**12**
Table (1. 4)	Hormones released by Adrenal glands	**13**
Table (1. 5)	Hormones released by Testes	**14**
Table (1. 6)	Hormones released by Ovary	**15**
Table (1. 7)	Histological features of thyroid gland	**21**
Table (1. 8)	Proteins that bind to free T3 and T4 in blood	**26**

List of Figures

No.	Title	Page No.
Figure (1.1)	Major endocrine glands	**17**
Figure (1.2)	Anatomy of thyroid gland	**20**
Figure (1.3)	Triiodothyronine (T3) structure	**28**
Figure (1.4)	Thyroxin (T4) structure	**28**
Figure (1. 5)	Comparison of amino acid homologies and their functional properties among TR isoforms	**55**
Figure (1. 6)	General organization of major TR domains and functional subregions	**56**
Figure (1. 7)	DNA-binding domains of human TRb	**57**
Figure (1. 8)	TRα-1 ligand-binding domain crystal structure	**59**
Figure (3.1)	Distribution of hypothyroidism in male and female patients in different ages	**94**

Figure (3.2)	Distribution of hyperthyroidism in male and female patients in different ages	**94**
Figure (3.3)	Comparison of goiter appearance among patients with thyroid dysfunction of both genders	**114**
Figure (3.4)	Papillary adenoma section in patients with hyperthyroidism suffering from fibrosis in thyroid gland	**115**
Figure (3.5)	Papillary carcinoma section in patients with thyroid cancer	**116**
Figure (3.6)	mRNA isolated from blood of patients with thyroid disorder	**122**
Figure (3.7)	RT – PCR amplification of mRNA isolated from patients with thyroid disorders using hexamere primers	**123**

Figure (3.8)	Analysis of DNA fragments resulted from RT – PCR of mRNA in figure 3.7 from subjects under study	**124**
Figure (3.9)	RT – PCR amplification of mRNA taken from subjects under study	**126**
Figure (3.10)	Analysis of DNA fragments resulted from RT – PCR of mRNA of subjects under study	**127**

Table (2.1)	Optimal values of various steps in the standardization of RT-PCR reactions	**87**
Table (2.2)	Optimal values of various steps in the standardization of RT-PCR reactions	**88**
Table (2.3)	Optimal protocol of RT - PCR reaction	**89**
Table (3.1)	Categorizing of patients with T 3 hypothyroidism	**91**
Table (3.2)	Categorizing of patients with T 4 hypothyroidism	**92**
Table (3.3)	Categorizing of patients with TSH hypothyroidism	**92**
Table (3.4)	Categorizing of cancer patients with T 3 hyperthyroidism	**92**
Table (3.5)	Categorizing of patients with T 4 hyperthyroidism	**93**

Table (3.6)	Categorizing of patients with TSH hyperthyroidism	**93**
Table (3.7)	Statistical analysis using two ways ANOVA of male and female showing hypothyroidism symptoms	**96**
Table (3.8)	Statistical analysis using two ways ANOVA of male and female showing hyperthyroidism symptoms	**98**
Table (3.9)	Determination of fertility hormone levels in males with hypothyroidism	**101**
Table (3.10)	Determination of fertility hormone levels in females with hypothyroidism	**101**
Table (3.11)	Determination of fertility hormone levels in males with hyperthyroidism	**102**
Table (3.12)	Determination of fertility hormone levels in females with hyperthyroidism	**102**

Table	Title	Page
Table (3.13)	Statistical analysis of Seminal fluid in male with hypothyroidism and hyperthyroidism using two ways ANOVA	**104**
Table (3.14)	Statistical analysis of fertility hormones using two ways ANOVA of patients with hypothyroidism	**106**
Table (3.15)	Statistical analysis of fertility hormones using two ways ANOVA of patients with hyperthyroidism	**107**
Table (3.16)	Thyroid goiter and benign tumor distribution in male with thyroid dysfunction	**112**
Table (3.17)	Thyroid goiter and benign tumor distribution in female with thyroid dysfunction	**113**
Table (3.18)	Identification of cancer type and it's manifestation among different age groups in male patients	**118**

Table (3.19)	Identification of cancer type and it’s manifestation among different age groups in female patients	**118**
Table (3.20)	Thyroid hormone concentration in patients with thyroid cancer	**119**
Table (3.21)	Statistical analysis of thyroid hormone levels in comparison with normal	**120**

List of abbreviations

ACTH	Adrenocorticotropic hormone
ADH	Antidiuretic hormone
AF-2	Activation function – 2
AMV	Avian myeloblastosis virus
bHLH	Basic helix – loop – helix
cDNA	Complementary DNA
CRH	Corticotrophin releasing hormone
DBD	DNA – binding domain
DDT	Dichlorodiphenyl trichloroethane
DEPC	Diethylpyrocarbonate
DIT	Diiodotyrosine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DSP	Daily sperm production
EDTA	Ethylendiaminetetra acetic acid
ELFA	Enzyme link fluorescent assay
EPO	Erythropoietin
ER	Estrogen receptor
FNAB	Fine needle aspiration biopsy
FSH	Follicle – stimulating hormone
fT_3	Free triiodothyronine
fT_4	Free thyroxine

GABA	Gamma – aminobutyric acid
GH	Growth hormone
GHIH	Growth hormone inhibiting hormone
GHRH	Growth hormone releasing hormone
GnRH	Gonadotropin releasing hormone
HDAC	Histon deacetylase
HPL	Human placental lactogen
HPT	Hypothalamic / pituitary / thyroid
IUGR	Intrauterine growth restriction
LBD	Ligand binding domain
LH	Luteinizing hormone
LH-RH	Luteinizing hormone – releasing hormone
MIT	Monoidotyrosine
M-MLV	Moloney murine leukemia virus
MOPS	(3 – [N – morpholino] propan sulfuric acid)
mRNA	messenger Ribonucleic Acid
MSH	Melanocyte stimulating hormone
NCoR	Nuclear corepressor receptor
NIS	Sodium / iodide Symporter
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PIH	Prolactin inhibition hormone
PRH	Prolactin hormone

PTH	Parathyroid hormones
PTU	Prorlthiouracil
RAREs	Retinoic acid response elements
RARs	Retinoic acid response
RIA	Radio immuno assay
RNA	Ribonucleic Acid
rpm	Rotation per minute
RT-PCR	Reverse Transcription – Polymerase Chain Reaction
RXRs	Retinoid X receptors
S	Secretin
SDS	Sodium dodecy sulfate
SFA	Seminal fluid analysis
SHBG	Sex hormone binding globulins
SPR	Solid Phase Receptacle
SS	Somatostatin
sTg	Serum Thyroglobulin
T_0a	Thyronamine
T_1a	Iodothyronamine
T3	Triiodothyronine
T4	Thyroxine
TAI	Thyroid autoimmunity
TBG	Thyroxine – binding globulin
TBPA	Thyroxine – binding prealbumin

Tg	Thyroglobulin
TgAb	Thyroglobulin antibodies
THs	Thyroid Hormones
TPO	Thyroid preoxidase
TPO-Ab	Thyroid preoxidase antibodies
TR	Thyroid receptors
TRH	Thyrotropin releasing hormone
TSH	Thyroid – Stimulating Hormone
TT_4	Total thyroxine
TTR	Transthyretin
UV	Ultra violet
VDRs	Vitamin D receptors
VIDAS	Vitric immuno diagnosis assay system
WBS	Whole body scan

1. Introduction and Literatures Review

1.1. Introduction

The thyroid gland in human consists of 2 lobes that are lateral and inferior to the anterior aspect of the larynx and are connected across the larynx by an isthmus to produce a U – shaped structure averaging 30 gm in weight in adults (Wartofsky, 1994).

Thyroid gland mobilizes dietary iodine, converting it to an organic compound that can accelerate metabolic processes. It is necessary to the development and function of all body cells (Wartofsky and Burman, 1994).

The iodine containing levorotatory amino acids, thyroxine and triiodothyronine, occur in the gland and are physiologically active upon oral administration. These metabolites also occur in the gland bound with globulin (thyroglobulins), and the exogenously administered amino acids could conceivably bind with serum protein to form physiologically active molecules that are responsible for the ultimate hormonal action (Biscolla *et al.*,2000).The thyroid gland can store thyroglobulins and other iodometabolites. The release of these thyroid hormones appears to be controlled by thyrotropin, a hormone of the anterior pituitary (Delange and Ermans, 1991).

Manifestations of hypothyroidism may be caused by an iodine deficiency and a resulting lack of precursor moieties for the hormonal substances (simple hyperplastic goiter, which is characterized by a compensatory enlargement of the gland), by a deficiency of thyrotropic factors, or by other metabolic irregularities. The first two causes may be corrected by adding iodine to the diet or administering thyrotropin, respectively. Naturally, replacement therapy with thyroid hormones can be used for a deficiency of any origin (Nuovu and Wartofsky, 1995).

A hypothyroid condition results in some degree of cretinism in infants and of myxedema in adults. Cretinism is characterized by retarded and abnormal growth; arrested sexual development; mental deficiency; thickened, dry skin; thickened tongue; coarsened features; and a fall in the metabolic rate. The features of myxedema include general lethargy; retarded mental processes; increased body fat; susceptibility to cold and fatigue; cardiac dilatation (Schneider, 1990).

Thyroid hyperactivity results in hypotoxicosis characterized by increased heart rate, blood pressure, nervous excitability, and metabolic rate; muscular weakness with tremor; loss of body weight and fat; and an increased tolerance to cold but intolerance to heat (Pinto and Glick, 2002).

Thyroid hormones (THs) play a critical role in differentiation, growth, and metabolism. Indeed, TH is required for the normal function of nearly all tissues, with major effects on oxygen consumption and metabolic rate (Mazzaferri *et al.*, 2003).

Disorders of the thyroid gland are among the most common endocrine maladies. Furthermore, endemic cretinism due to iodine deficiency remains a public health problem in developing countries at the advent of the third millennium. Thus the study of TH action has important biological and medical implications (Biscolla *et al.*, 2000).

Numerous laboratories have shown that triiodothyronine (T3) plays a key role in Sertoli cell development (McCoard *et al.*, 2003) and, ultimately, affects the establishment of adult Sertoli cell populations and the magnitude of adult sperm production. Triiodothyronine has a direct inhibitory effect on Sertoli cell proliferation and a concomitant stimulatory effect on Sertoli cell differentiation. For example, transient neonatal hypothyroidism extends the length of Sertoli cell proliferation, leading to significant increases in Sertoli

cell number, daily sperm production (DSP), and testis weight (Joyce *et al*., 1993). Conversely, hyperthyroidism induced by exogenous T3 injections results in a premature cessation of mitogenesis and precocious canalization of seminiferous tubules (Van Haaster *et al*., 1993).

At the beginning of last century thyroid preparations were already being administered to women with clinical signs of thyroiditis to improve ovarian function for fertility reasons and to treat menorrhagia. Later, after endocrine thyroid tests had been established, many euthyroid women were also treated with thyroid hormones on a routine basis merely because they were anovulatory (Griff and Vande Wiele, 1974).

Thyroid function and prolactin are closely interrelated. Long-standing, untreated thyroiditis is associated with ovulatory dysfunction (Del Pozo *et al.*, 1979) and, in 1–3% of cases, with galactorrhoea (Kleinberg *et al.*, 1977).

Thus, it is important to assess thyroid function in the investigation of galactorrhoea (Yamada *et al*., 1976). Increased thyrotrophin-releasing hormone (TRH) production, or more likely a diminished hypothalamic dopamine turnover, could account for hypersecretion of thyroid-stimulating hormone (TSH) and prolactin in thyroid dysfunction (Thomas and Reid, 1987). Hypothalamic deficiency of dopamine not only explains the prolactin and TSH abnormalities, but also luteinizing hormone (LH) overproduction (Scanlon *et al.*, 1981).

Infusion of dopamine leads to a rapid decline of LH, TSH and prolactin, while infusion of the dopamine antagonist metoclopramide results in an increase of TSH and prolactin (Rossmanith *et al.*, 1988).

Disturbed pulsatile release of LH, TSH and prolactin interferes with the normal hypothalamic-pituitary–ovarian function. This can result in menstrual dysfunction, ranging from inadequate corpus luteumprogesterone secretion to oligomenorrhoea or amenorrhoea. Subclinical thyroiditis can be characterized

by an exaggerated TSH response to TRH and normal thyroxine and triiodothyronine concentrations in the blood (Lazarus, 1996).

Preliminary data have suggested a possible link between this biochemical diagnosis, luteal phase defect and female infertility (Bohnet *et al.*, 1981). Subclinical thyroiditis has also been reported in a cohort of women with premenstrual syndrome. Subclinical thyroiditis is a frequent biochemical diagnosis in infertile women and, according to the prevailing perception of gynaecologists, should be treated with thyroxine (Brayshaw and Brayshaw, 1986).

Since the molecular cloning of thyroid receptors (TRs) in the last two decades, there has been an explosion of information on the molecular mechanisms of TR action. The power of molecular genetics has greatly aided the understanding of the roles of unliganded and liganded TRs in regulating target genes (Morreal *et al.*,2004). Researchers revealed that there are multiple TR isoforms that bind to TREs with variable orientation, spacing, and sequences for TRE half-sites. TRs also interact with other nuclear proteins such as corepressors or coactivators to form complexes that regulate local histone acetylation and interact with the basal transcriptional machinery. Additionally, the solution of the crystal structures of the TR ligand-binding domain (LBD) and other nuclear hormone receptors have provided insight into some of these complex interactions at the molecular level. The development of transgenic and knockout mouse models have shed light on the roles of TRs in the regulation of specific target genes and development. These findings have greatly aided our understanding of the molecular mechanisms of TH action in normal and disease states. In particular, much has been learned about the pathogenesis of the human genetic disorder of resistance to thyroid hormone (RTH) (Van Herle and Uller 1975, Spencer *et al.*, 1987).

Diagnostic tests for thyroid dysfunction included ultrasound tests to determine the presence of enlargement, nodules, and cysts. The nodules can be hot or cold depending on whether they are producing hormones or not. A fine needle aspiration may be necessary, if cancer is suspected. However, the development of molecular RT-PCR-based methods to diagnose viral diseases that identify specific mRNAs, rather than proteins, suggested that, a highly sensitive method for detecting thyroid cancer that would be unencumbered by antibody interference could be developed. After the initial promising report, it is an appropriate time to make a critical appraisal of the status of these assays for thyroid cancer and to determine whether we are able to detect a reliable "signal" over a more "noisy" background than was initially recognized (Ringgel, 2004).

Total thyriodectomy is recommended when cancer is suspected. It is crucial to recognize the hyperthyroidism at its early stage in order to prevent permanent changes in the eyes and heart (Lazar, 1999).

Since the incidence of thyroidal dysfunction (hyperthyroidism, hypothyroidism, and thyroid cancer) has increased in Iraq in the last twenty years, it has become an important issue that worth investigation. There is a real need to have a comprehensive study of this growing problem. Therefore, the aims of the study were as follows:

1. Determination of relationship of thyroid dysfunction with age and gender.
2. Studying the effect of abnormal thyroid hormones on the fertility of patients.

3. Identification of the abnormality and / or mutation in the gene responsible for encoding thyroid hormones.

1.2. Literature Review

1.2.1. The endocrine system

The endocrine system is an integrated system of small organs that involve the release of extracellular signaling molecules known as hormones. The endocrine system is instrumental in regulating metabolism, growth, development and puberty, tissue function, and also plays a part in determining mood (Kester *et al.*, 2004). The Endocrine system is an information signal system much like the nervous system. However, the nervous system uses nerves to conduct information, whereas the endocrine system mainly uses blood vessels as information channels. Glands located in many regions of the body release into the bloodstream specific chemical messengers called hormones. Hormones regulate the many and varied functions of an organism, e.g., mood, growth and development, tissue function, and metabolism, as well as sending messages and acting on them (Scientific American Mind, "Rhythm and Blues"; June/July 2007).

The typical mode of cell signaling in the endocrine system is endocrine signaling. However, there are also other modes, i.e., paracrine, autocrine, and neuroendocrine signaling. Purely neurocrine signaling between neurons, on the other hand, belongs completely to the nervous system (Kasper *et al.*, 2005).

A number of glands that signal each other in sequence is usually referred to as an axis, for example the Hypothalamic-pituitary-adrenal axis. Typical endocrine glands are the pituitary, thyroid, and adrenal glands as shown in tables (1.1; 1.2; 1.3; 1.4, 1.5; and 1.6). Features of endocrine glands are, in general, their ductless nature, their vascularity, and usually the presence of intracellular vacuoles or granules storing their hormones. In

contrast exocrine glands such as salivary glands, sweat glands, and glands within the gastrointestinal tract tend to be much less vascular and have ducts or a hollow lumen (Cŏnsole *et al.*, 1995).

1.2.2. Role in disease

Diseases of the endocrine system are common including diseases such as diabetes mellitus, thyroid disease, and obesity. Endocrine disease is characterized by dysregulated hormone release (a productive Pituitary adenoma), inappropriate response to signaling (Hypothyroidism), lack or destruction of a gland (Diabetes mellitus type 1, diminished erythropoiesis in Chronic renal failure), or structural enlargement in a critical site such as the neck (Toxic multinodular goitre). Hypofunction of endocrine glands can occur as result of loss of reserve, hyposecretion, agenesis, atrophy, or active destruction. Hyperfunction can occur as result of hypersecretion, loss of suppression, hyperplastic, or neoplastic change, or hyperstimulation (Kasper *et al.*, 2005).

Endocrinopathies are classified as primary, secondary, or tertiary. Primary endocrine disease inhibits the action of downstream glands, while secondary is associated with the gland itself. Tertiary endocrine disease is associated with dysfunction of the hypothalamus and its releasing hormones. Cancer can occur in endocrine glands, such as the thyroid, and hormones have been implicated in signalling distant tissues to proliferate, for example the estrogen receptor has been shown to be involved in certain breast cancers. Endocrine, paracrine, and autocrine signaling have all been implicated in proliferation, one of the required steps of oncogenesis (Kosfeld *et al.*, 2005).

Table (1.1). Hormones released by hypothalamus (Kosfeld *et al*., 2005).

Secreted hormone	Abbreviation	From cells	Role
Thyrotropin-releasing hormone	TRH	Parvocellular neurosecretory neurons	Releases thyroid-stimulating hormone from anterior pituitary (primarily) Stimulates prolactin released from anterior pituitary.
Gonadotropin-releasing hormone	GnRH	Neuroendocine cells of the Preoptic area	Release of FSH and LH from anterior pituitary.
Growth hormone-releasing hormone	GHRH	Neuroendocrine neurons of the Arcuate nucleus	Releases GH from anterior pituitary
Corticotropin-releasing hormone	CRH	Parvocellular neurosecretory neurons	Releases ACTH from anterior pituitary
Somatostatin, also growth hormone-inhibiting hormone	SS or GHIH	Neuroendocrince cells of the Periventricular nucleus	Inhibits release of GH and TSH from anterior pituitary
Prolactin inhibiting hormone or Dopamine	PIH or DA	Dopamine neurons of the arcuate nucleus	Inhibits release of prolactin and TSH from anterior pituitary
Prolactin-releasing hormone	PRH		Releases prolactin from anterior pituitary

Table (1.2). Hormones released by Pituitary gland (Kaushansky, 2006)

Anterior pituitary lobe (adenohypophysis)			
Secreted hormone	Abbreviation	From cells	Role
Growth hormone	GH	Somatotropes	stimulates growth and cell reproduction Releases Insulin-like growth factor I from liver
Prolactin	PRL	Lactotropes	milk production in mammary glands sexual gratification after sexual acts
Adrenocorticotropic hormone or corticotropin	ACTH	Corticotropes	synthesis of corticosteroids (glucocorticoids and androgens) in adrenocortical cells
Lipotropin		Corticotropes	lipolysis and steroidogenesis, stimulates melanocytes to produce melanin
Thyroid-stimulating hormone or thyrotropin	TSH	Thyrotropes	stimulates thyroid gland to secrete thyroxin (T4) and triiodothyronine (T3)
Follicle-stimulating hormone	FSH	Gonadotropes	In female: stimulates maturation of Graafian follicles in ovary. In male: spermatogenesis, enhances production of androgen-binding protein by the Sertoli cells of the testes

Luteinizing hormone	LH	Gonadotropes	In female: ovulation In male: stimulates Leydig cell production of testosterone
Posterior pituitary lobe (neurohypophysis)			
Secreted hormone	Abbreviation	From cells	Role
Oxytocin		Magnocellular neurosecretory cells	Contraction of cervix and vagina Involved in orgasm, trust between people and circadian homeostasis (body temperature, activity level, wakefulness) releases breast milk
Vasopressin or antidiuretic hormone	AVP or ADH	Magnocellular neurosecretory cells	retention of water in kidneys, moderate vasoconstriction
Intermediate pituitary lobe (pars intermedia)			
Secreted hormone	Abbreviation	From cells	Role
Melanocyte-stimulating hormone	MSH	Melanotroph	melanogenesis by melanocytes in skin and hair.

Table (1.3). Hormones released by Thyroid and parathyroid gland (Kazuki, 2005).

Secreted hormone	Abbreviation	From cells	Role
Triiodothyronine	T3	Thyroid epithelial cell	potent form of thyroid hormone: increases the basal metabolic rate and sensitivity to catecholamines, affects protein synthesis
Thyroxin or tetraiodothyronine	T4	Thyroid epithelial cells	less active form of thyroid hormone: increases the basal metabolic rate and sensitivity to catecholamines, affects protein synthesis, often functions as a prohormone
Calcitonin	-	Parafollicular cells	Constructs bone, and reduces blood Ca^{2+}
Parathyroid			
Parathyroid hormone	PTH	Parathyroid chief cell	increases blood Ca^{2+}: indirectly stimulate osteoclasts Ca^{2+} reabsorption in kidney, activates vitamin D, (Slightly) decreases blood phosphate, decreases reuptake in kidney but increases uptake from bones, activates vitamin D

Table (1.4). Hormones released by Adrenal glands (Massaro and Massaro, 2004).

Adrenal cortex		
Secreted hormone	From cells	Role
Glucocorticoids (chiefly cortisol)	zona fasciculata and zona reticularis cells	Stimulation of gluconeogenesis, Inhibition of glucose uptake in muscle and adipose tissue Mobilization of amino acids from extrahepatic tissues Stimulation of fat breakdown in adipose tissue anti-inflammatory and immunosuppressive
Mineralocorticoids (chiefly aldosterone)	Zona glomerulosa cells	Increases blood volume by reabsorption of sodium in kidneys (primarily), Potassium and H^+ secretion in kidney.
Androgens	Zona fasciculata and Zona reticularis cells	Virilization, anabolic
Adrenal medulla		
Adrenaline (epinephrine) (Primarily)	Chromaffin cells	Fight-or-flight response: Boosts the supply of oxygen and glucose to the brain and muscles (by increasing heart rate and stroke volume, vasodilation, increasing catalysis of glycogen in liver, breakdown of lipids in fat cells), Dilates the pupils, Suppresses non-emergency bodily processes (e.g., digestion), Suppresses immune system,

Noradrenaline (norepinephrine)	Chromaffin cells	Fight-or-flight response: Boosts the supply of oxygen and glucose to the brain and muscles (by increasing heart rate and stroke volume, vasoconstriction and increased blood pressure, breakdown of lipids in fat cells), Increases skeletal muscle readiness.
Dopamine	Chromaffin cells	Increases heart rate and blood pressure
Enkephalin	Chromaffin cells	Regulates pain

Table (1.5). Hormones released by Testes (Pentikäinen *et al.*, 2006).

Secreted hormone	From cells	Role
Androgens (chiefly testosterone)	Leydig cells	Anabolic: growth of muscle mass and strength, increases bone density, growth and strength, Virilizing: maturation of sex organs, formation of scrotum, deepening of voice, growth of beard and axillary hair.
Estradiol	Sertoli cells	Prevents apoptosis of germ cells
Inhibin	Sertoli cells Inhibit production of FSH	

Table (1.6). Hormones released by Ovary (Hould *et al*., 1988).

Secreted hormone	From cells	Role
Progesterone	Granulosa cells, theca cells, corpus luteum	Supports pregnancy: Converts endometrium to secretory stage, Makes cervical mucus permeable to sperm. Inhibits immune response, e.g., towards the human embryo, Decreases uterine smooth muscle contractility, Inhibits lactation, Inhibits onset of labor. Other: Raises epidermal growth factor-1 levels, Increases core temperature during ovulation, Reduces spasm and relaxes smooth muscle (widens bronchi and regulates mucus), Anti-inflammatory, Reduces gall-bladder activity. Normalizes blood clotting and vascular tone, zinc and copper levels, cell oxygen levels, and use of fat stores for energy, Assists in thyroid function and bone growth by osteoblasts, Increases resilience in bone, teeth, gums, joint, tendon, ligament, and skin, Promotes healing by regulating collagen, Provides nerve function and healing by regulating myelin, Prevents endometrial cancer by regulating effects of estrogen
Androstenedione	Theca cells	Substrate for estrogen

Estrogens (mainly estradiol)	Granulosa cells and theca	Structural: Promotes formation of female secondary sex characteristics, accelerates height growth, accelerates metabolism (burns fat), reduces muscle mass, stimulates endometrial growth, increases uterine growth, maintains blood vessels and skin, reduces bone resorption, increases bone formation, Protein synthesis: Increases hepatic production of binding proteins, Coagulation: increases circulating level of factors II, VII, IX, X, antithrombin III, plasminogen, increases platelet adhesiveness, increases HDL, triglyceride, height growth, decreases LDL, fat deposition, Fluid balance: regulates salt (sodium) and water retention, increases growth hormone, increases cortisol, SHBG. Gastrointestinal tract: reduces bowel motility, increases cholesterol in bile. Melanin: increases pheomelanin, reduces eumelanin. Cancer: Supports hormone-sensitive breast cancers (suppression of production in the body of estrogen is a treatment for these cancers.). Lung function: promotes lung function by supporting alveoli
Inhibin	Granulosa cells	Inhibits production of FSH from anterior pituitary

A schematic diagram representing endocrine gland distribution is shown in figure (1.1).

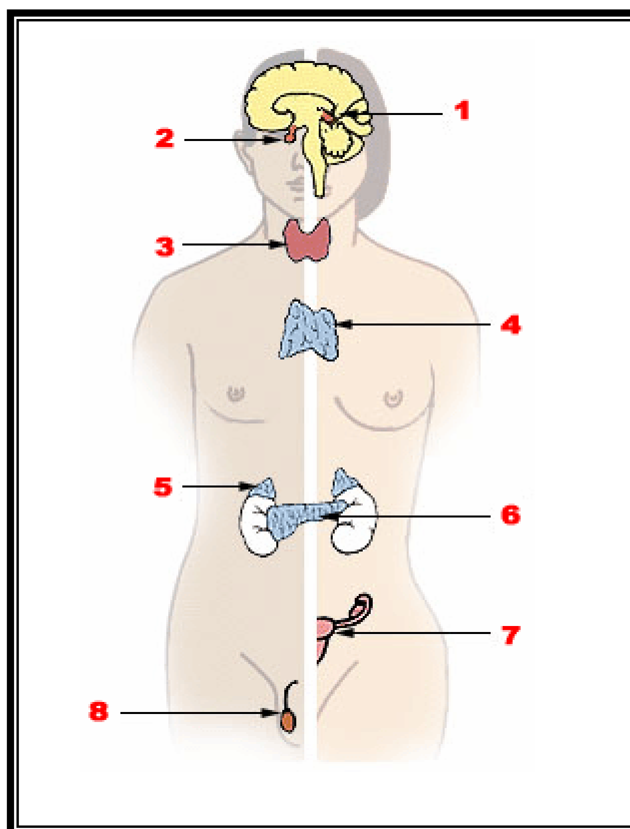


Figure (1.1). Major endocrine glands. (Male left, female on the right.) 1. Pineal gland 2. Pituitary gland 3. Thyroid gland 4. Thymus 5. Adrenal gland 6. Pancreas 7. Ovary 8. Testes (Mārcia *et al.*, 2008)

1.2.3. Thyroid gland

The thyroid is one of the largest endocrine glands in the body. This gland is found in the neck inferior to the thyroid cartilage (also known as the Adam's apple in men) and at approximately the same level as the cricoid cartilage (Gilleron *et al.*, 2006). The thyroid controls how quickly the body burns energy, makes proteins, and how sensitive the body should be to other hormones. The thyroid participates in these processes by producing thyroid hormones, principally thyroxin (T_4) and triiodothyronine (T_3). These hormones regulate the rate of metabolism and affect the growth and rate of

function of many other systems in the body. Iodine is an essential component of both T_3 and T_4. The thyroid also produces the hormone calcitonin, which plays a role in calcium homeostasis (Bianco *et al*., 2002).

The thyroid is controlled by the hypothalamus and pituitary. The gland gets its name from the Greek word for "door", after the shape of the related thyroid cartilage. Hyperthyroidism (overactive thyroid) and hypothyroidism (underactive thyroid) are the most common problems of the thyroid gland. The thyroid gland is a butterfly shaped organ and is composed of two cone like lobes or wings: *lobus dexter* (right lobe) and *lobus sinister* (left lobe), connected with the isthmus (Wajner *et al*., 2007). The organ is situated on the anterior side of the neck, lying against and around the larynx and trachea, reaching posteriorly the oesophagus and carotid sheath. It starts cranially at the oblique line on the thyroid cartilage (just below the laryngeal prominence or Adam's apple) and extends inferiorly to the fourth to sixth tracheal ring. It is difficult to demarcate the gland's upper and lower border with vertebral levels as it moves position in relation to these during swallowing. The thyroid gland is covered by a fibrous sheath, the *capsula glandulae thyroidea*, which is composed of an internal and external layer. The external layer is anteriorly continuous with the *lamina pretrachealis fasciae cervicalis* and posteriorolaterally continuous with the carotid sheath. The gland is covered anteriorly with infrahyoid muscles and laterally with the sternocleidomastoid muscle. Posteriorly, the gland is fixed to the cricoid and tracheal cartilage and cricopharyngeus muscle by a thickening of the fascia to form the posterior suspensory ligament of Berry (Yalçin and Ozan , 2006).

In variable extent, Zuckerkandl's tubercle, a pyramidal extension of the thyroid lobe, is present at the most posterior side of the lobe. In this region the

recurrent laryngeal nerve and the inferior thyroid artery pass next to or in the ligament and tubercle. Between the two layers of the capsule and on the posterior side of the lobes there are on each side two parathyroid glands (Mirilas and Skandalakis, 2003).

The thyroid isthmus is variable in presence and size, and can encompass a cranially extending pyramid lobe (*lobus pyramidalis* or *processus pyramidalis*), remnant of the thyroglossal duct (Jannini *et al.*, 1995). The thyroid is one of the larger endocrine glands, weighing 2-3 grams in neonates and 18-60 grams in adults, and is increased in pregnancy. The thyroid is supplied with arterial blood from the superior thyroid artery, a branch of the external carotid artery, and the inferior thyroid artery, a branch of the thyrocervical trunk, branching directly from the aortic arch (Cheng and Mruk, 2002). The venous blood is drained via superior thyroid veins, draining in the internal jugular vein, and via inferior thyroid veins, draining via the *plexus thyroideus impar* in the left brachiocephalic vein. Lymphatic drainage passes frequently the lateral deep cervical lymph nodes and the pre- and parathracheal lymph nodes. The gland is supplied by sympathetic nerve input from the superior cervical ganglion and the cervicothoracic ganglion of the sympathetic trunk, and by parasympathetic nerve input from the superior laryngeal nerve and the recurrent laryngeal nerve. Figure (1.2) illustrates the anatomy of thyroid gland (Yalçin *et al.*, 2007).

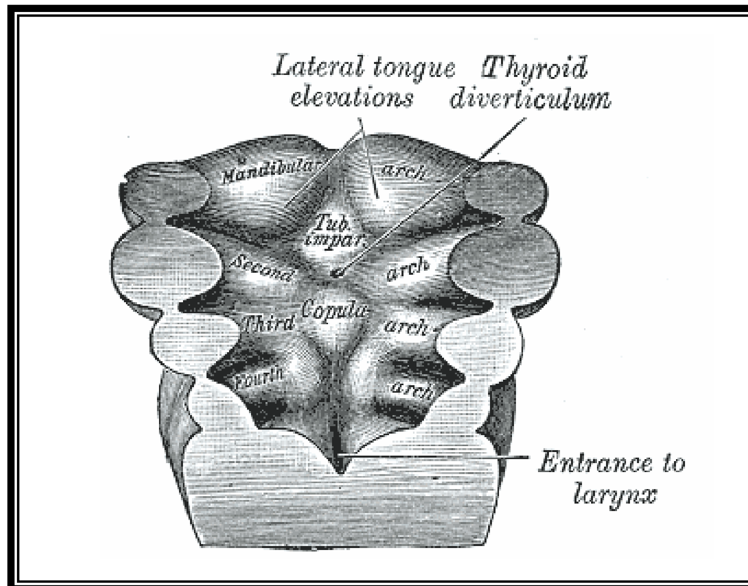


Figure (1.2). Anatomy of thyroid gland (Yalçin *et al.*, 2007).

1.2.3.1. Embryological development

In the fetus, at 3-4 weeks of gestation, the thyroid gland appears as an epithelial proliferation in the floor of the pharynx at the base of the tongue between the tuberculum impar and the copula linguae at a point latter indicated by the foramen cecum (Yen *et al.*, 2006). Subsequently the thyroid descends in front of the pharyngeal gut as a bilobed diverticulum through the thyroglossal duct. Over the next few weeks, it migrates to the base of the neck. During migration, the thyroid remains connected to the tongue by a narrow canal, the thyroglossal duct. The fetus starts making its own thyroid stimulating hormone (TSH) by week 8, and the follicles of the thyroid begin to make thyroxin by the 10th week (Bianco *et al.*, 2002).

1.2.3.2. Histology of thyroid gland

At the microscopic level, there are three primary features of the thyroid listed in table (1.7) (Carol *et al*., 2005):

Table (1.7). Histological features of thyroid gland.

Feature	Description
Follicles	The thyroid is composed of spherical follicles that selectively absorb iodine (as iodide ions, I^-) from the blood for production of thyroid hormones. Twenty-five percent of all the body's iodide ions are in the thyroid gland. Inside the follicles, colloids are rich in a protein called thyroglobulin which serve as a reservoir of materials for thyroid hormone production and, to a lesser extent, act as a reservoir for the hormones themselves.
Thyroid epithelial cells (or "follicular cells")	The follicles are surrounded by a single layer of thyroid epithelial cells, which secrete T3 and T4. When the gland is not secreting T3/T4 (inactive), the epithelial cells range from low columnar to cuboidal cells. When active, the epithelial cells become tall columnar cells.
Parafollicular cells (or "C cells")	Scattered among follicular cells and in spaces between the spherical follicles are another type of thyroid cell, parafollicular cells, which secrete calcitonin.

1.2.3.3. Physiology of thyroid gland

The primary function of the thyroid is production of the hormones thyroxin (T4), triiodothyronine (T3), and calcitonin. Up to 80% of the T4 is converted to T3 by peripheral organs such as the liver, kidney and spleen. T3 is about ten times more active than T4. Thyroxin (T4) is synthesised by the follicular cells from free tyrosine and on the tyrosine residues of the protein called thyroglobulin (TG). Iodine is captured with the "iodine trap" by the hydrogen peroxide generated by the enzyme thyroid peroxidase (TPO) (Ekholm R, Bjorkman, 1997) and linked to the 3' and 5' sites of the benzene ring of the tyrosine residues on TG, and on free tyrosine. Upon stimulation by the thyroid-stimulating hormone (TSH), the follicular cells reabsorb TG and proteolytically cleave the iodinated tyrosines from TG, forming T4 and T3 (in T3, one iodine is absent compared to T4), and releasing them into the blood. Deiodinase enzymes convert T4 to T3. Thyroid hormone that is secreted from the gland is about 90% T4 and about 10% T3 (Bianco *et al.*, 2002).

Cells of the brain are a major target for the thyroid hormones T3 and T4. Thyroid hormones play a particularly crucial role in brain maturation during fetal development (Kester *et al.*, 2004). A transport protein (OATP1C1) has been identified that seems to be important for T4 transport across the blood brain barrier. A second transport protein (MCT8) is important for T3 transport across brain cell membranes (Jansen *et al.*, 2005).

In the blood, T4 and T3 are partially bound to thyroxin-binding globulin, transthyretin and albumin (Santoro *et al.*, 2002). Only a very small fraction of the circulating hormone is free (unbound) T4 0.03% and T3 0.3%. Only the free fraction has hormonal activity. As with the steroid hormones and retinoic acid, thyroid hormones cross the cell membrane and bind to

intracellular receptors (α_1, α_2, β_1 and β_2), which act alone, in pairs or together with the retinoid X-receptor as transcription factors to modulate DNA transcription (Kester *et al.*, 2004).

1.2.3.4. T3 and T4 regulation

The production of thyroxin and triiodothyronine is regulated by thyroid-stimulating hormone (TSH). The thyroid and thyrotropes form a negative feedback loop: TSH production is suppressed when the T4 levels are high and vice versa (Chin and Yen, 1996). The TSH production itself is modulated by thyrotropin-releasing hormone (TRH), which is produced by the hypothalamus and secreted at an increased rate in situations such as cold (in which an accelerated metabolism would generate more heat). TSH production is blunted by somatostatin (SRIH), rising levels of glucocorticoids and sex hormones (estrogen and testosterone), and excessively high blood iodide concentration (Morreale *et al.*, 2004).

1.2.3.5. Calcitonin

An additional hormone produced by the thyroid contributes to the regulation of blood calcium levels. Parafollicular cells produce calcitonin in response to hypercalcemia (Orth *et al.*, 1988). Calcitonin stimulates movement of calcium into bone, in opposition to the effects of parathyroid hormone (PTH). However, calcitonin seems far less essential than PTH, as calcium metabolism remains clinically normal after removal of the thyroid, but not the parathyroids (Giannini *et al.*, 1987).

1.2.3.6. Significance of iodine

In areas of the world where iodine (essential for the production of thyroxin, which contains four iodine atoms) is lacking in the diet, the thyroid gland can be considerably enlarged, resulting in the swollen necks of endemic goitre. Thyroxin is critical to the regulation of metabolism and growth throughout the animal kingdom. Among amphibians, for example, administering a thyroid-blocking agent such as propylthiouracil (PTU) can prevent tadpoles from metamorphosing into frogs; conversely, administering thyroxin will trigger metamorphosis (Yamada *et al*., 1976). In humans, children born with thyroid hormone deficiency will have physical growth and development problems, and brain development can also be severely impaired, in the condition referred to as cretinism. Newborn children in many developed countries are now routinely tested for thyroid hormone deficiency as part of newborn screening by analysis of a drop of blood. Children with thyroid hormone deficiency are treated by supplementation with synthetic thyroxin, which enables them to grow and develop normally (Jansen *et al.*, 2005).

Because of the thyroid's selective uptake and concentration of what is a fairly rare element, it is sensitive to the effects of various radioactive isotopes of iodine produced by nuclear fission (Thomas and Reid, 1987). In the event of large accidental releases of such material into the environment, the uptake of radioactive iodine isotopes by the thyroid can, in theory, be blocked by saturating the uptake mechanism with a large surplus of non-radioactive iodine, taken in the form of potassium iodide tablets. While biological researchers making compounds labelled with iodine isotopes do this, in the wider world such preventive measures are usually not stockpiled before an accident, nor are they distributed adequately afterward. One consequence of

the Chernobyl disaster was an increase in thyroid cancers in children in the years following the accident. The use of iodised salt is an efficient way to add iodine to the diet (Li *et al*., 2000). It has eliminated endemic cretinism in most developed countries, and some governments have made the iodination of flour mandatory. Potassium iodide and Sodium iodide are the most active forms of supplemental iodine (Shomon, 2006).

1.2.4. Thyroid hormones

The thyroid hormones, thyroxin (T_4) and triiodothyronine (T_3), are tyrosine-based hormones produced by the thyroid gland (Salva *et al*., 2004) . The major form of thyroid hormone in the blood is thyroxin (T_4). The ratio of T_4 to T_3 released in the blood is roughly 20 to 1. Thyroxin is converted to the active T_3 (three to four times more potent than T_4) within cells by deiodinases (5'-iodinase). These are further processed by decarboxylation and deiodination to produce iodothyronamine (T_1a) and thyronamine (T_0a). Most of the thyroid hormone circulating in the blood is bound to transport proteins. Only a very small fraction of the circulating hormone is free (unbound) and biologically active, hence measuring concentrations of free thyroid hormones is of great diagnostic value. When thyroid hormone is bound, it is not active, so the amount of free T_3/T_4 is an important criterion (Rao *et al*., 2003). For this reason, measuring total thyroxin in the blood can be misleading (Verhaeghe *et al*., 2008). Proteins that bind to T3 and T4 are listed in table (1.8).

Table (1.8). Proteins that bind to free T3 and T4 in blood (Verhaeghe *et al.*, 2008).

Type	Percent
bound to thyroxin-binding globulin (TBG)	70%
bound to transthyretin or "thyroxin-binding prealbumin" (TTR or TBPA)	10-15%
para albumin	15-20%
unbound T_4 (fT4)	0.03%
unbound T_3 (fT_3)	0.3%

T_3 and T_4 cross the cell membrane, probably via amino acid importins, and function via a well-studied set of nuclear receptors in the nucleus of the cell, the thyroid hormone receptors. T_1a and T_0a are positively charged and do not cross the membrane; they are believed to function via the trace amine-associated receptor *TAAR1* (TAR1, TA1), a G-protein-coupled receptor located in the cell membrane. Another critical diagnostic tool is measurement of the amount of thyroid-stimulating hormone (TSH) that is present (Sherwood *et al.*, 2005).

The thyronines act on the body to increase the basal metabolic rate, affect protein synthesis and increase the body's sensitivity to catecholamines (such as adrenaline) by permissiveness (De Groot and Mayor, 1992). The thyroid hormones are essential to proper development and differentiation of all cells of the human body. These hormones also regulate protein, fat, and carbohydrate metabolism, affecting how human cells use energetic compounds. They also stimulate vitamin metabolism (American Medical Women’s Association, 1999a).

Numerous physiological and pathological stimuli influence thyroid hormone synthesis. Thyroid hormone leads to heat generation in humans. However, the thyronamines function via some unknown mechanism to inhibit neuronal activity; this plays an important role in the hibernation cycles of mammals and the moulting behaviour of birds. One effect of administering the thyronamines is a severe drop in body temperature (Ollikainen *et al.*, 2006).

1.2.4.1. Production of the thyroid hormones

Thyroxin (3,5,3',5'-tetraiodothyronine) is produced by follicular cells of the thyroid gland. It is produced as the precursor thyroglobulin (this is *not* the same as TBG), which is cleaved by enzymes to produce active T_4. Thyroxin is produced by attaching iodine atoms to the ring structures of tyrosine molecules. Thyroxin (T_4) contains four iodine atoms. Triiodothyronine (T_3) is identical to T_4, but it has one less iodine atom per molecule (Ishihara *et al.*, 2003).

Iodide is actively absorbed from the blood stream by a process called 'iodine trapping' and concentrated in the thyroid follicles. (If there is a deficiency of dietary iodine, the thyroid enlarges in an attempt to trap more iodine, resulting in goitre.) Via a reaction with the enzyme thyroperoxidase, iodine is covalently bound to tyrosine residues in the thyroglobulin molecules, forming monoiodotyrosine (MIT) and diiodotyrosine (DIT). Linking two moieties of DIT produces thyroxin. Combining one particle of MIT and one particle of DIT produces triiodothyronine (Ogasawara *et al.*, 2001).

- **DIT + MIT → r-T_3 (biologically inactive)**
- **MIT + DIT → triiodothyronine (usually referred to as T_3)**
- **DIT + DIT → thyroxin (referred to as T_4)**

Proteases digest iodinated thyroglobulin, releasing the hormones T_4 and T_3, the biologically active agents central to metabolic regulation (Dratman and Gordon, 1996). Thyroxin is supposedly a prohormone and a reservoir for the most active and main thyroid hormone T_3. T_4 is converted as required in the tissues by deiodinases. Deficiency of deiodinase can mimic an iodine deficiency. T_3 is more active than T_4 and is the final form of the hormone, though it is present in less quantity than T_4 (Boelaert and Franklyn, 2005).

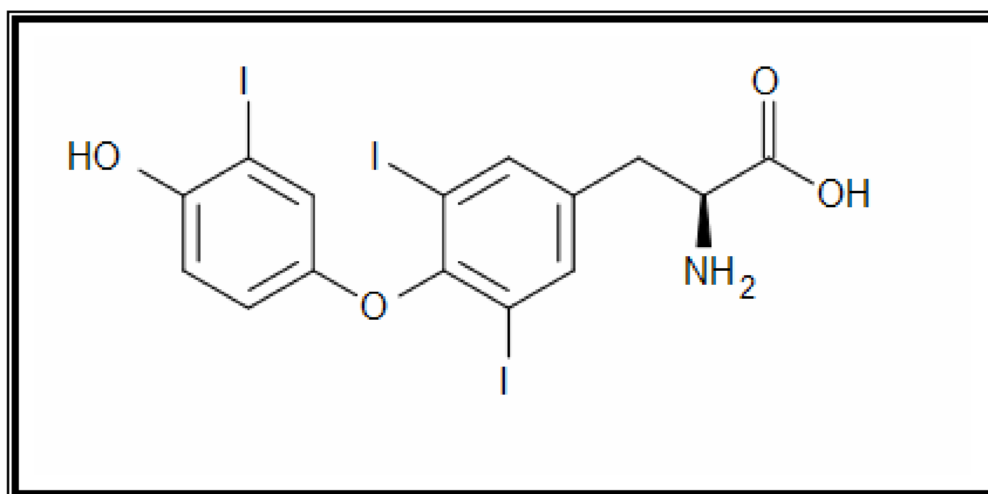


Figure (1.3). Triiodothyronine (T3) structure (Boelaert and Franklyn, 2005).

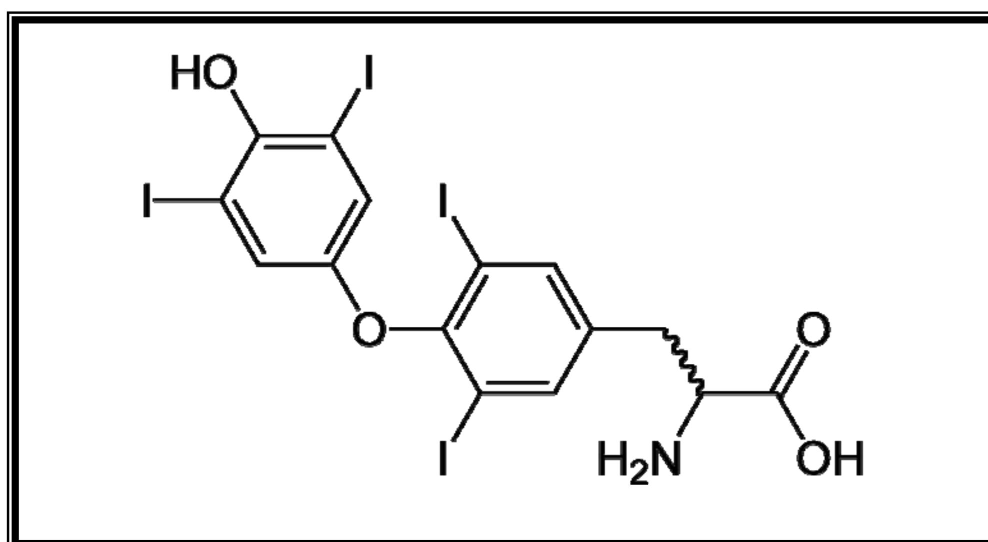


Figure (1.4). Thyroxin (T4) structure (Boelaert and Franklyn, 2005).

1.2.5. Thyroid disorders symptoms

Among the most common types of thyroid disorders are hypothyroidism (underactive thyroid), hyperthyroidism (overactive thyroid), benign nodules, inflammations of the thyroid, and malignant cancers (The Endocrine Society, 2000a).

If there is not enough thyroid hormone in the blood stream, the body's metabolism slows down (hypothyroidism or underactive thyroid). Complications of hypothyroidism may include an enlarged thyroid gland (goiter), increased risk of heart disease (primarily due to increased levels of LDL cholesterol among people with underactive thyroid), an enlarged heart, severe depression, decreased libido, slowed mental functioning, and rarely, a life threatening condition known as myxedema (symptoms including intense cold intolerance and drowsiness followed by profound lethargy and unconsciousness) (Mayo Clinic, 2002c).

If there is too much thyroid hormone in the bloodstream, the body's metabolism speeds up (hyperthyroidism or overactive thyroid) (American College of Obstetricians and Gynecologists, 1999). Complications of hyperthyroidism may include rapid heart rate, atrial fibrillation and congestive heart failure (a condition in which the heart becomes too weak to circulate enough blood to meet the needs of the body), osteoporosis (weak, brittle bones because too much thyroid hormone interferes with the body's ability to incorporate calcium), severe eye problems (known as Grave's opthalmopathy), and rarely severe skin problems causing redness and swelling of the shins and feet, as well as risk for thyrotoxic crisis (condition in which symptoms suddenly intensify, leading to fever, rapid pulse, and delerium) (Mayo Clinic, 2002b).

Risk for thyroid disorders if the following symptoms are observed:

• Previous thyroid dysfunction.

• A goiter.

• Surgery or radiotherapy affecting the thyroid gland.

• Diabetes mellitus.

• Pernicious anemia (vitamin B12 deficiency).

• Prematurely gray hair.

• Medications such as lithium carbonate and iodine-containing compounds.

• Family history of thyroid disease (American Association of Clinical Endocrinologists, 2003).

• Slower digestion and constipation.

• Weaker heart and slower heart rate.

• Decreased lung function and shortness of breath.

• An enlarged thyroid (i.e., goiter), which may appear as a swelling at the base of the neck (American College of Obstetricians and Gynecologists, 1999; American Foundation of Thyroid Patients, 1994; Mayo Clinic, 2002c; The Hormone Foundation, 2002a).

Symptoms of hypothyroidism include:

Anemia, Cardiomegaly, Cold intolerance, Dry hair, Elevated aspartate transaminase; alanine transaminase and lactate dehydrogenase levels, Elevated creatine, Goiter, Hyperlipidemia, Hypertelorism, Hypotension, Inverted T waves in electrocardiogram, Lethargy, Myxedema, Reduced cardiac output, Reduced respiratory rate, Weight gain (American Foundation of Thyroid Patients, 1994; Mayo Clinic, 2002b).

Symptoms of hyperthyroidism include:

Fatigue or tiredness, Weight loss, Hair loss, Bulging eyes, Soft nails, Nervousness and irritability, Rapid or irregular heartbeat (i.e., arrhythmia),

Increased perspiration, Feeling hot when others are not or trouble withstanding the heat, Irregular or scant menstrual periods, Trouble getting pregnant or infertility, More frequent bowel movements or diarrhea, Troubled or disturbed sleep, Weakness in muscles (e.g., can't easily walk up stairs or lift heavy objects), Tremors or trembling hands, Eye irritation or problems seeing, An enlarged thyroid (i.e., goiter), which may appear as a swelling at the base of the neck (American College of Obstetricians and Gynecologists, 1999; American Foundation of Thyroid Patients, 1994; Mayo Clinic, 2002b).

1.2.6. Types of Thyroid Disorders

Although most thyroid disorders fall into categories of hypo- or hyperthyroidism, there are particular types of thyroid disorders within each of these groups. There are also disorders that do not fit neatly within one of these categories.

1.2.6.1. Hashimoto's thyroiditis

The most common form of hypothyroidism is a condition known as Hashimoto's thyroiditis. Hashimoto's thyroiditis is a type of autoimmune thyroid disease in which the immune system attacks and destroys the thyroid gland (National Women's Health Information Center, 2001).

People with Hashimoto's thyroiditis may experience:

- A goiter (i.e., a noticeable bump or swelling at the front of the neck due to the swelling of the thyroid gland).
- A feeling of fullness or tightness in the throat.
- Trouble swallowing food or liquids.
- Tiredness; forgetfulness; depression; coarse, dry skin; slow heartbeat; weight gain; constipation; and intolerance to cold.

A person with Hashimoto's thyroiditis can also exhibit no symptoms; an ordinary blood test may be the only source of information to show that thyroid hormones are out of balance (American Academy of Family Physicians, 2001).

Hashimoto's thyroiditis has no cure, but can be treated and controlled with thyroid hormone replacement (a small pill, usually taken once daily) and regular blood tests to check levels of thyroid hormones.

Left untreated, Hashimoto's thyroiditis can cause further complications, such as menstrual changes, prevention of ovulation, and increased risk for miscarriage.

Additionally, too much hormone replacement left unnoticed can result in symptoms of hyperthyroidism (National Women's Health Information Center, 2001).

1.2.6.2. Graves' disease

The most common form of hyperthyroidism is a condition known as Grave's disease. Graves' disease is a type of autoimmune disease (in which the immune system over stimulates the thyroid gland (National Women's Health Information Center, 2000a).

People with Grave's disease may experience:

- Anxiety.
- Irritability.
- Difficulty in sleeping.
- Rapid, forceful, or irregular heartbeat.
- Fine tremor of the hands or fingers.
- Increase in perspiration.
- Sensitivity to heat.
- Weight loss, despite normal diet.

• Enlargement of the thyroid gland (i.e., goiter).

• Light menstrual periods.

• Frequent bowel movements.

Particularly related to Graves' disease are eye symptoms, known as Graves' opthalmopathy. In Graves' opthalmopathy, the eye protrudes beyond its protective orbit because tissues and muscles behind the eye swell. This condition can result in (Strawson *et al.*, 2004):

• Drying of the front of the eye.

• Excess tearing.

• Sensation of sand or grit in the eye(s).

• Reddened or inflamed eyes.

• Widening of the space between the eyelids.

• Swelling of the lids and tissue around the eyes.

• Bulging eyes.

• Decreased blinking and excessive staring.

• Light sensitivity.

• Blurry or double vision.

• Limited eye movement. Finally, a more uncommon symptom of

Grave's disease is reddening and swelling of the skin, often on the shins and on top of the feet (Mayo Clinic, 2002a).

Graves' disease is diagnosed using a simple blood test to identify if the body has the correct amount of thyroid hormones.

Treatments for Graves' disease include:

• Medications - anti-thyroid drugs, which inhibit production or conversion of the active thyroid hormone

• Radioactive iodine: iodine damages thyroid cells to shrink the thyroid gland, thus reducing hormone levels.

• Surgery: a subtotal thyroidectomy, in which a surgeon removes most of the thyroid gland and renders it incapable of overproducing thyroid hormone (National Women's Health Information Center, 2000b).

Medications are usually 20-30% effective; the latter two treatments are usually 90- 95% effective but almost always result in hypothyroidism, which would have to be managed with medication for the rest of the patient's life (National Graves' Disease Foundation, 2000).

1.2.6.3. Thyroid Nodules/Thyroid Cancer

A thyroid nodule is any abnormal growth of thyroid cells forming a lump within the thyroid gland. Though most thyroid nodules do not cause any symptoms, occasionally a nodule will cause pain, difficulty swallowing or breathing, hoarse- ness, or hyperthyroid symptoms. About 90-95% of thyroid nodules are benign (non-cancerous). (American Thyroid Association, 2003a; American Thyroid Association, 2003b).

To determine whether the nodule may be harmful or cancerous, the following tests should be performed:

• Fine needle aspiration.

• Thyroid scan.

• Blood tests.

• Thyroid hormone treatments to see if the nodule shrinks (which usually indicates that it is not malignant [cancerous]) (The Hormone Foundation, 2002b).

Risk factors of thyroid cancer:

• Low diet in iodine.

• Radiation exposure to the head and neck as a child.

• Radiation exposure during: a nodule may be cancerous if it grows quickly and feels solid or hard, if the lymph nodes under the jaw are swollen, or if it troubles swallowing or breathing or a hoarse voice (Hamburger, 1994).

Benign thyroid nodules should be checked regularly, at least annually to watch for any complications or development of cancerous cells.

Some women develop hypothyroidism (underactive thyroid) during or after pregnancy, often because they produce antibodies to their own thyroid gland. Left untreated, risk is increased for miscarriage, premature delivery, and preeclampsia (a condition that causes a significant increase in a woman's blood pressure during the last 3 months of pregnancy). Hypothyroidism during pregnancy can also affect the developing fetus (Mayo Clinic, 2002c).

During Pregnancy:

• Nearly 1 out of 50 women is diagnosed with hypothyroidism during pregnancy.

• Pregnant women with hypothyroidism have a four times greater risk for miscarriage during the second trimester.

• The rate of fetal death increases incrementally as TSH increases (which indicate a decrease in thyroid function).

• Six out of every 100 miscarriages can be attributed to thyroid deficiency during pregnancy.

• Untreated hypothyroidism during pregnancy may negatively impact a child's psychological development, resulting in a lower I.Q. score and a decrease in motor skills, attention, language and reading abilities (American Association of Clinical Endocrinologists, 2002a).

Postpartum

• Five to ten percent of women are diagnosed with postpartum thyroiditis within 1 year following delivery.

• Approximately 50 percent of women will develop permanent hypothyroidism within five years of being diagnosed with postpartum thyroiditis.
• Symptoms of postpartum thyroiditis include fatigue, depression, palpitations and irritability.
• Twenty percent of all new mothers experience postpartum depression following delivery (“the baby blues”), and seventy percent will experience some form of depression. Since depression is a flagship symptom of thyroid disease, women need to be aware that it could indicate the presence of an underlying thyroid condition (American Association of Clinical Endocrinologists, 2002b).

Both hypo and hyperthyroidism may result from medication. Taking too much medication to treat hypothyroidism can lead to an overactive thyroid (hyperthyroidism), while taking too high a dose of medication to treat hyperthyroidism can lead to underactive thyroid (hypothyroidism).

Additionally, hyperthyroidism can contribute to thinning bones (osteoporosis), so it is important to be sure you are getting enough calcium every day (usually about 1,500 milligrams daily from either diet or calcium supplements) (Mayo Clinic, 2002b; Carol *et al.*, 2003).

1.2.6.4 Mutations in thyroid cancer

In biology, a mutation is a randomly derived change to the nucleotide sequence of the genetic material of an organism. Mutations can be caused by copying errors in the genetic material during cell division, or by exposure to mutagens (ultraviolet or ionizing radiation, mutagenic chemicals, or viruses), or can be induced by the organism itself, by cellular processes such as hypermutation. In multicellular organisms with dedicated reproductive cells,

mutations can be subdivided into germ line mutations, which can be passed on to descendants through their reproductive cells, and somatic mutations, which involve cells outside the dedicated reproductive group and which are not usually transmitted to descendants. If the organism can reproduce asexually through mechanisms such as cuttings or budding the distinction can become blurred. For example, plants can sometimes transmit somatic mutations to their descendants asexually or sexually where flower buds develop in somatically mutated parts of plants. A new mutation that was not inherited from either parent is called a *de novo* mutation. The source of the mutation is unrelated to the consequence, although the consequences are related to which cells were mutated. Nonlethal mutations accumulate within the gene pool and increase the amount of genetic variation (Eyre-Walker and Keightley 2007).

As can be seen, clinically important missense mutations generally change the properties of the coded amino acid residue between being basic, acidic, polar or nonpolar, while nonsense mutations result in a stop codon. The sequence of a gene can be altered in a number of ways. Gene mutations have varying effects on health depending on where they occur and whether they alter the function of essential proteins. Mutations in the structure of genes can be classified as:

- Small-scale mutations, such as those affecting a small gene in one or a few nucleotides, including:
    - Point mutations, often caused by chemicals or malfunction of DNA replication, exchange a single nucleotide for another[3]. These changes are classified as transitions or transversions[4]. Most common is the transition that exchanges a purine for a

purine (A ↔ G) or a pyrimidine for a pyrimidine, (C ↔ T). A transition can be caused by nitrous acid, base mis-pairing, or mutagenic base analogs such as 5-bromo-2-deoxyuridine (BrdU). Less common is a transversion, which exchanges a purine for a pyrimidine or a pyrimidine for a purine (C/T ↔ A/G). An example of a transversion is adenine (A) being converted into a cytosine (C). A point mutation can be reversed by another point mutation, in which the nucleotide is changed back to its original state (true reversion) or by second-site reversion (a complementary mutation elsewhere that results in regained gene functionality). Point mutations that occur within the protein coding region of a gene may be classified into three kinds, depending upon what the erroneous codon codes for:

- Silent mutations: which code for the same amino acid.
- Missense mutations: which code for a different amino acid.
- Nonsense mutations: which code for a stop and can truncate the protein.

- Insertions add one or more extra nucleotides into the DNA. They are usually caused by transposable elements, or errors during replication of repeating elements (e.g. AT repeats). Insertions in the coding region of a gene may alter splicing of the mRNA (splice site mutation), or cause a shift in the reading frame (frameshift), both of which can significantly alter the gene product. Insertions can be reverted by excision of the transposable element.

- Deletions remove one or more nucleotides from the DNA. Like insertions, these mutations can alter the reading frame of the gene (Ciocci and German, 2001).

In theoretical population genetics, it is more usual to speak of such mutations as deleterious or advantageous. In the neutral theory of molecular evolution, genetic drift is the basis for most variation at the molecular level.

- A neutral mutation has no harmful or beneficial effect on the organism. Such mutations occur at a steady rate, forming the basis for the molecular clock.
- A deleterious mutation has a negative effect on the phenotype, and thus decreases the fitness of the organism.
- An advantageous mutation has a positive effect on the phenotype, and thus increases the fitness of the organism.
- A nearly neutral mutation is a mutation that may be slightly deleterious or advantageous, although most nearly neutral mutations are slightly deleterious.

- Inheritable generic in pro-generic tissue or cells on path to be changed to gametes.
- Non inheritable somatic (eg, carcinogenic mutation)
- Non inheritable post mortem a DNA mutation in decaying remains.

The human genome contains two copies of each gene – a paternal and a maternal allele.

- A heterozygous mutation is a mutation of only one allele.
- A homozygous mutation is an identical mutation of both the paternal and maternal alleles.

- Compound heterozygous mutations or a genetic compound comprises two different mutations in the paternal and maternal alleles. [6]
- A wildtype or homozygous non-mutated organism is one in which neither allele is mutated. (Just not a mutation)

- A frameshift mutation is a mutation caused by insertion or deletion of a number of nucleotides that is not evenly divisible by three from a DNA sequence. Due to the triplet nature of gene expression by codons, the insertion or deletion can disrupt the reading frame, or the grouping of the codons, resulting in a completely different translation from the original. The earlier in the sequence the deletion or insertion occurs, the more altered the protein produced is.

- A nonsense mutation is a point mutation in a sequence of DNA that results in a premature stop codon, or a *nonsense codon* in the transcribed mRNA, and possibly a truncated, and often nonfunctional protein product.

- Missense mutations or *nonsynonymous mutations* are types of point mutations where a single nucleotide is changed to cause substitution of a different amino acid. This in turn can render the resulting protein nonfunctional.
- A neutral mutation is a mutation that occurs in an amino acid codon which results in the use of a different, but chemically similar, amino acid. The similarity between the two is enough that little or no change is often rendered in the protein. For example, a change from AAA to AGA will encode lysine, a chemically similar molecule to the intended arginine.

- Silent mutations are mutations that do not result in a change to the amino acid sequence of a protein. They may occur in a region that does not code for a protein, or they may occur within a codon in a manner that does not alter the final amino acid sequence. The phrase *silent mutation* is often used interchangeably with the phrase *synonymous mutation*; however, synonymous mutations are a subcategory of the former, occurring only within exons. The name silent could be a misnomer. For example, a silent mutation in the exon/intron border may lead to alternative splicing by changing the splice site thereby leading to a changed protein.

- Conditional mutation is a mutation that has wild-type (or less severe) phenotype under certain "permissive" environmental conditions and a mutant phenotype under certain "restrictive" conditions. For example, a temperature-sensitive mutation can cause cell death at high temperature (restrictive condition), but might have no deleterious consequences at a lower temperature (permissive condition) (Doniger *et al.,* 2008).

Mutation rates also vary across species. Evolutionary biologists have theorized that higher mutation rates are beneficial in some situations, because they allow organisms to evolve and therefore adapt more quickly to their environments. For example, repeated exposure of bacteria to antibiotics, and selection of resistant mutants, can result in the selection of bacteria that have a much higher mutation rate than the original population (mutator strains) (Sawyer *et al.*, 2007).

Changes in DNA caused by mutation can cause errors in protein sequence, creating partially or completely non-functional proteins. To function correctly, each cell depends on thousands of proteins to function in the right

places at the right times. When a mutation alters a protein that plays a critical role in the body, a medical condition can result (Pfohl-Leszkowicz and Manderville , 2007).

If a mutation is present in a germ cell, it can give rise to offspring that carries the mutation in all of its cells. This is the case in hereditary diseases. On the other hand, a mutation may occur in a somatic cell of an organism. Such mutations will be present in all descendants of this cell within the same organism, and certain mutations can cause the cell to become malignant, and thus cause cancer (Ionov *et al.*, 1993).

1.2.7. Medical use of thyroid hormones

Both T_3 and T_4 are used to treat thyroid hormone deficiency (hypothyroidism). They are both absorbed well by the gut, so can be given orally (Kirkegaard and Faber, 1998). Levothyroxin, the most commonly used synthetic thyroxin form, is a stereoisomer of physiological thyroxin, which is metabolised more slowly and hence usually only needs once daily administration (Dratman and Gordon, 1996). Natural desiccated thyroid hormones, also under the commercial name Armour Thyroid, is derived from pig thyroid glands, it is a "natural" hypothyroid treatment containing 20% T_3 and calcitonin. Also available are synthetic combinations of T3/T4 in different ratios (such as Thyrolar) and pure-T3 medications (Cytomel) (Mayo Clinic, 2002c). Thyronamines have no medical usages yet, though their use has been proposed for controlled induction of hypothermia which causes the brain to enter a protective cycle, useful in preventing damage during ischemic shock. Synthetic thyroxin was first successfully produced by Charles Robert Harington and George Barger in 1926 (Farsetti *et al.*, 1992).

1.2.8. Thyroid and fertility

Infertility, is defined as the absence of pregnancy with in one year of regular unprotected intercourse, is present in 10 – 15% of couples in developed countries (American College of Obstetricians and Gynecologists, 1999). The usual causes of infertility include a male factor (30%), a female factor (35%), a combination of both (20%), and finally unexplained or 'idiopathic' infertility (15 %) (Hoxsey and Rinehart, 1997). Although thyroid disorders (i.e. hyperthyroidism, hypothyroidism and thyroid autoimmunity are less frequent in men than in women, they remain relatively common diseases. The prevalence of TAI in men of reproductive age (20 – 40 years) is 7% for thyroid peroxidase antibodies (TPO- Ab) and 5% for thyroglobulin antibodies. Increased serum thyrotropin (TSH. 4.5 mU/l) is present in 3.4 % of the men and decreased TSH (0.4 mU /l) in 1.8% of the men (Bjoro *et al.*, 2000).

However, in areas with a lower iodine intake (such as Belgium), the prevalence of TAI and supranormal serum TSH may be different. Associations between thyroid disorders and female infertility have been more extensively studied than associations between thyroid disorders and male infertility (Poppe and Velkeniers, 2004, Poppe *et al.* 2008.). Thyroid dysfunction can impair the quality of semen, and lower sperm motility and /or concentration (Knudsen *et al.*, 2002).

Furthermore, an association between TAI and as the no zoospermia has been described. Semen alterations were reversible after patients achieved euthyroidism. The impact of thyroid dysfunction on the semen is mainly due to an effect on central luteinizing hormone/follicle stimulating hormone and peripheral gonadal function and via sex hormone binding proteins (Krassas and Pontikides, 2004).

Physicians and Physiologists have long hypothesized the connections between hypothyroidism and adrenocortical dysfunction. The interaction of pituitary-thyroid and pituitary-adrenal functions has been studied, and the influences of thyroid hormones on adrenocortical function have been demonstrated (Bray and Jacobs, 1974; Steinetz and Beach, 1963; Sanchez-Franco *et al*., 1989).

After endocrine thyroid tests had been established, many euthyroid women were treated with thyroid hormones on a routine basis merely because they were anovulatory (Griff and Vande Wiele, 1974). Thyroid function and prolactin are closely interrelated. Long-standing, untreated hypothyroidism is associated with ovulatory dysfunction (Del Pozo *et al.*, 1979) and, in 1–3% of cases, with galactorrhoea (Kleinberg *et al.*, 1977). Thus, it is important to assess thyroid function in the investigation of galactorrhoea (Yamada *et al.*, 1976). Increased thyrotrophin releasing hormone (TRH) production, or more likely a diminished hypothalamic dopamine turnover, could account for hypersecretion of thyroid-stimulating hormone (TSH) and prolactin in hypothyroidism (Thomas and Reid, 1987). Hypothalamic deficiency of dopamine not only explains the prolactin and TSH abnormalities, but also luteinizing hormone (LH) overproduction (Feek *et al.*, 1980; Scanlon *et al.*, 1981).

Infusion of dopamine leads to a rapid decline of LH, TSH and prolactin while infusion of the dopamine antagonist metoclopramide results in an increase of TSH and prolactin (Rossmanith *et al.*, 1988). Disturbed pulsatile release of LH, TSH and prolactin interferes with the normal hypothalamic-pituitary–ovarian function. This can result in menstrual dysfunction, ranging from inadequate corpus luteum progesterone secretion to oligomenorrhoea or amenorrhoea (Bals-Pratsch *et al.*, 1997). Subclinical hypothyroidism can be an early stage of hypothyroidism and is characterized by an exaggerated TSH

response to TRH stimulation [in gynaecological practice are, 15 mU/ml] normal values for stimulated TSH (TSHs and normal thyroxin and triiodothyronine concentrations in the blood (Hoff and Obricht, 1988; Franklyn, 1995; Lazarus, 1996).

Preliminary data have suggested a possible link between this biochemical diagnosis, luteal phase defect and female infertility (Bohnet *et al.*, 1981).

Subclinical hypothyroidism has also been reported in a cohort of women with premenstrual syndrome (Brayshaw and Brayshaw, 1986). However, the relationship between subclinical hypothyroidism and ovarian function has never been proved by controlled studies. More over, serious questions remain about patient selection and premenstrual syndrome diagnostic criteria in those reports (Thomas and Reid, 1987; Pickardt, 1989). Subclinical hypothyroidism is a frequent biochemical diagnosis in infertile women and, according to the prevailing perception of gynaecologists, should be treated with thyroxin.

However, until 1993 there has been no proof that subclinical hypothyroidism is a cause of ovarian dysfunction and thus infertility. The study was performed to investigate the significance of subclinical hypothyroidism as an infertility factor using intensive endocrine examinations (Goldman *et al.*, 1993).

The aim was to assess whether subclinically hypothyroid infertile women have a disturbed early follicular LH pulse pattern in comparison to that of euthyroid controls. If this hypothesis was correct, subclinically hypothyroid infertile patients may have ovulatory disorders. Thus, 24 h blood sampling was performed to study the LH pulse pattern as an indirect determination of hypothalamic pulsatile gonadotrophin-releasing hormone (GnRH) release (Raber *et al.*, 2003). If the function of the GnRH pulse

generator in the subclinical hypothyroid patients was shown to be normal, the presence of normal ovarian function was likely, with normal follicular development, ovulation, corpus luteum function and normal conception rates. Thus there would be no basis for the continuation of thyroxin supplementation for fertility reasons in subclinical hypothyroid patients (Bals-Pratsch *et al.*, 1997).

Furthermore, fetal growth is dependent upon a number of endocrine, paracrine, and autocrine events within the fetoplacental unit the precise mechanisms of which remain to be defined. Babies born with intrauterine growth restriction (IUGR) are major casualties of both perinatal and neonatal mortality. This pathological process also causes significant morbidity, with 10% of low birth weight babies having physical handicaps and a further 5% showing neuro developmental delays at the age of 3–4 years (Gaffney, 1994). Thyroid status is one of several factors that have been postulated to play a critical role in the pathogenesis of such morbidity, especially with respect to growth and development of the central nervous system (Fisher *et al.*, 1977; Fisher, 1997).

Several studies have demonstrated that thyroid hormone determines the duration of Sertoli cell division and may be involved in them at urational changes that decrease and eliminate mitogenic responses to FSH (Holsberger *et al.*, 2005). Although hypothyroidism had no effect on testicular development during fetal life (Hamouli-Said *et al.*, 2007), when induced in newborn, it was associated, at puberty, with impaired testicular development including testicular growth, germ cell maturation, and seminiferous tubule formation (Francavilla *et al.*, 1991). However, as the animals made hypothyroid were allowed to recover back to the euthyroid state, a significant increase in testis size and daily sperm production (80 and 140% respectively, compared with control animals) was observed in adulthood (Cooke *et al.*,

1991). Subsequently, the mechanism underlying these unpredictable testicular changes was established. It has been shown that transient neonatal/prepubertal hypothyroidism extends the length of Sertoli cell proliferation by delaying their maturation, resulting in an increased number of Sertoli cells in the adult testis (De Franca *et al.*, 1995). The adult number of Sertoli cells that had been subjected to transient neonatal hypothyroidism was shown to increase 157% compared with control (Hess *et al.*, 1993). Conversely, transient juvenile hyperthyroidism resulted in an early cessation of Sertoli cell proliferation and had a concomitant stimulatory effect on their maturation, resulting in premature canalization of seminiferous tubules, decreased testis size, and sperm production (Cooke *et al.*, 1994).

1.2.9. Molecular aspects of thyroid hormones

Thyroid hormones (THs) play critical roles in differentiation, growth, and metabolism. Indeed, TH is required for the normal function of nearly all tissues, with major effects on oxygen consumption and metabolic rate (Oppenheimer *et al.*, 1987). Disorders of the thyroid gland are among the most common endocrine maladies. Furthermore, endemic cretinism due to iodine deficiency remains a public health problem in developing countries at the advent of the third millennium. Thus the study of TH action has important biological and medical implications. The story of TH action is interwoven with many of the major advances in biomedical science during the past century. Contributions from clinical medicine, physiology, biochemistry, and molecular genetics have had major impacts on our understanding of TH action (Oppenheimer *et al.*, 1997).

In the 1960s, Tata and co-workers first suggested that THs might be involved in the transcriptional regulation of target genes. These investigators observed that T3 treatment of hypothyroid rats induced a rapid increase in RNA synthesis in the liver which preceded new protein formation and mitochondrial oxidation (Farsetti *et al.*, 1992). The groups of Oppenheimer (Oppenheimer *et al.*, 1972) and Samuels (Samuels *et al.*, 1988) then used radiolabeled TH to demonstrate specific nuclear binding sites in different T3 sensitive tissues, and thus provided the first evidence for TH receptors (TRs). Moreover, T3binding was observed in almost all tissues (Oppenheimer *et al*, 1974). Attempts to purify these receptors biochemically were only partially successful; however, photoaffinity labeling of nuclear extracts demonstrated different sized receptors and raised the possibility of multiple TR isoforms (Dozin *et al.*, 1985). Studies on T3 induction of the rat growth hormone (GH) gene transcription suggested that TRs recognized enhancer sequences or TH response elements (TREs), similar to steroid hormone receptors (Lavin *et al.*, 1988). Thus TRs behaved similar to steroid hormone receptors with respect to nuclear site of action, recognition of specific DNA sequences, and ligand dependent regulation of transcription. In 1985, the glucocorticoid receptor was cloned and, surprisingly, had homology with a known viral oncogene product, v-*erbA* that in conjunction with v-*erbB*, can cause erythroblastosis in chicks (Hollenberg *et al.*, 1985).

Subsequent cloning of the estrogen receptor suggested that there was a family of nuclear hormone receptors (Green *et al.*, 1986). A year later, the laboratories of Evans (Weinberger *et al.*, 1986) and Vennstrom ushered in the molecular era of TH action when they cloned two different TR isoforms and showed they were the cellular homologs of v-*erbA*. Since the molecular cloning of TRs 15 years ago, there has been an explosion of information on the molecular mechanisms of TR action. The power of molecular genetics has

greatly aided our understanding of the roles of unliganded and liganded TRs in regulating target genes. There are multiple TR isoforms that bind to TREs with variable orientation, spacing, and sequences for TRE half sites. TRs also interact with other nuclear proteins such as corepressors or coactivators to form complexes that regulate local histone acetylation and interact with the basal transcriptional machinery. Additionally, the solution of the crystal structures of the TR ligand binding domain (LBD) and other nuclear hormone receptors have provided insight into some of these complex interactions at the molecular level (Sasaki *et al*., 1999).

1.2.9.1. Thyroid hormones synthesis

TH synthesis and secretion is exquisitely regulated by a negative feedback system that involves the hypothalamus, pituitary, and thyroid gland [hypothalamic/pituitary/thyroid (HPT) axis] (Shupnik *et al.*, 1989). Thyrotropin releasing hormone (TRH) is a tripeptide (PyroGlu-His-Pro) synthesized in the paraventricular nucleus of the hypothalamus. It is transported via axons to the median eminence and then to the anterior pituitary via the portal capillary plexus.

TRH binds to TRH receptors in pituitary thyrotropes, a subpopulation of pituitary cells that secrete thyroid stimulating hormone (TSH). TRH receptors are members of the seven transmembrane spanning receptor family and are coupled to G_{q11}. TRH stimulation leads to release and synthesis of new TSH in thyrotropes. TSH is a 28-kDa glycoprotein composed of α- and β-subunits designated as glycoprotein hormone α and TSH β subunits. The α subunit also is shared with other hormones such as luteinizing hormone, follicle stimulating hormone, and chorionic gonadotropin. Both TRH and TSH secretion are negatively regulated by TH. An important mechanism for the negative regulation of TSH may be the intrapituitary conversion of circulating

T4 to T3 by type II deiodinase. Additionally, somatostatin and dopamine from the hypothalamus can negatively regulate TSH secretion (Parmentier *et al.*, 1989).

TSH is the primary regulator of TH release and secretion. It also has a critical role in thyroid growth and development. TSH binds to the TSH receptor (TSHr), which also is a seven transmembrane spanning receptor coupled to G_{q11}. Activation of TSHr by TSH or autoantibodies in Graves' disease leads to an increase in intracellular cAMP and stimulation of protein kinase A mediated pathways. A number of thyroid genes, including Na/I symporter (NIS), thyroglobulin (Tg), and thyroid peroxidase (TPO), are stimulated by TSH and promote the synthesis of TH. It is noted that, activating mutations in TSHr and G_s have been described in autonomously functioning thyroid nodules and familial congenital hyperthyroidism (Parma *et al.*, 1997). The THs, T4 and the more potent T3, are synthesized in the thyroid gland. Iodide is actively transported and concentrated into the thyroid by NIS. The trapped iodide is oxidized by TPO in the presence of hydrogen peroxide and incorporated into the tyrosine residues of a 660 kDa glycoprotein, Tg. This iodination of specific tyrosines located on Tg yields monoiodinated and diiodinated residues (MIT, monoiodotyrosines; DIT, diiodotyrosines) that are enzymatically coupled to form T3 and T4. The iodinated Tg containing MIT, DIT, T3, and T4, then is stored as an extracellular storage polypeptide in the colloid within the lumen of thyroid follicular cells. Genetic defects along the synthetic pathway of THs have been described in humans and are major causes of congenital hypothyroidism in iodine replete environments (Kopp, 2000).

The secretion of THs requires endocytosis of the stored iodinated Tg from the apical surface of the thyroid follicular cell (Taurog, 1996). The internalized Tg is incorporated in phagolysosomes and undergoes proteolytic

digestion, recapture of MIT and DIT, and release of T4 and T3 into the circulation via the basal surface. The majority of released TH is in the form of T4, as total serum T3 is 40-fold higher than serum T4 (90 vs. 2 nM). Only 0.03% of the total serum T3 is free (unbound), with the remainder bound to carrier proteins such as thyroxin binding globulin (TBG), albumin, and thyroid binding prealbumin. Approximately 0.3% of the total serum T3 is free, with the remainder bound to TBG and albumin. It is the free TH that enters target cells and generates a biological response (Smanik *et al.*, 1996).

The major pathway for the production of T4is via 59-deiodination of the outer ring of T3 by deiodinases and accounts for the majority of the circulating T3 (Kohrle, 2000).

Type I deioidinase is found in peripheral tissues such as liver and kidney and is responsible for the conversion of the majority of T4 to T3 in circulation. Type II deiodinase is found in brain, pituitary, and brown adipose tissue and primarily converts T4 to T3 for intracellular use. These deiodinases recently have been cloned and demonstrated to be selenoproteins. 59-Deiodination by type I deiodinase and type III deioidinase, which is found primarily in placenta, brain, and skin, leads to the generation of rT3 , the key step in the inactivation of TH. rT3 and T3 can be further deiodinated in the liver and are sulfo and glucuronide conjugated before excretion in the bile. There is also an enterohepatic circulation of TH as intestinal flora deconjugates some of these compounds and promotes the reuptake of TH (Darling *et al.*, 1993).

Although THs may exert their effects on a number of intracellular loci, their primary effect is on the transcriptional regulation of target genes. Early studies showed that the effects of THs at the genomic level are mediated by nuclear TRs, which are intimately associated with chromatin and bind TH with high affinity and specificity (Samuels *et al.*, 1988). Similar to steroid

hormones that also bind to nuclear receptors, TH enters the cell and proceeds to the nucleus. It then binds to TRs, which may already be prebound to TREs located in promoter regions of target genes. The formation of ligand bound TR complexes that are also bound to TREs is the critical first step in the positive or negative regulation of target genes and the subsequent regulation of protein synthesis. Given their abilities to bind ligand and DNA as well as their ability to regulate transcription, TRs can be regarded as ligand regulatable transcription factors (Larsen, 1995).

1.2.9.2. Multiple thyroid hormone receptor isoforms

In 1986, the laboratories of Vennstrom (Sap *et al.*, 1986) and Evans (Sasaki *et al.*, 1999) independently cloned cDNAs encoding two different TRs from embryonal chicken and human placental cDNA libraries. Several unexpected findings stemmed from their landmark work. First, they demonstrated by amino acid sequence comparison that TRs are the cellular homologs of the viral oncogene product v-*erbA*. Second, TRs were shown to have amino sequence homology with steroid hormone receptors. This was initially surprising since T3 and cholesterol derived steroids are structurally different ligands. However, in the ensuing years, TRs have been shown to belong to a large superfamily of nuclear hormone receptors that include the steroid, vitamin D, and retinoic acid receptors as well as “orphan” receptors for which there are no known ligand or function (Lazar, 1999).

TRs share a similar domain organization with other family members as they have a central DNA binding domain containing two “zinc fingers” and a carboxy terminal LBD. These initial studies also suggested that there were multiple TR isoforms. Subsequent work by many groups has confirmed that there are two major TR isoforms encoded on separate genes, designated as TR α and TR β, encoded on human chromosomes 17 and 3, respectively.

Moreover, these multiple isoforms exist in different species such as amphibians, chick, mouse, rat, and human. Both TR isoforms bind T3 and mediate TH regulated gene expression (Wood *et al.*, 1996). In mammalian species, TR α -1 and TR β -1s range from 400 to slightly over 500 amino acids in size and contain highly homologous DNA binding domains and LBD (Lazar and Chin, 1990).

In addition to two separate genes that encode TRs, there is additional heterogeneity of TRs due to alternative splicing (Mitsuhashi *et al.*, 1988). Alternative splicing of the initial RNA transcript of the TR α gene generates two mature mRNAs that each encodes two proteins: TR α -1 and c-*erb A* α -2. In the rat, these proteins are identical from amino acid residues 1–370, but their respective sequences diverge markedly thereafter. Consequently, c-*erb A* α -2 cannot bind T3 because it contains a 122 amino acid carboxy terminus that replaces a region in TR α -1 that is critical for TH binding. Additionally, c-*erb A* α -2 binds TREs weakly but cannot transactivate TH responsive genes. Thus TR α -1, but not c-*erb A* α -2, is an authentic TR. Indeed, c-*erb A* α -2 may act as an inhibitor of TH action possibly by competing for binding to TREs (Koenig *et al.*, 1989). The TR α -1 and c-*erb A* α -2 system, then, represents one of the first examples in which multiple mRNAs generated by alternative splicing encode proteins that may be antagonistic to each other. Mitsuhashi *et al.* 1988 also have described a second TR α variant, c-*erb A* α -2V, in which the first 39 amino acids of the divergent sequence are missing. Its function currently is unknown. Yet another interesting feature of the TR α gene is the employment of the opposite strand to encode a gene product, rev-*erbA*. Rev*erbA* mRNA contains a 269-nucleotide stretch which is complementary to the c-*erbA* α -2 mRNA due to its transcription from the DNA strand opposite of that used to generate TR α -1 and c-*erbA* α -2. This protein also is a member of the nuclear hormone receptor superfamily. It is

expressed in adipocytes and muscle cells, and can bind to TREs and retinoic acid response elements (RAREs) and repress gene transcription (Zamir *et al.*, 1997). However, rev-*erbA* should be considered an orphan receptor since its cognate ligand and function are not known. One potential role for rev-*erbA* may be to regulate the splicing that generates c-*erbA* α -2 as increased levels of rev-*erbA* mRNA correlate with increased TR α -1 mRNA relative to c-*erbA* α -2 (Monden *et al.*, 1997).

There are also two TRs derived from the TR b gene (Hodin *et al.*, 1989). This gene contains two promoter regions each of which is vital for the transcription of an mRNA coding for a distinctive protein. By the use of alternate promoter choice, one or both of the coding mRNAs are generated. The resultant TR b isoforms are designated as TR β -1 and TR β -2. The amino acid sequences of the DNA binding, hinge region, and LBDs of these two TRβs are identical, but the amino terminal regions bear no homology. However, the evidence for isoform specific functions has been scant since most cotransfection experiments have failed to show important functional differences. Nonetheless, recent studies have suggested that TR β -1 may exhibit isoform specific regulation of the TRH and myelin basic protein genes, and TR β -2 may play an important role in the regulation of the GH and TSH b gene expression in the pituitary. Comparison of amino acid homologies and their functional properties are shown in figure (1.5) (Abel *et al.*, 1999).

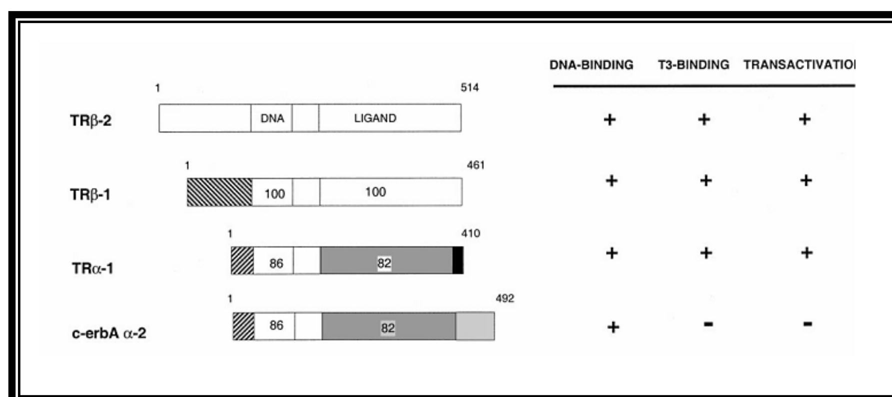


Figure (1.5). Comparison of amino acid homologies and their functional properties among TR isoforms. The length of receptors is indicated just above the receptor diagrams, and the percent amino acid homology with TRb-2 is included in the receptor diagrams (Abel *et al.*, 1999).

1.2.9.3. Thyroid hormone receptor functional domains

Mutational analyses of TRs and comparison with other members of the nuclear hormone receptor superfamily have yielded much information on the structural features of TRs. All TRs have a similar domain organization as that found in all nuclear hormone receptors: an amino-terminal A/B domain, a central DNA-binding domain containing two “zinc fingers” (DBD), a hinge region containing the nuclear localization signal, and a carboxy terminal LBD figure (1.6). It should be noted that each of these domains and regions may subserve multiple functions, and thus their names may only reflect the first function ascribed to them (Lazar, 1993).

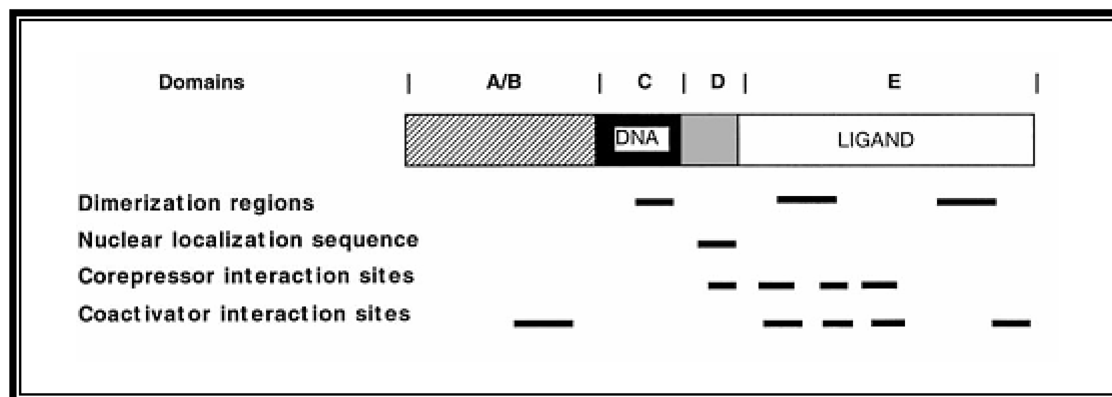


Figure (1.6). General organization of major TR domains and functional subregions (Lazar, 1993).

1.2.9.4. DNA-Binding Domain

The DBD is located in the central portion of TR and has two zinc fingers, each composed of four cysteines coordinated with a zinc ion. The integrity of each zinc finger is critical, as deletion of zinc fingers or amino acid substitution of these cysteine residues abrogates DNA binding and transcriptional activity of steroid hormone receptors and TRs (Nagaya *et al.*, 1992). Within the first zinc finger, there is a “P box,” comprised of amino acids located between and just distal to the third and fourth cysteines, which is similar to that of estrogen recetors (ERs), retinoic acid receptors (RARs), retinoid X receptors (RXRs), and vitamin D receptors (VDRs) (Danielson *et al.*, 1989). This critical region has been shown to be important in sequence specific recognition of hormone response elements by different members of the nuclear hormone superfamily and contacts nucleic acids and phosphate groups within the major groove of the TRE (Nelson *et al.*, 1995). Additionally, there are other important contact points within the minor groove of the TRE just downstream from the second zinc finger (A-box region). Also, as discussed below, TRs can heterodimerize with RXRs and can bind to TREs that are arranged as direct repeats separated by a four nucleotide gap. These TR/RXR heterodimers bind to TREs with a 5’ to 3’ polarity with TR in the

downstream position (Perlmann *et al.*, 1993). The ability to heterodimerize with RXR is critical for TR binding to the asymmetric TRE, as dimerization contacts stabilize the DNA binding and determine the spacing between half-sites. Within the DBD, there are dimerization interfaces in the TR just upstream of the first zinc finger, within the first zinc finger, and in a subregion distal to the second zinc finger (T box). The RXR dimerization surfaces are located in the second zinc finger including an arginine located in the D box, a region which previously has been shown to be important for distinguishing spacing between half-sites of hormone response elements. The DNA binding domain is shown in figure (1.7) (Luisi *et al.*, 1994).

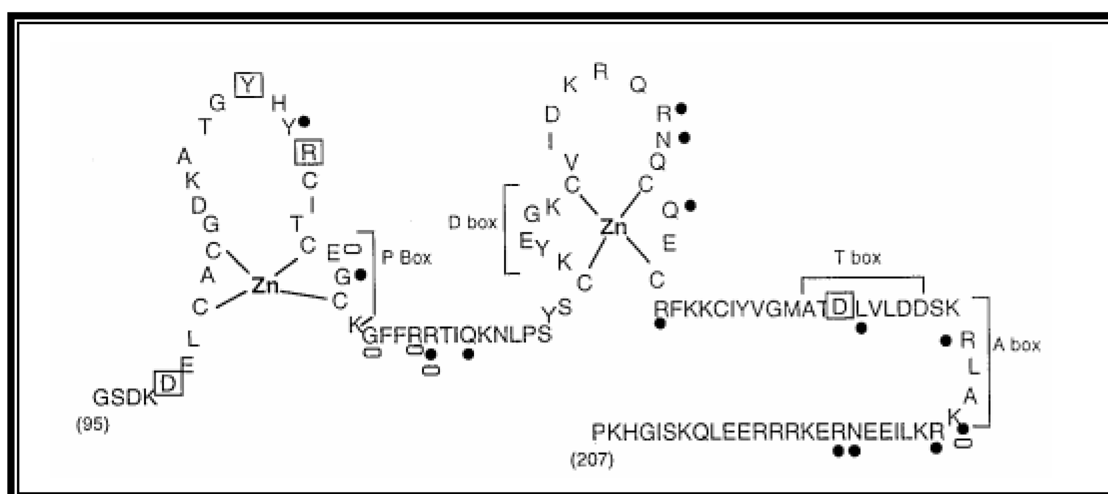


Figure (1.7). DNA-binding domains of human TRb. Schematic drawing of the two zinc fingers of human TRb and the various subregions within the DNA-binding domains. Squares, TR/RXR heterodimerization contacts; ovals, direct base contacts; solid circles, direct phosphate contacts (Luisi *et al.*, 1994).

1.2.9.5. Ligand-Binding Domain

The LBD not only is necessary for TH binding but also plays critical roles for dimerization, transactivation, and basal repression by unliganded TR. The recent solutions of the crystal structures of the liganded TR α-1,

unliganded RXRa, and RAR g LBDs have greatly aided the understanding of its role on these functions and the attendant conformational changes that occur when T3 binds to the receptor (Renaud *et al.*, 1995). Ligand is buried deep within a hydrophobic pocket in the LBD formed by discontinuous stretches that span almost the entire LBD. In particular, the most carboxy terminal region (Helix 12) contributes its hydrophobic surface as part of the ligand binding cavity. The hydrophobic residues face inward, whereas the conserved glutamate faces outward. The cavity also is bounded by hydrophobic surfaces from helices 3, 4, and 5. Although the crystal structure of unliganded TR has yet to be solved, the crystal structure of unliganded RXR a shows that helix 12 projects into the solvent. Thus it is likely that helix 12 undergoes major conformational changes upon ligand binding, from a more open conformation to a closed one, which has been likened to a "mouse trap" mechanism. In an analogous manner, estrogen bound LBD shows a similar structure as liganded TR LBD with helix 12 facing inward (Brzozowski *et al.*, 1997). However, helix 12 of raloxifene bound ER LBD is in a different position, lying in a groove between helices 3 and 5. Thus the relative positions of helix 12 and the boundary helices may determine whether coactivators can interact with TR. Indeed, studies using TR-LBD mutants based on the TR-LBD crystal structure have confirmed these regions for interacting with the coactivator GRIP-1. The X- ray crystallography of TR-LBD is given in figure (1.8) (Darimont *et al.*, 1998).

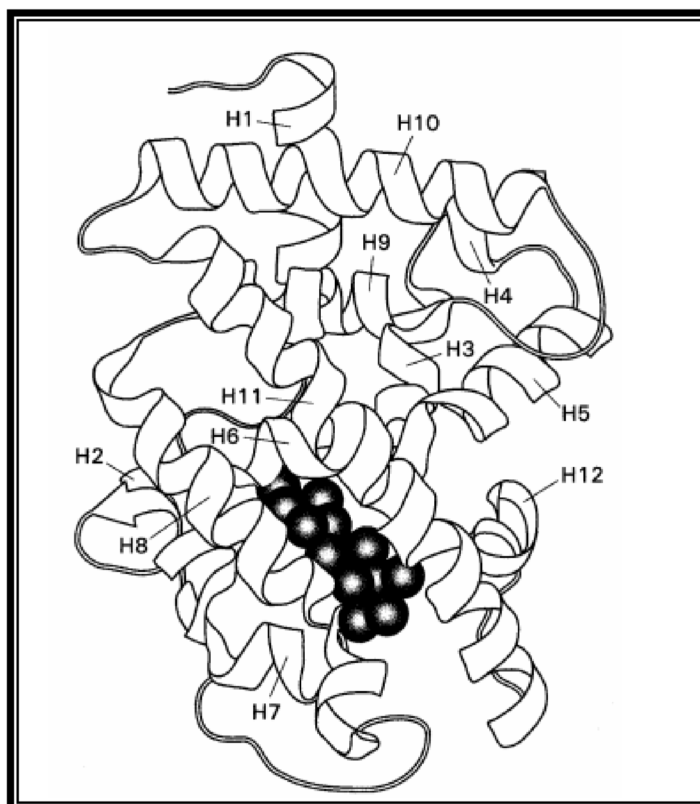


Figure (1.8). TRα-1 ligand-binding domain crystal structure. Dark mass represents ligand. α-Helices are indicated (Darimont *et al.*, 1998).

TRα and TRβ-1 isoforms can bind T3 and various TH analogs with subtle differences in affinity. TR α binds T3 with slightly higher affinity than TR β-1 (Schueler *et al.*, 1990). Triac (3,5,39 triiodothyroacetic acid) binds TR α-1 with similar affinity as T3 and binds TRb-1 with two to threefold higher affinity than T3. Several novel thyromimetics have been designed which bind TR β-1 (GC-1 and CGS 23425) with 10 to 50 fold higher affinity (Chiellini *et al.*, 1998). The transcriptional activities of these isoform specific compounds parallel their binding affinities and may offer novel therapeutic treatments of diseases such as hypercholesterolemia while sparing the heart (which contains mostly TR α) from side effects. The crystal structures of hTRα and hTRβ LBDs have been solved and may provide important information for designing even more selective thyromimetics in the future (Ribeiro *et al.*, 1998).

The LBD also is involved in several other important receptor functions. Scattered throughout the LBD are discontinuous heptad repeats that have been proposed to form hydrophobic interfaces for TR homo and heterodimerization. Mutations in the ninth heptad repeat region have selectively decreased TR homo and heterodimer formation, suggesting that there may be different subregions of the LBD that are important for TR dimerization. Indeed, the TR α-1 LBD crystal structure demonstrates that there is a hydrophobic surface in the ninth heptad repeat region that could serve as a potential dimerization interface. A natural TRβ mutation from a patient with resistance to TH at amino acid 316 also displayed decreased homodimer formation, suggesting that additional regions of the LBD may be important for dimerization (Yen *et al.*, 1995). The relative contributions to dimerization by the LBD and DBD interfaces may depend on the receptor. A recent study suggests that a region that contains the ninth heptad region called the "I box" may be important for RAR heterodimerization with RXR in solution and for binding to direct repeats of variable spacing. On the other hand, the DBD dimerization interface may be important for dictating binding to direct repeats of a specific spacing (in this case, a 5-nucleotide gap). Recent studies suggest that the ninth heptad region may be more important for heterodimerization of TR α-1, whereas the DBD may play the dominant role for c-*erbA* α-2 because it lacks a complete ninth heptad region due to alternative splicing (Reginato *et al.*, 1996).

Baniahmad *et al.* used a GAL4-fusion system to identify at least three transcriptional activation regions in the LBD and designated them as τ 2, τ 3, and τ4 (Baniahmad *et al.*, 1995). Uppalari and Towle have also used a yeast transfection system to describe several activation regions in TR β-1 LBD as well as in the hinge region (Uppalari and Towle, 1995). In particular, τ 4 located near the carboxy terminus has high homology with LBD sequences

found in other nuclear hormone receptors previously designated as the activation function-2 (AF-2) domain .

1.2.10. Diagnosis of thyroid diseases

Most would accept that the principal aim of thyroid testing in critically or acutely ill subjects is to identify, with maximum possible sensitivity and specificity, any abnormality that could be positively influenced by therapy (Heid *et al*., 1996). Whereas failure to identify previously unrecognized thyroid dysfunction is likely to have an adverse effect on outcome, the detection of transiently abnormal values that result from non thyroidal illness or medications is unlikely to lead to an important therapeutic intervention (Kaptein, 1994). The well-known unreliability of the clinical features of thyroid disease in the presence of another illness and the potentially serious consequences of missing associated thyroid dysfunction during critical illness would seem to be compelling reasons for widespread laboratory testing. However, if this approach is adopted and standard reference intervals are used, individual tests will show a high prevalence of apparently abnormal results, but the relation between thyrotropin (TSH) and thyroxin (T4) is often nondiagnostic. Many of these nonspecific changes (Kaptein, 1994) are related to sampling under non steady state conditions (Hamblin *et al.*, 1986), due to the large differences between the half lives of T4 (1 week) and TSH (1 h), leading to discordant or uninterpretable relations between these indicators. Such nonspecific abnormalities create uncertainty and generate further diagnostic activity of doubtful yield. The general hormonal response to critical illness involves activation of the pituitary adrenal axis, and inhibition of the pituitary-thyroid and pituitary- gonadal axes (Reichlin, 1993). These

normal responses distort standard reference intervals. In the case of the pituitary-thyroid axis, evaluation is further complicated by changes in nutrition and major effects of medications (Ohta *et al*., 1991).

1.2.10.1. Thyroid hormones and TSH changes during illness

Lowering of serum triiodothyronine (T3), generally regarded as a valuable calorie sparing economy, at least in the short term, is a normal acute response to illness (Docter *et al*., 1993). The evidence that these subnormal T3 concentrations lead to diminished thyroid hormone action rests mainly on studies during caloric restriction, which show lowered oxygen consumption and changes in hepatic enzyme and protein synthesis that resemble those of hypothyroidism (Carr *et al*., 1983). Supplementary T4 therapy does not overcome the block in T4-T3 conversion that occurs in severe illness (Brent and Hershman, 1986).

Although such an adaptive response may confer short-term calorie-sparing benefit, it is uncertain whether chronic lowering of T3 is beneficial or harmful. Since limited studies suggest no direct benefit from replacement therapy (Becker *et al*., 1982), there is no basis at present for routine documentation of low T3 states during illness. Immunoreactive TSH also tends to decrease in severe illness, a change often attributed in part to altered nutrition (Hughes *et al*., 1984), glucocorticoids (Re *et al*., 1976) (either endogenous or exogenous), or dopamine therapy (Kaptein *et al*., 1980), which, even in nonpressor doses, produces profound rapidly reversible suppression of serum TSH (Van den Berghe *et al*., 1994).

Lowered TSH leads to decreased T4 production; findings can then suggest hypothyroidism of central origin. With recovery, TSH concentrations become transiently increased, and if T4 is still subnormal (because of its much longer half-life), single samples may suggest primary hypothyroidism.

Changes may also result from alterations in protein binding of T4, effects of medications, or methodological quirks of particular free T4 estimates. Some currently available free T4 methods lack specificity for true thyroid dysfunction in the presence of associated illness (Stockigt *et al.*, 1994).

The effect of heparin on apparent free T4 is an important *in vitro* artifact. Although heparin has a low direct affinity for T4 binding sites in serum, it has long been known that measured free T4 is often increased in heparin treated hospitalized patients demonstrated that this effect is due to *in vitro* generation of nonesterified fatty acids as a result of heparin-induced increase in lipase activity. Any free T4 value from a heparin-treated subject is dubious because of possible *in vitro* generation of fatty acids during sample storage, processing, or assay incubation (Mendel *et al.*, 1987).

Numerous studies have even gone to the extreme of studying TSH responses to thyrotropin releasing hormone in an attempt to refine prognostic prediction this body of information has not so far been shown to improve management decisions for individual critically ill patients (Sumita *et al.*, 1994).

1.2.10.2. TSH/T4 relation

Because free T4 and TSH are in feedback relation, diseases of the thyroid or pituitary show predictable concordant relations between these indicators, either inverse in the case of primary thyroid dysfunction, or direct in the case of pituitary dependent abnormalities (Fisher, 1997). Subject to several assumptions, e.g., steady state and adequate assay sensitivity, this relation has high diagnostic sensitivity and specificity. However, in critical illness a high proportion of alterations reflect discordant nondiagnostic changes when TSH and T4 are considered together (Stockigt, 1991).

1.2.10.3. Free T4 assay

For preanalytical and analytical reasons it is difficult to establish true free T4 values in critically ill subjects. Various free T4 assays may give low, normal, or high values in euthyroid patients with nonthyroidal illness, due to effects of medications or method dependent artifacts. In general, two step free T4 methods that isolate a fraction of the T4 pool from binding proteins before the assay step are better validated and theoretically more sound, but tend to give high free T4 values in critical illness (Ekins, 1992). These methods are particularly vulnerable to the *in vitro* heparin effect, which falsely raises free T4, or to the influence of sample dilution, which attenuates the effect of binding competitors, thereby failing to show the true extent of the competitor-induced rise in free T4. With two-step free T4 methods, supranormal results are much more likely to be due to factors that inhibit protein binding of T4 than to true hyperthyroidism (Stockigt *et al.*, 1994).

In contrast, many of the one step or analog methods that attempt to measure free T4 in the presence of binding proteins are subject to analytical artifacts, due largely to protein differences between sample and calibrators. In critical illness such methods often give falsely low free T4 estimates. When hyperthyroidism coexists with another severe illness, falsely normal or low free T4 values may occur with abbreviated one-step free T4 estimates and also by equilibrium dialysis (Lum *et al.*, 1983).

1.2.10.4. TSH assay

The concentration of TSH may be low, normal, or high in euthyroid patients with nonthyroidal illness. In contrast to free T4 variations, these deviations generally indicate true physiological responses or the effects of medications, rather than methodological artifacts (Thorpe-Beeston *et al.*, 1991). Abnormalities often reflect non steady state conditions, because of the

short half-life of TSH. In considering the distinction between the subnormal TSH values of illness and those of hyperthyroidism, there is a marked difference in specificity and positive predictive value between second-generation and third-generation TSH assays. Thirdgeneration assays, based on a functional sensitivity limit of 0.0 1-0.02 IU/L, are generally well able to separate the suppressed TSH values of hyperthyroidism from the values seen in nonthyroidal illness. In an analysis of samples from hospitalized subjects preselected on the basis of TSH values <0.1 mIU/L, Spencer *et al.* (Spencer *et al.*, 1990) found that almost all hyperthyroid subjects had TSH values <0.01 mIU/L, whereas most critically ill subjects with low TSH had values between 0.01 and 0.1 mIU/L. Nevertheless, Franklyn *et al.* found that as many as 4% of patients with nonthyroidal illness had values below the functional sensitivity of a third generation TSH assay (Franklyn *et al.*, 1994).
Hence, a diagnosis of hyperthyroidism should be based on the relation between free T4 and TSH, rather than the latter alone (Bianco *et al*., 2002).

In thyroid, the fine needle aspiration biopsy (FNAB) technique is recommended (Ryu *et al.*, 1998). Aspiration of material for cytological examination through a fine bore needle (approximately 23-25 gauge) can be performed from virtually any site.

Tissue is obtained following puncture of the lesion and careful movement of the tip of the needle within the lesion; gentle suction may be applied. For superficial palpable lesions the needle may be moved back and forth with a fanning action providing wider sampling and cell dislodgement. In most cases, the aspirated material remains in the needle (unless cyst fluid present), and is then expressed onto pre-labelled slides and smeared out rapidly (Spencer *et al.*, 1987). Care needs to be followed when expressing material onto slides in patients with suspected infection e.g. tuberculosis. This should be undertaken in a controlled biohazard cabinet. Both air-dried and 70-

95% ethanol or commercial spray wet fixed smears are preferred, but may depend on the tissue being sampled and on the preference of the reporting pathologist (Escobar-Morreale *et al.*, 2005).

If there is clinical suspicion of lymphoma or reactive lymph node, material may also be sent for cell surface markers. Fine needle aspiration biopsy is valuable in the diagnosis of superficial and readily accessible lesions eg, skin and breast (Escobar-Morreale *et al.*, 1996). With organ imaging techniques, deeply situated organs can be sampled. The technique is used mainly in the primary diagnosis of neoplasia and for the assessment of disease recurrence. Infections and benign lesions are also readily evaluated. Some neoplasms and well differentiated lesions may result in an abnormal but equivocal diagnosis requiring histological confirmation (eg, follicular neoplasms of thyroid, well differentiated hepatocellular carcinoma) (Wakim *et al.*, 1993).

Thyroid tumors are often diagnosed by FNAB as well as by ultrasonography. Cytological examination of FNAB by a skillful pathologist who is an expert in thyroid tumors provides the most reliable information for the diagnosis of thyroid neoplasms (Hamburger, 1994). A new method of preoperative molecular-based diagnosis of thyroid carcinomas may also be used this technique, ABRP, allows to perform cytological and molecular-based diagnoses simultaneously by extracting RNA from leftover cells within the needle used for FNAB (Takano *et al.*, 1997). Thus, ABRP provides both RNA information and a cytological diagnosis without further invasion to the patient. onfFN mRNA is abundantly expressed only in papillary and anaplastic carcinomas (Ryu *et al.*, 1998). Thus, by using ABRP to measure the relative expression level of onfFN mRNA in FNABs, these carcinomas

may be accurately diagnosed preoperatively. An automated system that measures the quantity of a small amount of mRNA by monitoring the amplification rate in each PCR cycle (real-time quantitative RT-PCR) was developed (Heid *et al.*, 1996). By using this system in measuring onfFN mRNA, a fully automated system used in clinical laboratories may be established. Because thyroglobulin mRNA is expressed in most of the differentiated thyroid tumors of epithelial ancestry and its expression was reportedly decreased in some of the thyroid carcinomas (Brabant *et al.*, 1991), this gene is considered to be one of the most important for the internal control of thyroid cells. Thus, by measuring the ratio of onfFN and thyroglobulin mRNA, the researcher may be able to distinguish thyroid carcinomas from benign tumors using only a small number of tumor cells. In light of the above, the researcher calculated the relative expression level of onfFN to thyroglobulin mRNA by real-time quantitative RT-PCR in RNAs extracted from tumor tissues and FNABs (Takano *et al.*, 1997).

Reports have demonstrated that reverse transcription RT – PCR can be used to detect circulating cancer cells in the peripheral blood of patients with malignancies such as differentiated thyroid cancer, melanoma, and adenocarcinomas of the prostate (Heid *et al.*, 1996) and breast (Ohta *et al.*, 1991).

The researchers have reported the detection of circulating thyroid cells in whole blood samples of individuals with no known thyroid disease, using a qualitative RT-PCR assay (Hayashi *et al.*, 1999). Furthermore, it was possible to recover cells expressing TSH receptor and Tg protein from the peripheral blood of these same healthy subjects. This surprising finding of circulating thyroid cells in healthy subjects may limit the usefulness of the qualitative RT-PCR assay technique in the diagnosis and follow up of thyroid cancer patients and in the management of patients with benign thyroid diseases.

Therefore, the primary objective is to study, develop and optimize a quantitative RT-PCR assay for Tg mRNA that would circumvent the limitations of the qualitative assay. Moreover, quantification of the circulating Tg mRNA would allow for monitoring response to therapy in patients with metastases (Camargo *et al*., 2001).

To facilitate the collection of samples in a busy thyroid clinic, the researchers second objective was to optimize a simplified sample collection and processing technique that would require minimal support from the research laboratory. The researcher believe the combination of a simplified sample collection and extraction technique with a reliable quantitative RT – PCR assay will improve the clinical usefulness of the Tg mRNA assay (Takano *et al.*, 1999).

2. Materials and Methods

2.1. Materials

2.1.1. Apparatus

Various apparatus used in this work are shown below

Apparatus	Company / country
Analytical balance	OHAUS / France
Autoclave	Tomy / Japan
Bench centrifuge	VEB / Germany
Cooled centrifuge	Sanyo / Japan
Deionizer	CFL / Germany
Electrophoresis equipment	Consort / Belgium
Heater – magnetic stirrer	Stuart / England
Incubator	Sanyo / Japan
Laminar flow hood	Heraeus / Germany
Light microscope	Olympus / Japan
Microcentrifuge	Sigma / U,S.A
Microscope camera	Olympus / Japan
Microtom	Lieca / Sweden
Minividas	Bio mearex / France
Oven	Sanyo / Japan
PCR master cycler gradient	Techne / U.K.
pH meter	WTW/ Germany
Sensitive balance	Mettlev / Switzerland

Spectrophotometer	Shimadzu / Japan
U.V. – transilliuminator and Camera	Flowgen / U.K.
Vortex	Scientific industries / U.S.A.
Water bath	Memmert / Germany

2.1.2. chemicals

Pharmacia / Sweden		
Ethanol	Eosin	
Sigma / U.S.A.		
Canada balsam	Diethyl pyrocarbonate (DEPC)	EDTA
Ethidium bromide	Hematoxyline	L – glutamine
Mycostatine	3-(N- morpholino) propan sulforic acid (MOPS)	Pencillin G
RPMI – 1640 (media)	Streptomycin	Sodium bicarbonate
Tris – HCL		
Fluka / Switzerland		
Boric acid	Chloroform	Formaldehyde
Isoproponal	Sodium hydroxide	Tris – base
Trichloroacetic acid	Xylol	
BDH / England		
Bromophenol blue	Bromocresol purple	Glycerol

Iodine	Sodium – hydrosulphate	Sodium acetate
Merck / Germany		
Formamide	Hydrochloric acid	Methanol

2.1.3. kits

Kits	Company	Countary	Cat – NO.
FSH	Bio - Merieux	France	06268I
LH	Bio - Merieux	France	06267K
Testosterone	Bio - Merieux	France	09345B
Progesterone	Bio - Merieux	France	08593C
T3	Bio - Merieux	France	067626
T4	Bio - Merieux	France	09345D
TSH	Bio - Merieux	France	06268J
isolation of RNA RNX^{TM}(- Plus)	CinnaGen	Iran	RN7713C
RNA marker	Sigma	U.S.A	R-7644
Cyclin A primer set	Sigma	U.S.A	P-8092
Hexanucleotide primers	Sigma	U.S.A	H-0268
M-MLV Revers transcriptase	Sigma	U.S.A	M-1302
AMV Revers transcriptase	Sigma	U.S.A	R-9376

10X PCR buffer	Sigma	U.S.A	P-2192
dNTPS mixture	Sigma	U.S.A	C-1141
Taq polymerase	Sigma	U.S.A	D-1806
Ribonuclease inhibitor	Sigma	U.S.A	R-4380
Lambda DNA EcoR I Hind III digest	Sigma	U.S.A	D-3398
DNA Ladder (123bp)	Sigma	U.S.A	D-5042

2.1.4. Blood samples collection from subjects

2.1.4.1. Collection of blood from hypothyroidism, hyperthyroidism, thyroid cancer patients, and control (healthy) subjects

Blood samples were collected from 100 patients with hypothyroidism, 100 patients with hyperthyroidism, 15 patients with thyroid cancer, and 20 healthy persons as control. Their ages ranged between (20-70) years. A volume 5ml of peripheral blood was collected, by vein puncture into sterile tube for 30 min. to allow blood clotting in test tubes. The serum was separated from blood cells by centrifugation at 2000 rpm for 10 min. at room temperature, then the serum was stored at -20°C until used. These samples were obtained from AL- Yarmok Teaching Hospital (Baghdad), AL-Kadhymia Teaching Hospital (Baghdad), St. Rafael Hospital (Baghdad), Marjan General Surgery Hospital (Babylon), AL-Zahra Hospital (Najaf), AL-Basra General Hospital (Basra), AI- Ramadi General Hospital (Ramadi), AL-Shifaa General Hospital (Mousel), and Karkuk General Hospital (Karkuk).

2.1.4.2. Collection of tumor tissue samples

Tumor tissue samples from 15 patients were collected into sterile tube containing a transport media (section 2.6). The ages of patients ranged between (40-70) years. These samples were obtained from the operation theater of AL- Yarmok Teaching Hospital (Baghdad), AL- Kadhymia Teaching hospital (Baghdad), St. Rafael hospital (Baghdad), Marjan /general surgery hospital (Babylon), AL-Zahra hospital (Najaf), AL-Basra general hospital (Basra), AI- Ramadi general hospital (Ramadi), AL- Shifaa general hospital (Mousel), and Karkuk general hospital (Karkuk).

2.1.5. Buffers and solutions

2.1.5.1. Sterile DEPC- treated solution

A volume of 0.1 % DEPC was added to water, mixed over night, and then autoclaved for 20 min to destroy DEPC by hydrolysis.

2.1.5.2. NaOH - EDTA washing solution

The solution was prepared from 0.1 N NaOH , and 1mM EDTA

2.1.5.3. Thyroid Histopathological Examination (Bancroft and Stevens, 1992)

The following buffers and solutions were used for histopathological examination.

2.1.5.3.1. Sodium bicarbonate solution (NaHCO3)

It was prepared by dissolving 4.4 gm of (NaHCO3) in 100 ml of sterile D.W. this solution was kept at $4^{o}C$ until use.

2.1.5.3.2. Acid alcohol

Ethanol 70%	400 ml
HCL 1%	4 ml

2.1.5.4. Hormones kit

2.1.5.4.1. Fertility hormones (Butt and Blunt, 1988)

The following kits were used for fertility hormones.

2.1.5.4.1.1.FSH

FSH levels were measured by the Bio – Merieux kit Sa. 69230 marcy I' Etoile – France, as instructed by the manufacturer.

2.1.5.4.1.2. LH

LH levels were measured by the Bio – Merieux kit Sa. 69230 marcy I' Etoile – France , as instructed by the manufacturer.

2.1.5.4.1.3. Testosterone

Testosterone levels were measured by the Bio – Merieux kit Sa. 69230 marcy I' Etoile – France , as instructed by the manufacturer.

2.1.5.4.1.4. Progesterone

Progesterone levels were measured by the Bio – Merieux kit Sa. 69230 marcy I' Etoile – France, as instructed by the manufacturer.

2.1.5.4.2. Thyroid hormones (Biersact and Hotze, 1991)

The following kits were used for thyroid hormones.

2.1.5.4.2.1. T3

T3 levels were measured by the Bio – Merieux kit Sa. 69280 marcy I' Etoile – France, as instructed by the manufacturer.

2.1.5.4.2.2. T4

T4 levels were measured by the Bio – Merieux kit Sa. 69280 marcy I' Etoile – France, as instructed by the manufacturer.

2.1.5.4.2.3. TSH

TSH levels were measured by the Bio – Merieux kit Sa. 69280 marcy I' Etoile – France, as instructed by the manufacturer.

2.1.5.5. RNA isolation system kit (Chomczynski and Sacchi, 1987)

RNX^{TM} total RNA isolation system kit from CinnaGen Iran (RN7713C) , ready – to – use that contain the RNX^{TM} (- plus) : 1 bottle 25 ml containing solution.

2.1.5.6. Determination of RNA yield and purity (Sambrook and Russel , 2001)

2.1.5.6.1. Gel electrophoresis analysis of RNA

The following buffers and solutions were used for gel electrophoresis analysis of RNA.

2.1.5.6.1.1. 10X MOPS electrophoresis buffer

MOPS pH 7.0	0.2 M
Sodium acetate	20 mM
EDTA pH 8.0	10 mM

Three solutions were prepared separately for MOPS electrophoresis buffer. The first solution was prepared by dissolving 41.8 gm of MOPS in 700ml DEPC – treated water. The pH was adjusted to 7.0 by addition 2N of NaOH. The second solution was prepared from 1M sodium acetate. The third solution was composed of 0.5M EDTA with pH 8.0. The final buffer was made by adding 20ml from the second solution and 20ml of the third solution to the first one, and the volume was completed to 1000ml. The final buffer was sterilized by filtration through a 0.22µm Millipore filter then kept at room temperature.

2.1.5.6.1.2. RNA sample buffer

RNA sample buffer was prepared by mixing 10 ml formamide , 3.5 ml (37%) formaldehyde and 2 ml 10X MOPS buffer . This buffer was stored at – 20 ^{o}C.

2.1.5.6.1.3. RNA loading buffer

RNA loading buffer was prepared by mixing 50% glycerol , 1mM EDTA and 0.4% Bromophenol blue.

2.1.5.6.1.4. RNA Marker

RNA Marker range 0.28 – 6.6 KB, Ready – to – use, it was used for agarose (formaldehyde) gel electrophoresis.

2.1.5.7. Standardization one step – RT – PCR reaction

The tests were carried out in accordance with manufacture's instruction and standardization was carried out according to; Gerard, *et al*., 1993, and Sambrook and Russel , 2001.

2.1.5.7.1. Hexanucleotide containing all possible deoxynucleotide

Hexanucleotide primers (primers hexamers 5́ – OH dn_6) are mixture of random synthetic 5́ – hydroxyl hexanucleotides or sequences.
Random primers are commonly used in priming single stranded DNA templates to produce sites for the initiation of DNA synthesis by DNA polymerase.

2.1.5.7.2. Cyclin A primer set

Cyclin A PCR primer set includes 2 synthetic oligonucleotides to be used in PCR reaction for the detection of cyclin A mRNA.

The " sense " oligonucleotide represents the sequence between nucleotides 458-479 and the " antisense " oligonucleotide represents the sequence between nucleotides 737-758 on the human cyclin A mRNA coding reactions.

These primers are detection of cyclin A mRNA and its sequence is (5́ to 3́) as follows:-

- Cyclin A sense primer : 5́ – GTCACCACATACTATGGACATG – 3́
- Cyclin A antisense primer: 5́ -AAGTTTTCCTCTCAGCACTGAC-3́

2.1.5.7.3. Reverse Transcriptase

In this work two reverse transcriptase were used.

2.1.5.7.3.1. AMV Reverse Transcriptase

AMV (Avian Myeloblastosis Virus) reverse transcriptase catalyzes the polymerization of DNA using template DNA, RNA or RNA: DNA hybrids. It requires a primer (DNA primers are more efficient then RNA primers) as well as Mg^{+2} or Mn^{+2}.

2.1.5.7.3.2. M-MLV Reverse Transcriptase

M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase is a DNA polymerase that uses single – stranded RNA , DNA or RNA:DNA hybrid (using a primer) to synthesize a complementary DNA strand . M-MLV is used for the preparation of cDNA libraries of for first strand cDNA synthesis for use in RT-PCR reactions.

2.1.5.7.4. (10X) PCR buffer

This buffer was prepared from 100 mM Tris-HCL (pH 8.3), 500mM KCL, 15 mM MgCL2, and 0.01% gelatin.

2.1.5.7.5. dNTPs mixture

It was a mixtur of 100 mM of dATP, dCTP, dGTP and dTTP.

2.1.5.7.6. Taq polymerase

Taq polymerase containing 5.0 units/µl was used in this study.

2.1.5.7.7. Ribonuclease inhibitor

A vial containing 900 µl equivalent to 500 units.

2.1.5.8. DNA gel electrophoresis (Maniatis *et al.*, 1982)

The following buffers and solutions were used for electrophoresis of DNA.

2.1.5.8.1. (0.5 M) EDTA (pH 8.0)

A weight of 2.922 gm of EDTA was dissolved in 15 ml deionized distilled water, and stirred vigorously on a meagnetic stirrer. The pH was adjusted to 8.0 using (10N) NaOH, the volume was completed to 20 ml using deionized distilled water then sterilized by autoclaving.

2.1.5.8.2. (5X) TBE buffer

Tris – base	54 gm
Boric acid	27.5 gm
EDTA (0.5M) pH 8.0	20 ml
deionized distilled water	1000 ml

Then was sterilized by autoclaving

2.1.5.8.3. Loading buffer

Tris – base pH 8.0	0.025 M
Glycerol	50%.
Bromocresol purple	0.25%

2.1.5.8.4. Ethidium bromide stock solution

Ten milligram of ethidium bromide dye was dissolved in 1 ml of deionized distilled water stirrer on magnetic stirrer for several hours until the dye has dissolved.

The container wrapped with aluminum foil or kept in a dark bottle and stored at 4 ^{o}C

2.1.5.8.5. DNA marker

Two types of DNA markers were used in this work:

2.1.5.8.5.1. Lambda DNA EcoR I, Hind III digest

DNA marker ranged 125-21226 bp. Ready – to – use was more suitable as an electrophoresis marker for DNA contain 13 fragment supplied in storage buffer 10mM Tris-HCL pH 8.0 , 1mM EDTA.

2.1.5.8.5.2. DNA ladder (123 bp)

DNA ladder 123 – 4182 bp containing 34 fragments supplied in storage buffer 10mM Tris-HCL pH (7.5) , 0.1 mM EDTA , 5 mM NaCl.

2.1.6. Preparation of cell culture medium for transportation and washing of tumor tissue samples

The preparation of this medium was preformed using the method of (Freshney, 2000).

RPMI - 1640	10.4 gm
Penicillin G	10^{6} U/ml
Streptomycin	1 mg/ml
Mycostatine	250 U/ml
L- glutamine	0.5 gm
Sodium bicarbonate	1%

All these ingredients were completely dissolved in one liter of sterile D.W., the pH was adjusted to 7.2 and the medium was sterilized by filtration through Millipore filter (0.22µm) under aseptic conditions. Then 10 ml of the medium was transferred into sterile tubes and kept at -20°C until use.

2.2. Methods

2.2.1. Sterilization methods

- Autoclaving: Buffers and solutions were sterilized by pressure vessel (autoclave) at 121 ^{0}C and 15 bar for 15 minutes.
- Dry heat: A laboratory oven was used for glassware sterilization. Glassware was placed in the oven at 200 ^{0}C for 2 hours.
- Filtration: some media and solutions that cannot stand heat were sterilized by filtration through Millipore filters (0.45 and 0.22 µm diameter).

2.2.2. Histopathological Examination

This was performed by using method of (Bancroft and Stevens, 1992).

2.2.2.1. Preparation of histological section

Samples taken for histopathological examination were immediately preserved in 10% formaldehyde. After washing with tap water, they were fixed and processed with a set of increasing alcohols concentrations (70%, 80%, 90%, and 100%). Tissues were embedded in paraffin blocks and sectioned at (5-6)µm . All the sections were stained with hematoxyline and eosin stain, and histopathological changes were observed.

2.2.2.2. Hematoxyline – Eosin Method

Histological sections were placed in the following solutions and reagents as follows

1. Xylol for 5 min.
2. Absolute alcohol for 1 min.
3. Grade of series of ethanol (80%, 70%, 50%, and 35%) to be dried and then rinsed in D.W. for 1 min.
4. Iodine staining for 1 min.
5. Sodium – hydrosulphate to erase the iodine and turn the color of tissue to white and rinsed in tap water for few min.
6. Hematoxyline for (1-5) min., then rinsed in tap water to wipe off the excess dye.
7. Acid alcohol was added until the color turned to pink then rinsed in tap water.
8. Sodium – bicarbonate was added until the color turned to blue, then rinsed in tap water.
9. Eosin for 5 min, then rinsed in tap water.
10. Xylol for 3 min., then mounted in Canada balsam.

2.2.3. Determination of FSH, LH, Testosterone, and Progesterone levels (Butt and Blunt, 1988)

In FSH test the assay principle combines an enzyme immunoassay sandwich method with a final fluorescent detection (ELFA).

The solid phase receptacle (SPR), serves as the solid phase as well as the pipetting device for the assay. Reagents for the assay are ready – to – use and predispensed in the sealed reagent strips

All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times.

The sample is taken and transferred into the well containing alkaline phosphate- labeled anti – FSH (conjugate). The sample / conjugate mixture is cycled in and out of the SPR several times to increase the reaction speed. The antigen binds to antibodies coated on the SPR and to the conjugate forming a "sandwich ".

Unbound components are eliminated during the washing steps. During the final detection step, the substrate (4 – Methyl – Umbelliferyl phosphate) is cycled in and out of the SPR . The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescence of which is measured at 450nm. The intensity of the fluorescence is proportional to the concentration of antigen present in the sample.

At the end of the assay, results are automatically calculated by the VIDAS in relation to the calibration curve stored in memory, and then printed out.

All the previous steps were also applied LH, Testosterone, and Progesterone hormones in order to measure the concentration in all PCOS samples unit.

2.2.4. Determination of T3, T4 and TSH levels (Biersact and Hotze, 1991)

VIDAS T4 is an automated assay for the VIDAS system, which enables total thyroxin (T4) in human serum or plasma to be quantitatively measured. The assay principle combines the enzyme immunoassay method with a final fluorescent detection (ELFA).

All reaction steps are performed by the VIDAS instrument. The disposable solid phase receptacle serves both as a solid phase and pipetting device during the assay. Reagents for the assay are all contained in the sealed reagent strips.

All the previous steps were also applied to T3, and TSH hormones order to measure the concentration in all samples unit.

2.2.5. RNA extraction (Chomczynski and Sacchi, 1987)

1. 1ml of RNX solution was added to an eppendorf tube containing 50 100 mg of tissue (or 3-5 million cells, RNX may be added directly to the monolayer of cells in the culture flask) or 100µl 0f serum and homogenized, mixed well and incubated at room temperature for 5 min.
2. 200 µl of chloroform was added to the mixture,mixed well for 15 seconds by shaking, then Incubated in ice for 5 min.
3. The mixture was centrifuged at 12,000 rpm at 4°C for 15 min
4. The upper phase was transferred to RNase – free tube and an equal volume isoproponal was added.
5. The mixture was gently moved and incubated in ice for 15 min.
6. The mixture was centrifuged at 12,000 rpm at 4°C for 15 min.
7. The supernatant was discarded and 1ml of 75% ethanol was added, and vortexed to dissolve the pellet and centrifuged at 4°C for 8 min at 7500 rpm.
8. The supernatant was discarded and the pellets were left to dry at room temperature for few min.
9. Pellets were dissolved in 50 µl of 0.5% S.D.S or 1mM EDTA pH 7.0.

2.2.6. Determination of RNA yield and purity (Sambrook and Russel , 2001)

2.2.6.1. Gel electrophoresis analysis RNA

1. 1.0% agarose / formaldehyde was prepared by mixing: 72 ml sterile deionized, and 1 gm agarose .
2. The gel was heated, cooled to about 55°C, 10 ml of 10X MOPS electrophoresis buffer then added to18 ml formaldehyde (37%) and 5µl of (10mg/ml) ethidium bromide were mixed with the gel.
3. The gel was poured in clean glass mold (16X12X4 cm) and allowed to solidify at room temperature (25 °C).
4. RNA samples were prepared by mixing one part RNA sample with two parts RNA sample buffer to total volume 10-30µl .These samples were heated to 65 °C for 5 minutes and then cooled on ice for two minutes . 2 µl of RNA loading buffer was added.
5. The RNA samples were loaded, the gel was run at 5 v/cm. The electrophoresis was continued until bromophenol blue migrated at least 2/3 of length of the gel.
6. The gel was viewed under U.V. light using U.V. transilluminator.

2.2.6.2. Determination of RNA yield and purity spectrophotometrically

Solubilized RNA was diluted in respective solution as described below and spectrophotometrically analyzed , absorbance values were measured using different wave lengths (230, 260, and 280 nm), and a nuclease – free – water was used as blank, determination was carried out as follows :

1. A volume of 495 µl of nuclease free water was added into sterile ependorf tube, 5 µl of RNA sample was added and mixed gently by pipetting.
2. RNase – free cuvette was prepared by washing the cuvette briefly with washing solution section (2.5.2) followed by a brief rinsing with RNase free water.
3. Diluted sample was added into RNase – free cuvatte. Absorbency was read at wavelengths 230, 260, and 280.
4. RNA concentration was calculated according to the following formula : RNA concentration (µg/ µl) = [A260 nm X 40X(Dilution factor)] / 1000
5. RNA purity = A260/A280, RNA purity = A260/A230

The yield of total RNA extracts was determined spectrophotometrically at wave length 260 nm (A260) , such that one absorbance unit equal 40 µg/ml of single stranded RNA (Manchester, 1996) . The purity of RNA preparation was estimated by relate absorbance at 260 nm wave length (A260) to the absorbance at wave length 280nm (A280) and 230nm (A230) respectively[i.e.A260/A280 ratio and A260/A230 ratio] , the A260/A280 ratio a sensitive indicator of nucleic acid (RNA) contamination (Sambrook *et al*., 2001).

2.2.7. One step – RT – PCR reaction (Sambrook and Russel , 2001; Gerard , *et al.*, 1993)

RT – PCR reaction was processed as follows:

1. Sterile thin walled micro-centrifuge tubes and reaction tubes were placed on ice; the experimental reagents were thawed in ice and any unused portion was returned to the freezer.

2. In sterile 1.5 ml micro-centrifuge tubes a master – mix was prepared by combining appropriate multiples of each components of RT-PCR system on ice Tables (2.1 and 2.2) except RNA sample or control.

Table (2.1) .Optimal values of various steps in the standardization of RT-PCR reactions.

Test step	Optimal values	
	Volume	Concentration
Nuclease – free – water	29 µl	-
AMV reaction buffer	10 µl	1X
dNTP Mixture	1 µl	0.2 mM
Ribonuclase inhibitor (40 unit/ µl)	1 µl	1 unit/ µl
Hexanucleotide primer/ Cyclin A primer set	2 µl	1mM
10X PCR buffer	3 µl	1.5mM
AMV reverse transcriptase (20 unit/ µl)	1 µl	0.1 U/ µl
Taq DNA polymerase	1 µl	0.1 U/ µl
RNA sample (template)	2 µl	0.36 – 0.4 µg/ µl
Total volume	50 µl	

Table (2.2) .Optimal values of various steps in the standardization of RT-PCR reactions.

Test step	Optimal values	
	Volume	Concentration
Nuclease – free – water	27.5 µl	-
M-MLV reaction buffer	10 µl	1X
dNTP Mixture	1 µl	0.2 mM
Ribonucleas inhibitor (40 unit/ µl)	1 µl	1 unit/ µl
Hexanucleotide primer/ Cyclin A primer set	2 µl	1mM
10X PCR buffer	3 µl	1.5mM
M-MLV reverse transcriptase (200 unit/ µl)	2.5 µl	0.24 U/ µl
Taq DNA polymerase	1 µl	0.1 U/ µl
RNA sample (template)	2 µl	0.36 – 0.4 µg/ µl
Total volume	50 µl	

3. The components were vortexed for 30 seconds, each tube spun for 10 seconds in micro – centrifuge to collect the condensates and maintain the original volume.
4. Master mix was distributed to each labeled thin walled micro – centrifuge tube with specimen number or controls.
5. 2µl of RNA target or control was added to final volume 50 µl.

6. The tubes were incubated in thermal cycler using the protocol shown in table (2.3).
7. The PCR products were analyzed by agarose gel electrophoresis of 2% of the total reaction.

Table (2.3). Optimal protocol of RT - PCR reaction

No.	Thermal cycler protocol	No. of cycles	Temperature/ Time
A.	First strand cDNA synthesis :		
1.	Reverse transcription	1 cycle	45°C for 45 min.
2.	AMV / M-MLV RT inactivation and RNA/cDNA / primer denaturation	1 cycle	94°C for 2 min.
B.	Second strand cDNA synsthesis and PCR amplification		
1.	Denaturation	40 cycles	94°C for 30 second.
2.	Annealin		60°C for 1 min.
	Extension		68°C for 2 min.
C.	Final extension	1 cycle	68°C for 7 min.

2.2.8. DNA (RT – PCR) product gel electrophoresis (Maniatis *et al.* , 1982)

1. 2% agarose gel was prepared by mixing: 100ml of 0.5X TBE buffer and 2 gm agarose in glass bottle. A glass bottle was heated in a magnetic stirrer with heater until the agarose was dissolved.
2. This solution was cooled to 70°C, 5 µl ethidium bromide was added from stock solution and mixed thoroughly.
3. The clean glass mold (17X12X4 cm) was set on a horizontal section of the bench. The comb was set in position 0.5-1.0 mm above the

surface of mold so that a complete well was formed when agarose was added.

4. The warm agarose – solution was poured into the mold.
5. After the gel was completely set (20-30 min. at room temperature), the comp was carefully removed and the gel mounted in the electrophoresis tank which contain previously small amount of 1X TBE buffer.
6. A volume of 600ml of 1X TBE was added to cover the gel to depth about 1mm.
7. A volume of 10 µl of the sample of DNA (RT – PCR product) was mixed with 2 µl of loading buffer. The mixture was set slowly into the slots of the submerged gel using an automatic micropipette.
8. A volume of 5 µl of DNA marker was mixed with 1 µl of loading buffer. The mixture was set slowly into the slots of the submerged gel using an automatic micropipette.
9. The gel was subjected to electrophoresis at 80 volts until the bromophenol blue tracking dye, migrated at least two-thirds of the way down the gel.
10. The gel was examined by ultraviolet light using UV transilluminator then photographed.

2.3. Statistical analysis

Statistical analysis was done using Minitab 15 statistical analysis software. Two way ANOVA test was used to compare different groups among each other and with control. All values were expressed as Mean ± Standard Deviation of the mean (M ± SD). P value < 0.05 was regarded as statistically significant.

3.Results and discussion

3.1. Patients categorizing and thyroid hormone concentrations measurements

The study focused on the three categories of thyroid dysfunction that are, hypothyroidism, hyperthyroidism, and thyroid cancer. Patients were divided according to their gender, age, and thyroid symptoms using the formula $R = X\max - X\min$ where R is the range and *Xmax* and *Xmin* represent the upper and lower limit of the age. The number of categories enlisted in each table M was determined by $M = 1 + 3.3\log n$ where n is the number of patients with the same trait and age. The length of each trait is determined by $L = R / M$ (Paulson, 2003). Results are shown in tables (3.1, 3.2, 3.3, 3.4, 3. 5, and 3.6.)

***Table (3.1)**. Categorizing of patients with T 3 hypothyroidism.

T 3 (normal value 1.2 – 2.8 nmol/l)					
No.	Gender	Age (years)	Hormone concentration	Deviation in hormone concentration	No. of cases
1.	Male	30 – 70	0.2 – 0.7	1 – 2.1	36
2.	Female	20 – 70	0.1 – 1.6	1.1 – 1.2	64

*= detailed data are listed in appendix (5)

***Table (3.2)**. Categorizing of patients with T 4 hypothyroidism.

T 4 (normal value 60 – 160 nmol/l)					
No.	Gender	Age (years)	Hormone concentration	Deviation in hormone concentration	No. of cases
1.	Male	30 – 70	10 – 50	50 – 110	36
2.	Female	20 – 70	7.5 –50	52.5 – 110	64

*= detailed data are listed in appendix (5)

***Table (3.3).** Categorizing of patients with TSH hypothyroidism.

TSH (normal value 0.25 – 0.5 nmol/l)					
No.	Gender	Age (years)	Hormone concentration	Deviation in hormone concentration	No. of cases
1.	Male	30 – 70	0.4 – 0.9	0.15 – 0.4	36
2.	Female	20 – 70	0.5 – 1	0.25 – 0.5	64

*= detailed data are listed in appendix (5)

***Table (3.4)**. Categorizing of cancer patients with T 3 hyperthyroidism.

T 3 (normal value 1.2 – 2.8 nmol/l)					
No.	Gender	Age (years)	Hormone concentration	Deviation in hormone concentration	No. of cases
1.	Male	30 – 70	4 – 22	2.8 – 19.2	68
2.	Female	20 – 70	3 – 8	1.8 – 5.2	32

*= detailed data are listed in appendix (5)

***Table (3.5).** Categorizing of patients with T 4 hyperthyroidism.

T 4 (normal value 60 – 160 nmol/l)					
No.	Gender	Age (years)	Hormone concentration	Deviation in hormone concentration	No. of cases
1.	Male	30 – 70	170 – 200	110 – 80	68
2.	Female	20 – 70	180 – 190	120 – 30	32

*= detailed data are listed in appendix (5)

***Table (3.6).** Categorizing of patients with TSH hyperthyroidism.

TSH (normal value 0.25 – 0.5 nmol/l)					
No.	Gender	Age (years)	Hormone concentration	Deviation in hormone concentration	No. of cases
1.	Male	30 – 70	0.2 – 0.4	0.05 – 0.1	68
2.	Female	20 – 70	0.08 – 0.3	0.17 – 0.2	32

*= detailed data are listed in appendix (5)

Depending on the previous tables, the distribution of both types of thyroid dysfunction is presented in figures (3.1) and (3.2).

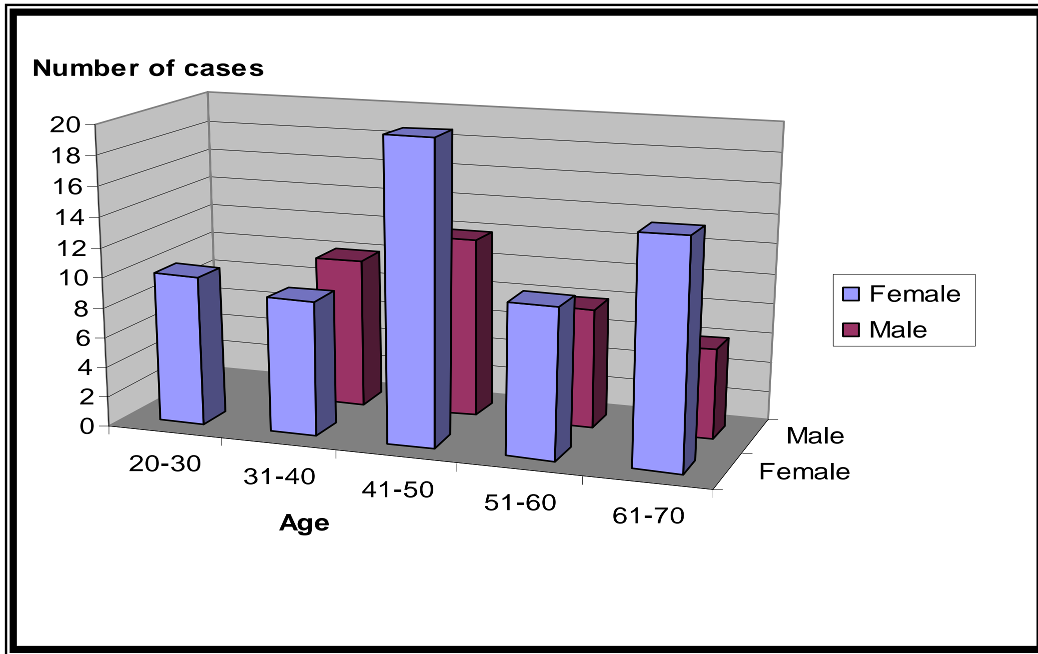


Figure (3.1). Distribution of hypothyroidism in male and female patients in different ages. The figure shows that hypothyroidism is more frequent in female patients than male patients.

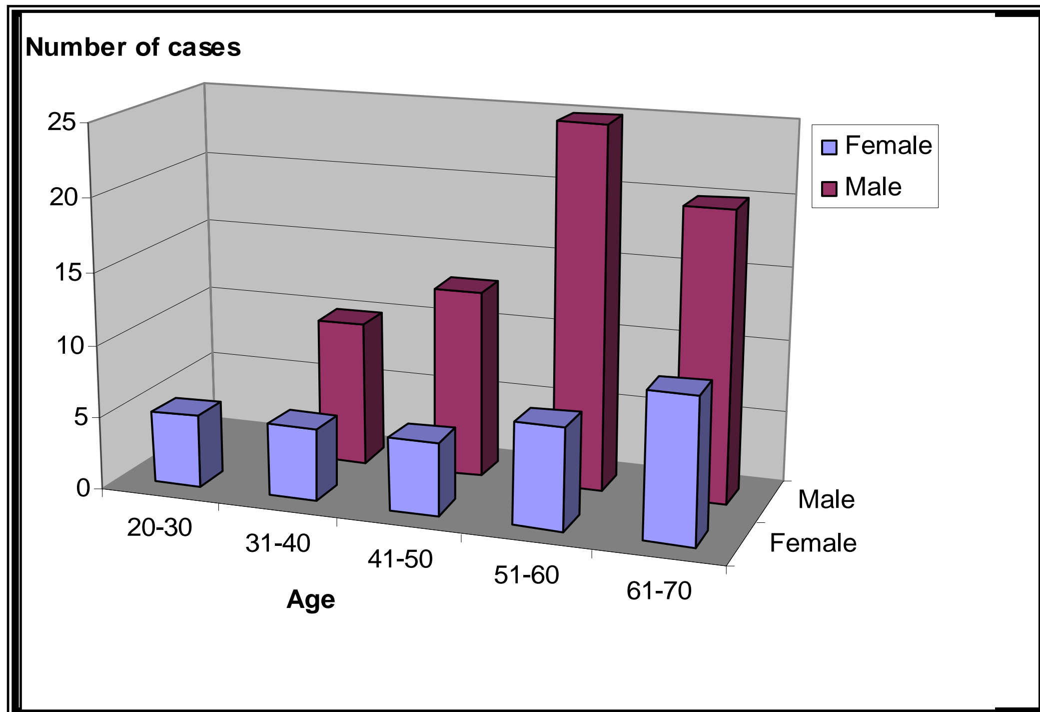


Figure (3.2). Distribution of hyperthyroidism in male and female patients in different ages. The figure shows that hyperthyroidism is more frequent in male patients than female patients.

The conclusion that can be drawn from figure (3.1) that hypothyroidism is more frequent in female than male and most of the cases appear to happen at age of 40 – 50 years. This can be attributed to hormonal changes at that age, while figure (3.2) shows that hyperthyroidism is more frequent in male especially at age 50 – 60 years. There are many theories about the processes that might be involved in aging. The endocrine system is often referred to as being affected, since aging alters the function of many endocrine glands, including the pituitary and thyroid glands (Morley, 1995).

Aging significantly decreases serum T4 in male and female, as previously reported (Klug and Adelman, 1979; Corrêada Costa and Rosenthal 1996). Normally, decreased thyroid hormone serum levels should stimulate TSH secretion and result in increased TSH serum levels (Pekary *et al.*, 1983).

3.1.1. Statistical analysis of patients with hypothyroidism

Statistical analysis of patients with dysfunction is shown in table (3.7).

***Table (3.7).** Statistical analysis using two ways ANOVA of male and female showing hypothyroidism disorder.

Age / years	T3 (nmol/l+SD)		T4 (nmol/l+SD)		TSH (nmol/l+SD)	
	Male	Female	Male	Female	Male	Female
20-30		A 0.63±0.437		A 28.75±15.6		A 0.78±0.1751
31-40	A, a 0.455±0.1691	A, a 0.644±0.482	A, a 27.5±12.08	A, a 29.17±17.23	A, a 0.625±0.162	A, a 0.7667±0.1581
41-50	A, a 0.425±0.1545	A, a 1.13±2.316	A, a 30.83±13.46	A, a 26.8±16.54	A, a 0.6542±0.1602	A, a 0.745±0.1791
51-60	A, a 0.4187±0.1731	A, a 0.66±0.448	A, a 31.87±13.35	A, a 24.35±16.35	A, a 0.625±0.1732	A, a 0.78±0.1687
61-70	A, a 0.45±0.1871	A, a 0.653±0.484	A, a 31.67±15.71	A, a 23.77±16.58	A, a 0.6167±0.2137	A, a 0.8067±0.1751
Control	B, a 2.12±0.4638	A, a 1.83±0.49	B, a 114±30.62	B, a 106±34.06	A, a 0.38±0.0789	B, a 0.36±0.0876

A and B = Comparison of hormone concentration of patients of the same gender but differ in age.

a and b= Comparison of hormone concentration of patients of the same age category but differ in gender.

Different letter means the difference is considered significant when ($P<0.05$).

*= detailed data are listed in appendix (6)

Table 3.7 shows the presence of significant decrease in male hormone concentration of T3 and T4 when compared with normal value while no significant difference is noticed in TSH measurements. In female patients, the difference was noticed in T4 and TSH. The explanation for this can be given on the following basis: In old females, the TSH unresponsiveness to decreased T4 could be due to the unaltered T3 serum levels, but the same explanation would not be acceptable to explain the unaltered serum TSH in aged males, which had significant decrease of serum T4 and T3. These observations showed that, directly or through a decrease of TRH stimulus, the aged thyrotrophs are unable to respond efficiently to the decreased negative modulation by thyroid hormones. Neither basal nor TRH-stimulated TSH release differed between young and old female pituitary explants. Pituitary responsiveness to TRH is maintained in old persons (Borges *et al.,* 1998).

Thus, the decrease in basal TSH secretion by the aged male pituitary would indicate a decrease of endogenous TRH, possibly related to diminished serum testosterone. There is a decreased hypothalamic TRH and pituitary TSH mRNA levels in old versus young male, but no difference in hypothalamic TRH mRNA between young and old females, although the circulating thyroid hormone levels were decreased in elderly of both genders. In their view, aging appears to be associated with central hypothyroidism in both genders, although the magnitude of the alteration is less in female than in male (Cizza *et al.*, 1992).

3.1.2. Statistical analysis of patients with hyperthyroidism

Hyperthyroidism was also investigated during this study and statistical analysis was performed for patients showing this disease as listed in table (3.8).

***Table (3.8).** Statistical analysis using two ways ANOVA of male and female showing hyperthyroidism disorders.

Age / years	T3 (nmol±SD)		T4 (nmol±SD)		TSH (nmol±SD)	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
20-30		A 5.4±2.074		A 186±3.81		A 0.154±0.09476
31-40	A, a 13.20±5.94	A, a 5.60±2.074	A, a 182.5±10.34	A, a 186±3.81	A, a 0.288±0.07743	A, b 0.154±0.09476
41-50	A, a 12.077±6.474	A, a 5.4±2.074	A, a 185.83±9.46	A, a 186±3.81	A, a 0.30769±0.07596	A, b 0.154±0.09476
51-60	A, a 11.92±5.438	A, b 5.429±1.718	A, a 184.8±10.15	A, a 185.71±4.27	A, a 0.308±0.07455	A, b 0.16714±0.08558
61-70	A, a 11.8±5.444	A, b 5.7±2.003	A, a 184.5±10.25	A, a 187±3.09	A, a 0.31±0.07539	A, b 0.182±0.08217
Control	B, a 2.12±0.464	B, a 1.83±0.49	B, a 114±30.62	B, a 106±34.06	A, a 0.38±0.07888	B,a 0.36±0.08756

A and B = Comparison of hormone concentration of patients of the same gender but differ in age.

a and b= Comparison of hormone concentration of patients of the same age category but differ in gender.

Different letter means the difference is considered significant when ($P<0.05$).

*= detailed data are listed in appendix (6).

Table 3.8 shows presence of a significant increase in T3 and T4 in male compared with normal value while in female the increase is noticed in all thyroidal hormones compared with normal. The explanation for this is due to the presence of a preferential T3 release from the thyroid gland has been suggested to occur in aged people of both genders (Pekary *et al.*, 1983). It was found that no change in thyroidal T3 concentration in aged females, even when very old, and a slight, non significant decrease in aged males. Furthermore, no significant differences in thyroidal T3 concentration was detected between males and females. Notwithstanding, the T4 levels were higher in the female thyroid glands than in males. These differences suggest some gonadal hormone modulation on either thyroidal T4 expression or total thyroidal T3 activity. A stimulating effect of estrogens on T3 expression would agree with the greater hepatic T3 activity in females and the unchanged T3 in the old female thyroids, since the aging murine ovary decreases progesterone production but maintains estrogen release for some time (Woodman, 1997). This hypothesis also agrees with the findings of Lisbôa *et al.*, (1997), who reported young adult male have lower hepatic, pituitary and thyroidal T3 activity than females (Bates *et al.*, 1999).

Furthermore, it was noticed that the disease is more frequent in young women than young men since during this study no men with thyroid dysfunction came forward for test or treatment. This may be attributed to difference in sex hormones. The presence of testosterone in higher concentration in male than female may generate a higher level of tolerance to the symptoms and they may appear if a change takes place in male hormone concentration. In addition, taking in to consideration the social habits and many of males in this age are at their work or jobs, they take longer time to come for test and treatment. In another meaning the

appearance of the disease may occur in young male of ages of 20 – 30 at the same frequency but still there is a difference in the type of thyroid dysfunction.

3.2. Effect of thyroid dysfunction on fertility

Many researchers had pointed out the relation of thyroid dysfunction with fertility and pregnancy (Takser *et al.*, 2005).

Hormones, by their very nature, are potent bioactive compounds. The importance of hormones for a wide variety of reproductive processes is common knowledge; these processes include development, puberty, behavior, gametogenesis, and integrated sexual function. In some cases there is a tight coupling of stimulus and response. In others (FSH action in the male), this coupling is so loose as to make it difficult to ascertain any connection at all.

The general case study of patients examined in this part of study is listed in tables (3.9, 3.10, 3.11, and 3.12)

***Table (3.9).** Determination of fertility hormone levels in males with hypothyroidism.

No.	Hormone	Age (year)	Hormone normal concentration	Measured concentration	Deviation in hormone concentration	No. of cases
1.	Testosterone	30 – 70	3 – 10 ng/ ml	5 – 9	2 – 1	36
2.	LH		0.6 – 12 mU/ ml	4 – 8	3.4 – 4	
3.	FSH		1 – 8 mU/ ml	1 – 8	0 – 0	
4.	Progesterone		0.13 – 1.26 ng/ ml	0.2 – 0.4	0.11 – 1.22	

*= detailed data are listed in appendix (7)

***Table (3.10).** Determination of fertility hormone levels in females with hypothyroidism.

No.	Hormone	Age (year)	Hormone normal concentration	Measured concentration	Deviation in hormone concentration	No. of cases
1.	Testosterone	20 – 70	0.2 – 0.9 ng/ml	0.2 – 0.5 ng/ ml	0 – 0.4	64
2.	LH		3 – 6 mU/ml	0.09 – 6 mU/ ml	2.91 – 0	
3.	FSH		2 – 8 mU/ml	5 – 50 mU/ ml	3 – 42	
4.	Progesterone		2.5 – 25 ng/ml	0.5 – 15 ng/ ml	2 – 10	

*= detailed data are listed in appendix (7)

***Table (3.11)**. Determination of fertility hormone levels in males with hyperthyroidism.

No.	Hormone	Age (year)	Hormone normal concentration	Measured concentration	Deviation in hormone concentration	No. of cases
1.	Testosterone	30 – 70	3 – 10 ng/ ml	0.4 – 10	2.6 – 0	68
2.	LH		0.6 – 12 mU/ ml	2 – 5	1.4 – 7	
3.	FSH		8 – 15 mU/ ml	4 – 6	4 – 9	
4.	Progesterone		0.13 – 1.26 ng/ ml	0.2 – 0.8	0.11 – 1.8	

*= detailed data are listed in appendix (7)

***Table (3.12).** Determination of fertility hormone levels in females with hyperthyroidism.

No.	Hormone	Age (year)	Hormone normal concentration	Measured concentration	Deviation in hormone concentration	No. of cases
1.	Testosterone	20 – 70	0.2 – 0.9 ng/ml	0.9 – 2 ng/ ml	0.7 – 0.11	32
2.	LH		3 – 6 mU/ml	3 – 24 mU/ ml	0 – 18	
3.	FSH		2 – 8 mU/ml	3 – 10 mU/ ml	1 – 2	
4.	Progesterone		2.5 – 25 ng/ml	1.5 – 3 ng/ ml	1 – 22	

*= detailed data are listed in appendix (7)

The overview of the general case shows there is a relation between thyroid dysfunction and fertility in both genders since there is a change in measured values of fertility hormones from that in normal. In the case of hypothyroidism, FSH and LH showed a deceased level in blood than normal in female and male, while in patients with hyperthyroidism, a slight increase in LH and FSH was observed in female while decrease in LH and progesterone was measured in male with a slight increase in FSH.

The first attempt made is to determine Seminal Fluid Analysis (SFA) and fertility in patients showing thyroiditis. The test depends on measurement of total count, motile sperms, sluggish, and non – motile and being compared to each other at different ages, then among patients showing different thyroiditis (hypothyroidism or hyperthyroidism). The detailed statistical analysis is shown in table (3.13).

Table (3.13). Statistical analysis of Seminal fluid in male with hypothyroidism and hyperthyroidism using two ways ANOVA.

Age / years	Total count (mean± SD)		Motile (mean± SD)		Sluggish (mean± SD)		Non motile (mean± SD)	
	Hyper	Hypo	Hyper	Hypo	Hyper	Hypo	Hyper	Hypo
31-40	A, a 33200000 ± 29491242	A, a 40700000 ± 32414160	A, b 7500000 ± 13630440	A, b 12960000 ± 15456333	A, a 12200000 ± 8299933	A, a 15990000 ± 18639650	A, a 13500000 ± 14597945	A, a 11750000 ± 8408091
41-50	A, a 26538462 ± 26152168	A, a 46090909 ± 37189930	A, a 10846154 ± 17439639	A, a 22727273 ± 30672760	A, a 7169231 ± 5735327	A, a 13251818 ± 13819321	A, a 8523077 ± 6170785	A,a 10090403 ± 10269027
51-60	A, a 33840000 ± 29191437	A, a 34750000 ± 29936838	A, a 207508000 ± 61925256	A, a 11375000 ± 14451273	A, a 7742000 ± 7634149	A, a 9366667 ± 6945142	A, a 5624000 ± 17161300	A, a 13462500 ± 10821795
61-70	A, a 27550000 ± 28087223	A, a 33000000 ± 27294688	A, a 11450000 ± 20048559	A, a 7666667 ± 11535453	A, a 7810000 ± 6511035	A, a 11816667 ± 8546442	A, a 8290000 ± 5355361	A, a 12516666 ± 7002976
Control	B, b 77000000 ± 12516656	B, b 77000000 ± 12516656	A, b 37860000 ± 22914925	A, a 37860000 ± 22914925	A, a 18560000 ± 6703929	A, a 18560000 ± 6703929	A, a 20580000 ± 4897573	A, a 20580000 ± 4897573

A and B = Comparison between patients of the same gender but differ in age.

a and b= Comparison between patients of the same age category but differ in thyroid dysfunction type.

Different letter means the difference is considered significant when ($P<0.05$).

Table (3.13) gives a clear idea about the relation between thyroiditis and fertility concerning seminal fluid production. In all cases of hypothyroidism or hyperthyroidism, the total count showed a significant difference in patients from that in normal cases. In all cases studied, almost all patients showed less total count than normal. However, no significant difference in motile, sluggish, and non – motile sperms was found when compared with normal.

It is noteworthy to say that both types of thyroiditis showed the same effect on seminal fluid development when comparing data of each type of thyroiditis with the other with one observation, that motile sperms in both types of thyroid dysfunction at age 31 – 40 showed a significant difference when compared with other age groups. The reason for that, most of men at this age come for diagnosis and treatment only when the disease shows severe symptoms. Thus, SFA showed a decreased number of seminal count when compared to normal and patients under medication.

More work was performed to study the relation between thyroid dysfunction and fertility hormones. The study at this point focused on both genders showing both types of thyroiditis and measurement of testosterone, Leutinizing hormone (LH), follicle stimulating hormone (FSH), and progesterone. The results of effect of hypothyroidism and hyperthyroidism on fertility hormones is shown in tables (3.14 and 3.15).

***Table (3.14).** Statistical analysis of fertility hormones using two ways ANOVA of patients with hypothyroidism.

Age / years	**Testosterone (ng/ml±SD)**		**LH (mU± SD)**		**FSH (mU± SD)**		**Progesterone (ng/ml±SD)**	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
20-30		A 0.32±0.1229		A 3.809±1.854		A 6.7±1.64		A 3.47±4.491
31-40	A, a 6.7±1.767	A, b 0.3222±0.1202	A, a 6.5±1.65	A, b 2.677±1.985	A, a 5.6±2.797	A, a 8.89±4.59	A, a 0.3±0.0943	A, a 2.033±2.346
41-50	A, a 7.417±1.564	A, b 0.34±0.1273	A, a 6.417±1.73	A, b 1.858±2.132	A, a 4.5±2.876	A, b 19.15±15.26	A, a 0.3167±0.0835	A, b 4.275±3.873
51-60	A, a 6.375±1.506	A, b 0.32±0.1033	A, a 5.25±1.581	A, b 1.466±1.903	A, a 3.5±2.673	A, b 18.5±16.1	A, a 0.3125±0.0835	A, a 1.07±0.814
61-70	A, a 6.5±1.517	A, b 0.3333±0.1175	A, a 5±1.673	A, b 1.304±1.575	A, a 3.667±2.875	A, b 27.33±17.71	A, a 0.3±0.0894	A, a 3.9±3.374
Control	A, a 7.3±2.751	A, b 0.46±0.2675	A, a 4.78±4.437	A, a 4.3±0.949	A, a 3.7±2.669	A, a 5.3±1.89	A, a 0.578±0.4829	B, b 15.7±7.889

A and B = Comparison of hormone concentration of patients of the same gender but differ in age.

a and b= Comparison of hormone concentration of patients of the same age category but differ in gender.

Different letter means the difference is considered significant when ($P<0.05$).

*= detailed data are listed in appendix (8)

*__Table (3.15).__ Statistical analysis of fertility hormones using two ways ANOVA of patients with hyperthyroidism.

Age / years	Testosterone (ng/ml±SD)		LH (mU± SD)		FSH (mU± SD)		Progesterone (ng/ml±SD)	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
20-30		A, 1.38±0.5675		A, 9.6±8.444		A, 5.4±2.074		A, 2.1±0.652
31-40	A, a 4.06±3.351	A, a 1.38±0.5675	A, a 3.6±1.174	A, a 10.4±8.019	A, a 4.8±0.823	A, a 5.8±2.588	A, a 0.48±0.2044	A, b 2.2±0758
41-50	A, a 4.638±3.29	A, a 1.16±0.4722	A, a 3.385±1.193	A, b 12.8±7.662	A, a 5.115±0.795	A, a 5±1.581	A, a 0.4462±0.2066	A, b 2.3±0.671
51-60	A, a 3.636±3.443	A, a 1.2571±0.5094	A, a 3.48±1.229	A, b 11.429±8.223	A, a 4.9±.791	A, b 6.857±2.41	A, a 0.492±0.2499	A, b 2.286±0.636
61-70	A, a 3.71±3.707	A, a 1.16±0.4452	A, a 3.4±1.142	A, b 10.9±6.082	A, a 5.05±0.793	A, a 6±2.449	A, a 0.465±0.23	A, b 2.2±0.715
Control	A, a 7.3±2.751	A, b 0.46±0.2675	A, a 4.78±4.437	A, a 4.3±0.949	A, a 4.2±2.573	A, a 5.3±1.889	A, a 0.578±0.4829	B, b 15.7±7.889

A and B = Comparison of hormone concentration of patients of the same gender but differ in age.

a and b= Comparison of hormone concentration of patients of the same age category but differ in gender.

Different letter means the difference is considered significant when ($P<0.05$).

*= detailed data are listed in appendix (8)

The explanation that can be drawn from tables (3.14), and (3.15) is as follows: testosterone levels in male had not been affected by hypothyroidism since it is maintained at normal levels, whereas a significant difference is noticed when compared with female, which is natural since testosterone is less in concentration than in male. In addition, the progesterone in female showed a significant difference when compared with male and normal. At age groups 20 – 30, 31 – 40, and 41 – 50 the change may be attributed to hypothyroiditis effect, since the female at these age groups is able to conceive a child if hormones levels at normal value, whereas female at age groups 51 – 60, and 61 – 70 are at menopause, and it is normal to see the change in LH and FSH. So it is not conclusive to attribute LH and FSH change in the latest group of ages to hypothyroidism or hyperthyroidism. In human, altered thyroid status is known to adversely affect many organs and tissues. Nevertheless, for many years, the impact of thyroid disorders on male reproduction remained controversial. This was partly due to the demonstration that the adult testis of experimental animals was metabolically unresponsive to thyroid hormones and to the low number of thyroid hormone-binding sites found in the adult organ (Oppenheimer *et al.*, 1974).

These early reports led to the widespread view that the testis was unaffected by iodothyronines. Additionally, clinical data correlating male sexual function with thyroid disorders are limited, probably because thyroid diseases are more common in females than in males. However, in the past two decades, several experimental and clinical studies have demonstrated that thyroid hormone plays an important role in testicular development and function. It is now established that triiodothyronine (T3) regulates the maturation and growth of testis, controlling Sertoli cell and Leydig cell

proliferation and differentiation during testicular development in rats and other mammal species (Holsberger *et al.*, 2005; Mendis-Handagama and Siril Ariyaratne, 2005).

Furthermore, changes in thyroid hormone levels during early testicular development have been shown to affect testicular maturation and reproduction later in life (Jannini *et al.*, 1995).

Although hypothyroidism had no effect on testicular development during fetal life (Hamouli-Said *et al.*, 2007), it was associated, at puberty, with impaired testicular development including testicular growth, germ cell maturation, and seminiferous tubule formation (Francavilla *et al.*, 1991).

However, as the male with hypothyroid were treated to recover back to the euthyroid state, a significant increase in testicular size and daily sperm production was observed in adulthood (Cooke *et al.*, 1991).

Subsequently, the mechanism underlying these unpredictable testicular changes was established. It has been shown that transient neonatal/ prepubertal hypothyroidism extends the length of Sertoli cell proliferation by delaying their maturation, resulting in an increased number of Sertoli cells in the adult testis (De Franca *et al.*, 1995).

Conversely, transient juvenile hyperthyroidism resulted in an early cessation of Sertoli cell proliferation and had a concomitant stimulatory effect on their maturation, resulting in premature canalization of seminiferous tubules, decreased testis size, and sperm production (Palmero *et al.*, 1995).

The above data together with the reported high levels of expression of functional T3 receptors in proliferating Sertoli cells indicate that Sertoli cells are a major testicular target for thyroid hormone. It appears that thyroid

hormone acts directly on Sertoli cells to inhibit proliferation while stimulating differentiation (Jannini *et al*., 1995; Buzzard *et al*., 2000).

Although several factors are presumed to play a role in proliferation and maturation of Sertoli cells, T3 is likely to represent a major hormonal signal involved in the establishment of the adult Sertoli cell population (Jansen *et al.*, 2007).

Thyroid hormones interact with both oestrogens and progesterone to maintain a normally functioning uterus and are necessary for the normal maturation of the oocytes. The impact of thyroid hormones has been reported to be both direct through the presence of thyroid hormone receptors on the ovaries and indirect through an impact on the secretion of sex hormonebinding globulin (SHBG), prolactin and luteinizing hormone-releasing hormone (LH-RH) (Poppe *et al.*, 2008).

The prevalence of hypothyroidism in women of reproductive age varies between 2 % and 4 % and, in most cases, is due to chronic autoimmune thyroiditis (Hollowell *et al.*, 2002).

Hypothyroidism can be associated with menstrual irregularities and other types of reproductive disorders and, thus, may finally lead to infertility (Joshi *et al.*, 1993).

Thyroid hormone receptors have been described in human oocytes, where they synergize with the LH/hCG receptor, mediated by follicle-stimulating hormone to exert direct stimulatory effects on granulosa cell function (i.e. progesterone production) and on trophoblastic differentiation (Wakim *et al.*, 1993).

In an *in vitro* fertilization setting, Cramer *et al.* 2003 showed that serum TSH levels were significantly higher among women who produced oocytes that failed to be fertilized, and that among women who had at least

one oocyte inseminated, the likelihood that they would have fewer than 50% of their eggs fertilized was significantly related to higher TSH levels. These data might, however, reflect an abnormally low value for T4 concentrations in patients with SH and could thus be interpreted as overt hypothyroidism (Andersen *et al.*, 2002; Poppe *et al.*, 2008).

Thus, besides age, a history of treatment for infertility may be an additional argument toward lowering the upper limit of the serum TSH reference range (Bohnet *et al.*, 1981).

A positive correlation between basal TSH, LH and testosterone concentrations in the early follicular phase. Women with elevated serum TSH levels had a lower pregnancy rate than women with a normally stimulated serum TSH. The prevalence of FSH is considerably higher in studies in which the TRH test was used than in those in which only the upper limit of basal serum TSH was used. This difference might once more indicate that, in older studies using less sensitive measurements of serum TSH, the actual TSH reference levels are perhaps slightly too high in the setting of infertility. In the general population, the prevalence of subclinical hyperthyroidism is ~1.5 % (Bjoro *et al.*, 2000).

Treatment of hyperthyroidism appeared to frequently correct these cycle changes. Several aspects of the reproductive axis influenced by an excess of thyroid hormones are comparable with the situation in hypothyroid women. In hyperthyroidism, SHBG production, the conversion of androgens to oestrogens and the gonadotrophin response to GnRH are increased (Krassas, 2000).

Despite these metabolic changes, hyperthyroid women usually maintain ovulation, according to endometrial biopsies. Women with hyperthyroidism and fertility problems should be treated with antithyroid

drugs and/or surgery according to the cause of hyperthyroidism. Treatment with radioiodine is not recommended, especially when women plan to start an ART procedure, with the possibility of an early pregnancy (Mestman, 2004).

3.3. Histopathology of thyroid tumors.

Of all patients included in this study, 93% did not show malignant tumors (200 patient), of this percentage 70% with thyroid goiter (140 patient), 65% female (91 patient) and 35 % male (49 patient), where as 7 % (15 patient) with thyroid carcinoma. Table (3.16) shows the number of cases and distribution of thyroid goiter and benign tumors among different age groups in male patients, whereas table (3.17) shows the number of cases and distribution of thyroid goiter and benign tumors among different age groups in female patients.

Table (3.16). Thyroid goiter and benign tumor distribution in male with thyroid dysfunction. Cases listed were a result of sonography and radiology photography.

Age / years	Patients with Follicular adenoma
31 – 40	27
41 – 50	12
51 – 60	9
61 – 70	1

Table (3.17). Thyroid goiter and benign tumor distribution in female with thyroid dysfunction. Cases listed were a result of sonography and radiology photography.

Age / years	Patients with Follicular adenoma
20 – 30	37
31 – 40	28
41 – 50	17
51 – 60	7
61 – 70	2

Tables (3.16) and (3.17) show that thyroid goiter is a trait of thyroid dysfunction and it is noticeable in patients at early stages of the disease since the thyroid hormones at unusual levels and patients are at the early stage of medication. However, at late ages (51 – 60 and 61 – 70 years) thyroid goiter is less common because of the treatment that reduces the size of thyroid gland. In addition, comparison of both genders, goiter appears more frequent in female patients than in male. This may be due to physiological differences of both genders.

Further illustration is given in figure (3.3) that compares thyroid goiter appearance between female and male patients.

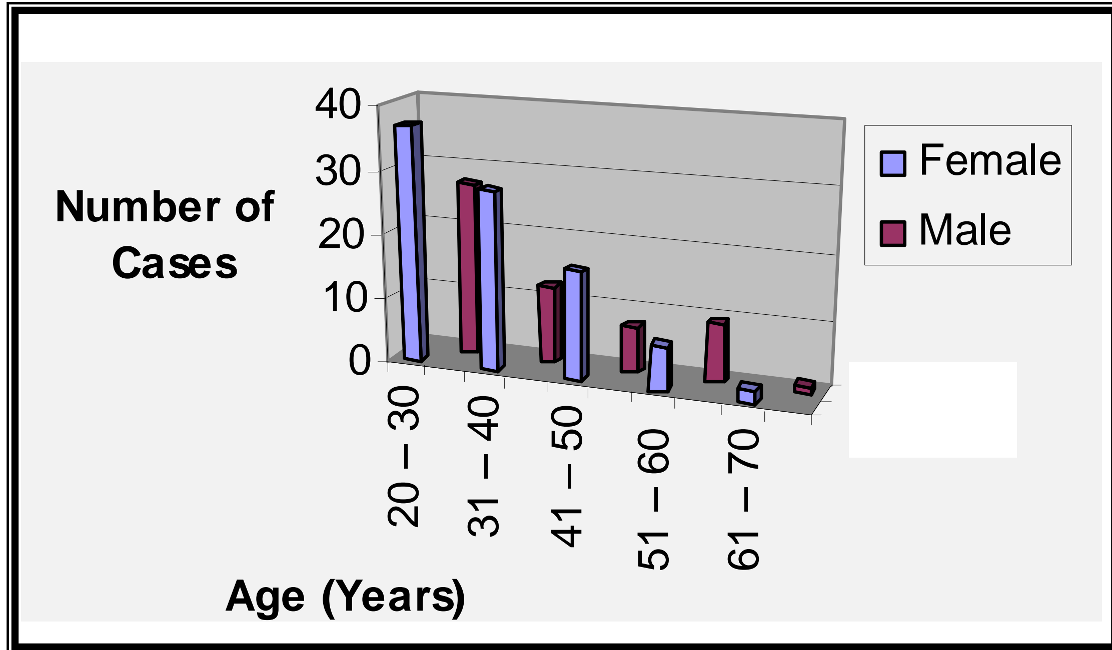


Figure (3.3). Comparison of goiter appearance among patients with thyroid dysfunction of both genders. The figure shows that the frequency of thyroid enlargement in female is more than male.

The figure shows that thyroid tumor is more frequent in female than male especially in younger patients since it exceeds about 10% at this age and comes closer in older patients with continuous medication.

Thyroid enlargement occurs with greater frequency in females, which may relate to the influence of estrogens on the immune system, particularly the B cell repertoire (Da Silva, 1995). Although it has been suggested previously that males suffer worse biochemical hyperthyroidism with less severe symptoms (Reed *et al.*, 1998), little evidence exists to suggest any difference in treatment outcome. Although another study of Italian subjects reported a small excess in the long term relapse rate after medical therapy in male patients (Vitti *et al.*, 1997). Age related differences in clinical presentation of goiter have also been reported (Nordyke *et al.*, 1988), with

severity of hyperthyroidism and prevalence of antibodies shown to decrease with advancing age (Aizawa *et al.*, 1989).

Some patients with thyroid goiter in this study who suffered a fibrosis in thyroid gland had reported to histopathology laboratories for biopsy, where as thyroid cancer patients were subjected for thyrodectomy. Samples from those patients were used for histopathological examination as shown in figure (3.4) that shows papillary adenoma in patients with hyperthyroidism and fibrosis, and figure (3.5) for patients with papillary carcinoma.

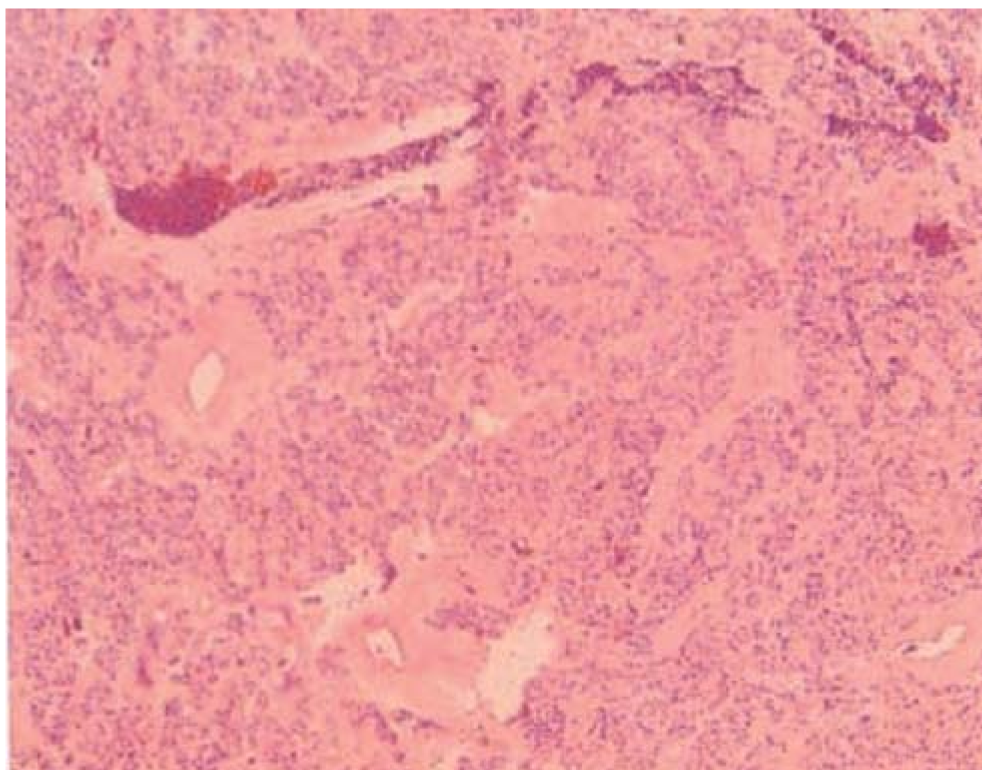


Figure (3.4). Papillary adenoma section in patients with hyperthyroidism suffering from fibrosis in thyroid gland.

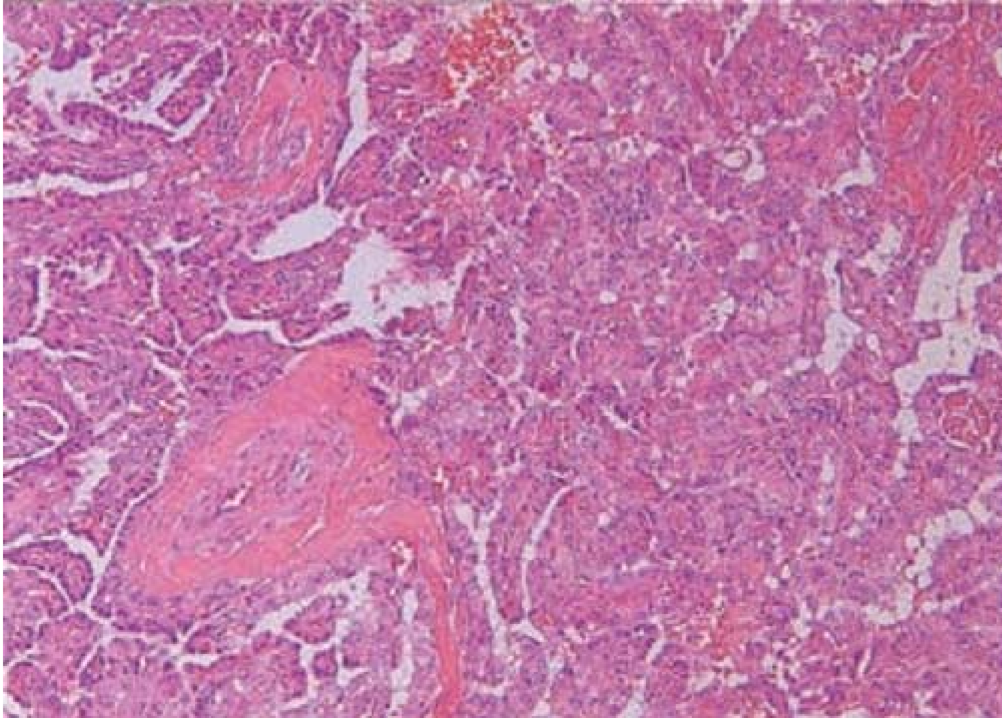


Figure (3.5). Papillary carcinoma section in patients with thyroid cancer.

Nodular lesions of the thyroid are very common among general populations. The incidence of palpable thyroid nodule in the adult population is about 4% to 7% (Carol *et al.*, 2003).

Thyroid tumors are often diagnosed by FNAB as well as by ultrasonography. Cytological examination of FNAB by a skillful pathologist who is an expert in thyroid tumors provides the most reliable information for the diagnosis of thyroid neoplasms (Yokozawa *et al.*, 1996).

In some clinical situations, however, slide samples are not adequate for cytological examination because of poor fixation, and a well-trained expert pathologist is not always available for diagnosis. In such cases, a more objective method is required for exact diagnosis. Gene diagnosis by analyzing nucleic acids from aspirates may be used for this purpose. Most previous trials, however, have failed to provide much information on the clinical features of thyroid tumors (Weiss *et al.*, 1996).

3.3.1.Thyroid cancer

Thyroid cancer is a common endocrine malignancy. The majorities, derived from follicular epithelial cells, represent a model of malignant transformation from benign adenomas and well differentiated carcinomas to poorly differentiated thyroid carcinoma and the rare but rapidly lethal undifferentiated thyroid carcinoma. This spectrum of progression has been linked with a pattern of cumulative intragenic defects that correlates with tumor differentiation, aggressiveness, and metastatic potential. Gene rearrangements involving the RET proto-oncogene or activating point mutations along the Ras / BRAF pathway account for the majority of these carcinomas. These intragenic abnormalities are considered to be early events in thyroid carcinogenesis; cancer behavior and progression are further modified by dysregulation of growth factors and their cognate receptors (Yen *et al.*, 2006). However, during this study, 15 patients with thyroid carcinomas were subjected for investigation. All of them suffered from hyperthyroidism and evolved to cancer with neglecting treatment in some of them, while others especially elderly patients developed cancer as a result of change in their body physiology and deterioration of immune and DNA repair system.

Categorizing cancer type with age and gender is listed in tables (3.18) and (3.19).

Table (3.18). Identification of cancer type and it's manifestation among different age groups in male patients.

Age / years	Patients with Papillary carcinoma	Patients with Medullary carcinoma
31 – 40	Non	Non
41 – 50	2	Non
51 – 60	2	1
61 – 70	3	2

Table (3.19). Identification of cancer type and it's manifestation among different age groups in female patients.

Age / years	Patients with Papillary carcinoma	Patients with Medullary carcinoma
20 – 30	Non	Non
31 – 40	Non	Non
41 – 50	1	Non
51 – 60	2	Non
61 – 70	1	1

Tables (3.18) and (3.19) show that cancer initiation was more in male than female and all of those patients were identified as hyperthyroidism patients as shown in table (3.20).

***Table (3.20)**. Thyroid hormone concentration in patients with thyroid cancer.

T 3 (normal value 1.2 – 2.8 nmol/l)			
Gender	Age (years)	Hormone concentration	No. of cases
Male	40 – 70	5 – 7	10
Female	40 – 70	4 – 10	5
T 4 (normal value 60 – 160 nmol/l)			
Gender	Age (years)	Hormone concentration	No. of cases
Male	40 – 70	400 – 700	10
Female	40 – 70	300 – 600	5
TSH (normal value 0.25 – 0.5 nmol/l)			
Gender	Age (year)	Hormone concentration	No. of cases
Male	40 – 70	0.01 – 0.25	10
Female	40 – 70	0.1 – 0.3	5

*= detailed data are listed in appendix (9)

Table (3.20) shows the unusual elevation in T3 and T4 in cancer patients serum while TSH reduced in blood stream. More analysis to determine the significance of thyroid hormone elevation and comparison between the two genders is given in table (3.21).

***Table (3.21)**. Statistical analysis of thyroid hormone levels in comparison with normal using two ways ANOVA.

Age / years	T3 (nmol±SD)		T4 (nmol±SD)		TSH (nmol±SD)	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
41-50	A, a 5.5±0.7071	AB, a 4 ± 1	A, a 500±141.42	A, a 500±100	A, a 0.13±0.16971	A, a 0.2±0.1
51-60	A, a 6.3333±1.1547	A, a 8.5±2.121	A, a 566.67±152.75	A, a 450±212.13	A, a 0.12±0.12124	A, a 0.2±0.14142
61-70	A, a 6±1	A, a 7.5±3.536	A, a 580±130.38	A, a 500±141.42	A, a 0.126±0.09762	A, a 0.25±0.07071
Control	B, a 2.12±0.4638	B, a 1.830±0.49	B, a 114±30.62	B, a 106±34.06	B, a 0.38±0.07888	A, a 0.36±0.08756

A and B = Comparison of hormone concentration of patients of the same gender but differ in age.

a and b= Comparison of hormone concentration of patients of the same age category but differ in gender.

Different letter means the difference is considered significant when (P<0.05).

*= detailed data are listed in appendix (10)

Table (3.21) shows there is a significant difference in thyroid hormones concentration when compared with normal in both genders. It is at an elevated level in all cases; whereas TSH in female did not show significant difference when compared with normal.

Thyroid cancer accounts for approximately 1% of total cancer cases in developed countries. It affects all age groups, although it is rare in children. Thyroid tumors are more frequent in women than in men. Despite their relative rarity, they exhibit a wide range of morphological patterns and biological behavior, which may explain the great interest in these neoplasms of both pathologists and clinicians (Chrisoula, 2004).

In cancer cases studied, papillary adenoma had over rated medullary carcinoma. The reason for this may be attributed to the genetic alterations, some of which are seen only in this cancer. The classical oncogenic genetic alterations commonly seen in thyroid cancer include Ras mutations (Fagin ,2002), RET / PTC rearrangements (Nikiforov, 2002), and PAX8 peroxisome proliferators activated receptor g (PPARg) fusion oncogene (McIver *et al.*, 2004). Various activating Ras mutations, widely seen in other cancers as well, occur mainly in FTC and the follicular variant of PTC (Vasko *et al.*, 2003).

There are few known thyroid cancer risk factors except female gender and the reasons for the increasing incidence and gender differences are unknown. Age-specific rates were higher among men than women across all age groups, and the male-to-female rate ratio declined quite consistently from more than five at ages 20-24 to 3.4 at ages 35-44 and approached one at ages over 80. Models for papillary thyroid cancers confirmed statistically different age-specific effects among women and men ($P < 0.001$ for the null hypothesis of no difference by gender), adjusted for calendar-period and

birth-cohort effects. Gender was an age-specific effect modifier for papillary thyroid cancer incidence (Briseis *et al.*, 2009).

3.4. Molecular analysis of thyroid mRNA from blood

Blood stream contains many components; some of them are proteins, minerals, lipids, and cells that the body is composed of. Some literatures referred that thyroid cells are circulating blood and can be isolated by magnetic cell sorting technique (Takano *et al.*, 1997).

Exploiting this fact, blood samples from patients of thyroid dysfunction was used to search for markers that can be useful to determine the genetic change in their thyroid and the possibility to diagnose patients that may develop thyroid cancer at early stage. This required isolation of mRNA from blood and generating DNA by using it as a template for RT – PCR. This was made by using a kit developed for this purpose, and photo of mRNA isolated from blood is shown in figure (3.6).

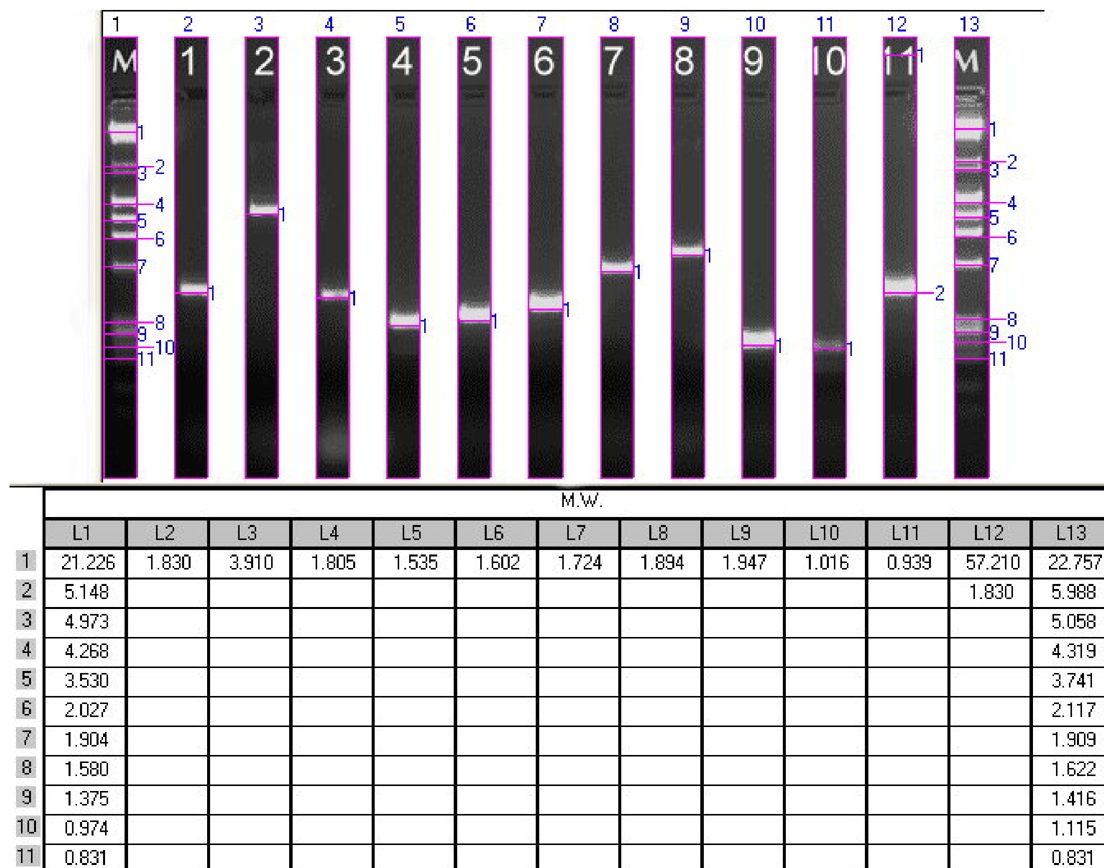
Figure (3.6). mRNA isolated from blood of patients with thyroid disorder. Lane 1 and 3 mRNA from healthy persons, lane 2, 6, 7 is mRNA from patients with thyroid cancer, lane 4, and 5 mRNA from patients with hyperthyroidism, lane 8, and 10 mRNA from patients with hypothyroidism, lane 9 and 11 is RNA markers. Samples were subjected to electrophoresis in 1% agarose / formaldehyde for 2 hours at 5 v/cm.

The figure shows that the kit used for mRNA extraction is proper for this work since RNA concentration was 30 – 60 µg / sample and the purity was high for the extended work.

RNA samples shown in figure (3.6) were used for RT – PCR. Result of using random hexamere primers is shown in figure (3.7).

Figure (3.7). RT – PCR amplification of mRNA isolated from patients with thyroid disorders using hexamere primers. M is a marker of Lambda DNA digested with *EcorI* and *HindIII*, lane 1, 3, and 11 is the result of healthy persons, lane 2 the result of patient of medullary carcinoma, lane 4, 5, 6 is the result of patients with hyperthyroidism, lane 7 and 8 is the result of patients with hypothyroidism, lane 9 and 10 is the result of patients with papillary carcinoma. Gel electrophoresis was performed at 10 v/cm for 2 hrs in 1 % agarose.

Determination of amplified bands size and analysis of DNA amplification was done using PhotoCapt analysis software. Results are shown in figure (3.8).



	M.W.												
	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13
1	21.226	1.830	3.910	1.805	1.535	1.602	1.724	1.894	1.947	1.016	0.939	57.210	22.757
2	5.148											1.830	5.988
3	4.973												5.058
4	4.268												4.319
5	3.530												3.741
6	2.027												2.117
7	1.904												1.909
8	1.580												1.622
9	1.375												1.416
10	0.974												1.115
11	0.831												0.831

Figure (3.8). Analysis of DNA fragments resulted from RT – PCR of mRNA in figure 3.7 from subjects under study. The figure shows the position and size of DNA band in kbp photographed from electrophoresis. Analysis was performed using PhotoCapt DNA analysis software.

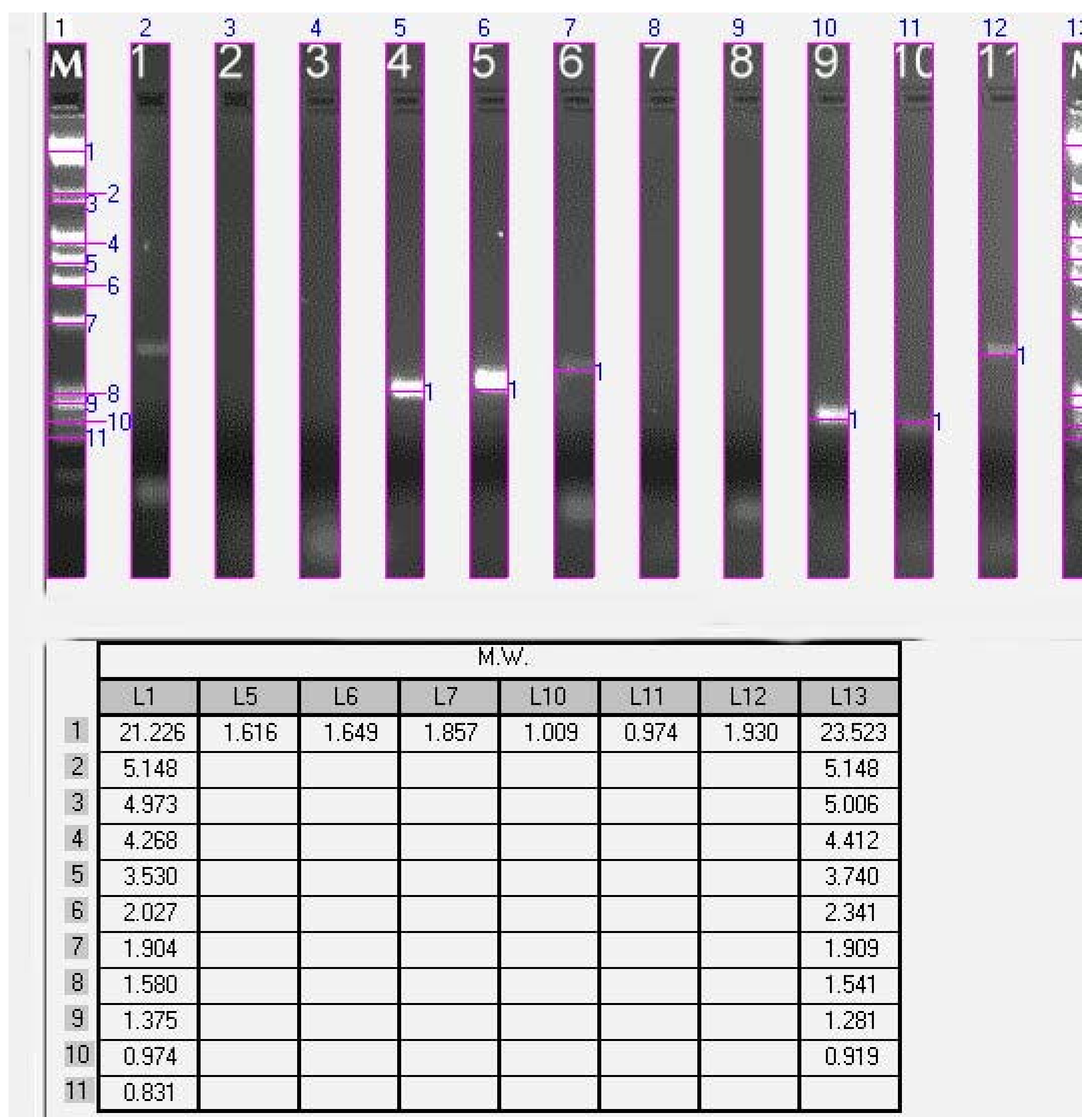
Figure (3.7) and the analysis in figure (3.8) can be interpreted as follows: rapid hexamere primers that were designed for analysis of thyroid genes had succeeded in pairing with target sites giving different patterns of DNA bands. In healthy persons lane 1, 3, and 11 showed a DNA band of the same size without significant difference that are useful as a reference point

for comparison of other bands. Lane 2 which is a DNA amplification of patients with medullary carcinoma showed a DNA fragment of size 3.91 kbp whereas lane 9, and 10 showed DNA fragments of patients with papillary carcinoma of size 1.016 and 0.939 kbp. Comparing these fragments with each other and with normal showed a significant difference relating their size and distance traveled. The reason for this may be explained as the presence of mutation within genes controlling thyroid function of different types in both disorders. The mutation in medullary carcinoma patients may be of amplification and rearrangement type resulted in presence of multiple DNA fragments of the same sequence that increased the specificity of primers used for amplification to associate with the target site. The result was a DNA fragment of large size whereas in case of papillary carcinoma, a point mutation or deletion in gene(s) controlling thyroid function reduced the specificity of primers to associate with target size resulted in a faint and less size fragment. In addition, no significant difference was found among patients with hyperthyroidism and hypothyroidism when compared with normal suggesting the change only occurred at physiological level.

For further confirmation of the results, another RT – PCR process was performed using Cycline A primers designed specifically for thyroid gene analysis. The result is shown in figure (3.9).

Figure (3.9). RT – PCR amplification of mRNA taken from subjects under study. Lane 1, 2, and 3 patients with papillary carcinoma amplified DNA, lane 4, 5, and 6 healthy persons amplified DNA, 7 negative control, lane 8 patient with meduulary carcinoma amplified DNA, lane 9, and 10 patients with hyperthyroidism and hypothyroidism amplified DNA respectively, lane 11 positive control. M is a marker of Lambda DNA digested with *EcorI* and *HindIII*. Gel electrophoresis was performed at 10 v/cm for 2 hrs in 1 % agarose.

Analysis of figure (3.9) is given in figure (3.10)



	M.W.							
	L1	L5	L6	L7	L10	L11	L12	L13
1	21.226	1.616	1.649	1.857	1.009	0.974	1.930	23.523
2	5.148							5.148
3	4.973							5.006
4	4.268							4.412
5	3.530							3.740
6	2.027							2.341
7	1.904							1.909
8	1.580							1.541
9	1.375							1.281
10	0.974							0.919
11	0.831							

Figure (3.10). Analysis of DNA fragments resulted from RT – PCR of mRNA of subjects under study. The figure shows the position and size of DNA band in kbp photographed from electrophoresis. Analysis was performed using PhotoCapt DNA analysis software.

Results obtained can be explained as follows: the Cycline A primer set was specific for thyroid gene analysis. The comparison among patients with hyperthyroidism and hypothyroidism with normal (healthy) did not yield a significant difference, suggesting no DNA alteration occurred at the molecular level. However, no DNA band was observed in patients with

thyroid carcinomas (lanes 1, 2, 3, and 8) from RT – PCR amplification. This can be attributed to the presence of a mutation within gene(s) controlling thyroid function and normal development and response. This mutation had altered the gene(s) in a way that the primer used for this purpose (Cycline A) was not sensitive to the target site, thus no amplification occurred as a result of the process.

In most patients with previously treated well differentiated thyroid carcinoma, this highly sensitive technique can detect small amounts of thyroid tissue even during TSH-suppressive thyroid hormone therapy. The assay of using circulating mRNA developed derives its sensitivity from the combined use of RT-PCR, a technique that allows specific amplification of small numbers of mRNA molecules.

RT-PCR has been used to detect small foci of malignant tissue as well as circulating cancer cells in patients with metastatic prostate cancer (Ghossein *et al.*, 1995), malignant melanoma, breast cancer, and thyroid cancer (Kruger *et al.*, 1996).

The assay for circulating thyroglobulin mRNA used in this study appears to have performance characteristics different from those of other assays recently reported. Ditkoff *et al.*, 1996 detected circulating thyroglobulin mRNA in all 7 patients with metastatic thyroid cancer whom they studied, but in less than 10% of 78 patients without metastases (Ditkoff *et al.*, 1996). Moreover, they did not detect thyroglobulin mRNA in blood from normal subjects. Tallini *et al.*, 1998 analyzed thyroglobulin mRNA in blood samples obtained from patients with thyroid cancer before surgery (Tallini *et al.*, 1998). Although these patients had intact thyroid glands, circulating thyroglobulin mRNA was detected in only 4 of 7 patients with

thyroid cancer and in 4 of 17 patients with benign thyroid nodules before thyroidectomy.

The differences between these investigators' findings and results of this study probably relate to the greater sensitivity of our thyroglobulin mRNA assay used in this work. In the absence of TSH stimulation, Ditkoff *et al.* 1996 could not detect thyroglobulin mRNA in samples with fewer than 200 thyroid cells/ml blood. The detection limit for Tallini *et al.*, 1998was equivalent to approximately 50–100 thyroid cells/ml blood. By contrast, this study calculated detection of thyroglobulin mRNA in as few as 10 normal thyroid cells/ml blood, a sensitivity similar to experimental observation of approximately 3 thyroid cells / ml blood. The sensitivity of the assay in patients with thyroid cancer will depend on the level of thyroglobulin expression in the circulating cancer cells and may differ from the calculated sensitivities. The difference in analytical sensitivity between these assays may reflect differences in the design of the oligonucleotide primers, conditions for synthesis of first strand cDNA and PCR, and preparation of RNA.

Several lines of evidence argue that the thyroglobulin mRNA detected in peripheral blood arises from circulating thyroid cells rather than from lymphocytes, in which thyroglobulin gene expression might represent ectopic transcription. First, negative and positive control lane did not show amplified bands after electrophoresis. Second, thyroglobulin mRNA can be detected in peripheral blood RNA after just one round of amplification. By contrast, detection of the extremely low levels of ectopically expressed mRNAs typically requires two consecutive rounds of nested amplification (Matthew *et al.*, 1998).

4. Conclusions and Recommendations

4.1. Conclusions

1. Thyroid dysfunction is a disease that affects both genders. Hyperthyroidism is more frequent in male than female patients in Iraq, whereas hypothyroidism is mostly diagnosed in female patients.
2. Thyroid dysfunction can affect fertility in both genders. It was found to affect seminal fluid formation in male, whereas it affected fertility hormones in female especially progesterone. This reduced fertility in both genders.
3. Goiter is a result of papillary adenoma and fibrosis in thyroid tissues. Most of thyroid tumors were found in female patients, and it is less in male patients.
4. In the case of thyroid cancer, most of the cases had developed in patients with hyperthyroidism. The ratio of cancer manifestation is higher in female patients although it seemed higher in men since most male patients showed hyperthyroidism symptoms that made them candidates for cancer if medication is neglected.
5. Most cases of thyroid cancer were papillary carcinoma and medullary carcinoma is less frequent.
6. The molecular analysis of thyroid dysfunction (hyperthyroidism, hypothyroidism, and cancer) revealed the following results: (i) no genetic alteration was found in patients with hyperthyroidism and hypothyroidism, and the illness may be attributed to physiological changes, (ii) in case of medullary carcinoma, the genetic alteration may be amplification or rearrangement of thyroid gene(s) at specific location, whereas papillary carcinoma was interpreted as a point mutation or deletion in thyroid gene(s).

7. RT – PCR technique for mRNA collected from blood serum of patients proved to be a reliable method and can be accommodated for diagnosis of thyroid cancer.

4.2. Recommendations

1. People should always perform periodic checkups of their thyroid and its hormonal level.
2. In case of infertility in normal people, THs should be considered when hormonal check is made.
3. The procedure of RT – PCR should be employed for early diagnosis of thyroid cancers.
4. Further study should be made to locate the position and the sequence undergoing mutation in thyroid cancer gene(s) in Iraq population in particular.

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Appendix -1-

Case profile

Name				
Sex	Female		Male	
Age				
Address				
Occupation				
Material Status				
Education				
No. of children				
Time of disease diagnosis				
Any more symptoms				
Nutrition				
Medication				

Appendix -2-

No. **Date**

Name **Age**

Sex

Fertility hormones

Testosterone	(ng/ml)
LH	(mU/ml)
FSH	(mU/ml)
Progesterone	(ng/ml)

Thyroid hormones

Triiodothyronin (T3)	(ng/ml)
Thyroxin (T4)	(µg/dl)
Thyroid stimulate hormone (TSH)	

Appendix -3-

Name…….:

Age……… :

Date …….:

Seminal Fluid Examination

Colour……...:	
Appearance..:	
Volume…….:	
Reaction…...:	(PH=8) (N= Alkaline)
Liquefaction:	min (N=15-30 min.)
Count………:	Million / ml (N=50-200)

Motility

Motile………:	%
Sluggish…….:	%
Non Motile…:	%

Morphology

Normal……..:	%
Abnormal…..:	%
Pus cell……...:	/ H.P.F.
R.B.C……….:	/ H.P.F.
Others………:	

Appendix -4-

Governorate	Hospital name	Hypothyroidism No. of patients	Hyperthyroidism No. of patients	Cancer tissue No. of patients
Baghdad	AL- Yarmok Teaching Hospital	5	10	2
	AL- Kadhymia Teaching Hospital	10	5	1
	St. Rafael hospital	10	5	2
Hilla	Marjan /general surgery hospital	10	15	2
Najaf	AL-Zahra hospital	10	15	2
Basra	AL-Basra general hospital	10	30	3
Ramadi	AI- Ramadi general hospital	10	5	-
Mousel	AL- Shifaa general hospital	10	10	1
Karkoak	Karkoak general hospital	25	5	2

Appendix 5

Male hypothyroidism data

T3

age	no.of cases	1	2	3	4	5	6	7	8	9	10	11	12
30-40	10	0.2	0.4	0.6	0.5	0.5	0.45	0.65	0.7	0.3	0.25		
41-50	12	0.5	0.7	0.55	0.2	0.3	0.6	0.4	0.25	0.3	0.55	0.4	0.3
51-60	8	0.4	0.6	0.2	0.7	0.3	0.25	0.4	0.5				
61-70	6	0.6	0.2	0.4	0.5	0.3	0.7						

T4

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12
30-40	10	10	20	30	20	25	30	40	15	35	50		
41-50	12	20	15	40	35	50	20	10	30	30	45	25	50
51-60	8	20	40	35	25	10	45	50	30				
61-70	6	25	50	40	20	45	10						

TSH

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12
30-40	10		0.4	0.8	0.65	0.7	0.4	0.7	0.6	0.5	0.9		
41-50	12		0.8	0.4	0.6	0.9	0.75	0.5	0.6	0.55	0.9	0.65	0.7
51-60	8	0.6	0.45	0.9	0.8	0.5	0.7	0.65	0.4				
61-70	6	0.4	0.5	0.7	0.4	0.8	0.9						

Female hypothyroidism data

T3

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
20-30	10	0.2	0.4	0.8	0.3	0.9	0.5	0.1	0.8	0.7	1.6										
31-40	9	0.3	1	0.1	1.6	1	0.2	0.5	0.4	0.7											

41-50	20	0.9	0.5	0.4	0.2	1	0.3	0.4	0.2	0.1	0.7	0.6	0.5	0.3	0.1	1.6	10.8	0.9	1.6	0.8	0.7
51-60	10	0.3	1	0.1	0.8	0.2	0.7	0.6	0.4	0.9	1.6										
61-70	15	1.6	0.4	0.2	0.3	0.5	1	0.3	0.9	0.1	0.9	1.6	0.4	0.8	0.1	0.7					

T4

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
20-30	10	30	45	7.5	15	40	50	20		25	10										
31-40	9	10	15	50	40	7.5	25	20	45	50			9	35	10	20	50	40	50	45	25
41-50	20	35	9	50	25	20		7.5	7.5	8	50	30									
51-60	10	45	30	8	40	50	25	20	8	7.5	10	45	50	50	25	40					
61-70	15	8	10	9	35	7.5	8	9	10	20	30										

TSH

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
20-30	10	0.8	1	0.5	0.9	0.6	1	0.9	0.7	0.8	0.6										
31-40	9	0.8	0.6	0.9	0.7	0.9	0.5	0.7	1	0.8											
41-50	20	0.7	1	0.5	0.7	0.8	0.9	0.8	0.6	0.7	1	1	0.7	0.5	1	0.6	0.5	0.5	0.7	0.9	0.8
51-60	10	0.9	0.6	0.7	0.8	1	0.5	0.9	0.7	1	0.7	0.9									
61-70	15	1	0.8	0.8	0.9	0.6	0.7	0.7	0.9	0.8	0.5										

Male hyperthyroidism data

T3

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
30-40	20	20	15	22	17	4	12	10	11	5	16															
41-50	12	12	4	7	4	20	18	11	5	10	8	16	22													
51-60	15	15	9	4	14	8	7	18	11	6	5	10	12	6	15		13	16	4	8	19	22	17	20	9	10
61-70	5	5	13	10	19	8	17	12	4		15	9	6		20	14	18	14	7	10	22					

T4

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
30-40	10	185	190	175	170	175	180	195	170	200																
41-50	13	195	185	200	200	175	190	180	180	190	175	185	170													
51-60	25	190	180	180	200	175	190	170	185	190	175	195	170	185	175	180	175	200	190	195	170	200	175	185	200	190
61-70	20	170	200	185	180	185	175	195	180	190	185	190	200	170	175	175	200	195	170	190	180					

TSH

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
30-40	10	0.28	0.3	0.2	0.25	0.2	0.2	0.4	0.35	0.3	0.4														
41-50	13	0.2	0.35	0.3	0.4	0.25	0.35	0.3	0.2	0.4	0.3	0.2	0.35	0.4											
51-60	25	0.4	0.35	0.2	0.2	0.4	0.25	0.3	0.35	0.4	0.35	0.3	0.2	0.25	0.35	0.4									
61-70	20	0.3	0.2	0.4	0.25	0.3	0.35	0.25	0.2	0.35	0.4	0.35	0.4	0.25	0.2	0.4	0.3	0.2	0.25	0.4	0.3	0.35	0.2	0.25	0.4

Female hyperthyroidism data

T3

age	no. of cases	1	2	3	4	5	6	7	8	9	10
20-30	5	7	3	5	4	8					
31-40	5	8	4	6	3	7					
41-50	5	5	8	3	7	4					
51-60	7	8	5	7	3	5	6	4			
61-70	10	6	5	4	8	7	3	3	8	5	8

T4

age	no. of cases	1	2	3	4	5	6	7	8	9	10
20-30	5	180	185	188	187	190					
31-40	5	190	187	180	185	188					
41-50	5	185	188	190	187	180					
51-60	7	187	190	180	185	188	180	190			
61-70	10	185	185	180	190	188	187	187	190	188	190

TSH

age	no. of cases	1	2	3	4	5	6	7	8	9	10
20-30	5	0.09	0.08	0.3	0.1	0.2					
31-40	5	0.09	0.1	0.3	0.2	0.08					
41-50	5	0.3	0.09	0.2	0.08	0.1					
51-60	7	0.08	0.15	0.3	0.1	0.09	0.2	0.25			
61-70	10	0.3	0.2	0.15	0.08	0.2	0.25	0.09	0.1	0.3	0.15

Appendix 6 statistical analysis

Male hyper analysis appendix

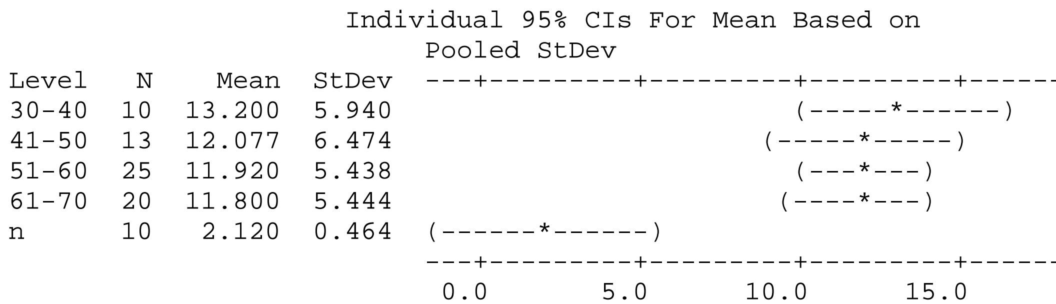
C1, C2, C3, C4, C5 represent age groups 30 – 40, 41 – 50, 51 – 60, 61 – 70, and normal respectively.

T3

One-Way Analysis of Variance

Analysis of Variance

Source	DF	SS	MS	F	P
Factor	4	866.2	216.6	7.69	0.000
Error	72	2027.5	28.2		
Total	76	2893.7			



```
                          Individual 95% CIs For Mean Based on
                                Pooled StDev
Level    N    Mean  StDev  ---+---------+---------+---------+------
30-40   10  13.200  5.940                          (-----*------)
41-50   13  12.077  6.474                        (-----*-----)
51-60   25  11.920  5.438                          (---*---)
61-70   20  11.800  5.444                         (----*---)
n       10   2.120  0.464  (------*------)
                           ---+---------+---------+---------+------
                             0.0       5.0      10.0      15.0
```

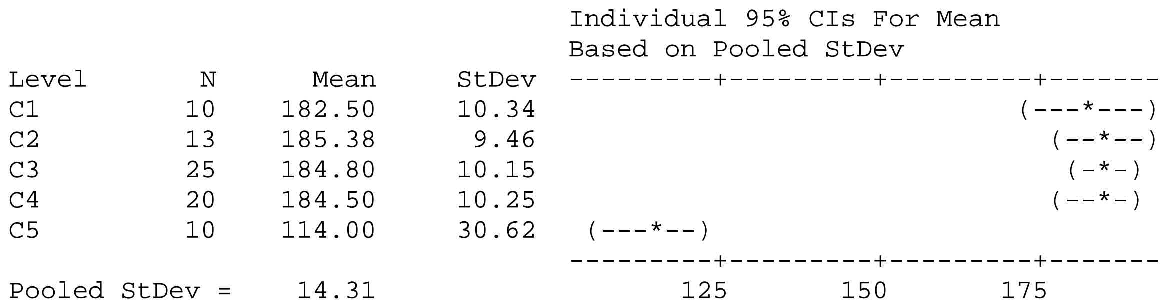
Pooled StDev = 5.358

T4

One-Way Analysis of Variance

Analysis of Variance

Source	DF	SS	MS	F	P
Factor	4	43365	10841	52.96	0.000
Error	73	14945	205		
Total	77	58309			



```
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean      StDev  ---------+---------+---------+-------
C1         10    182.50      10.34                              (---*---)
C2         13    185.38       9.46                                (--*--)
C3         25    184.80      10.15                                 (-*-)
C4         20    184.50      10.25                                (--*-)
C5         10    114.00      30.62   (---*--)
                                    ---------+---------+---------+-------
Pooled StDev =    14.31                    125       150       175
```

TSH

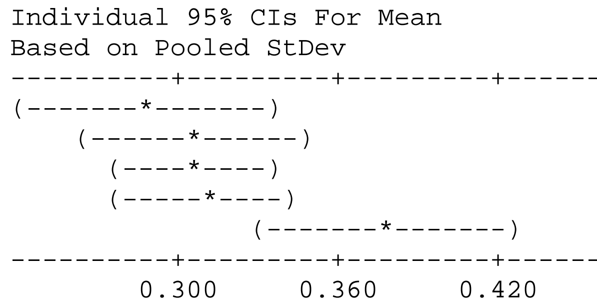
One-Way Analysis of Variance

Analysis of Variance

Source	DF	SS	MS	F	P

Factor	4	0.05196	0.01299	2.25	0.071
Error	73	0.42059	0.00576		
Total	77	0.47255			

Level	N	Mean	StDev
C1	10	0.28800	0.07743
C2	13	0.30769	0.07596
C3	25	0.30800	0.07455
C4	20	0.31000	0.07539
C5	10	0.38000	0.07888



Pooled StDev = 0.07590

Female hyper analysis appendix
C1, C2, C3, C4, C5, C6 represent age groups 20 – 30, 31 – 40, 41 – 50, 51 – 60, 61 – 70, and normal respectively.

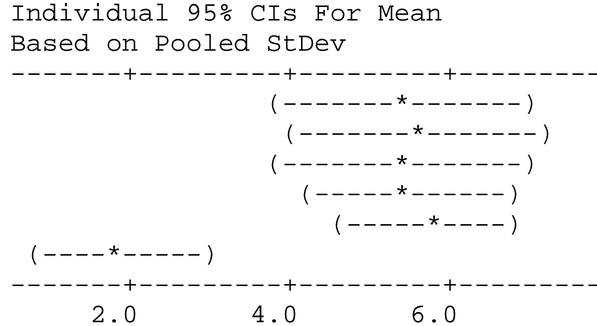
T3

One-Way Analysis of Variance

Analysis of Variance

Source	DF	SS	MS	F	P
Factor	5	104.93	20.99	7.02	0.000
Error	36	107.58	2.99		
Total	41	212.50			

Level	N	Mean	StDev
C1	5	5.400	2.074
C2	5	5.600	2.074
C3	5	5.400	2.074
C4	7	5.429	1.718
C5	10	5.700	2.003
C6	10	1.830	0.490



Pooled StDev = 1.729

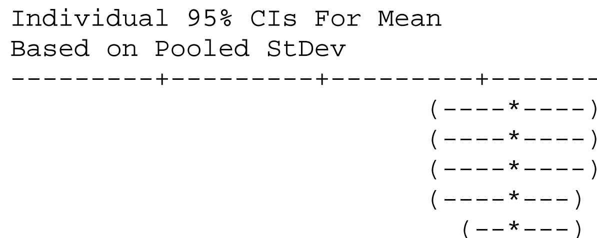
T4

One-Way Analysis of Variance

Analysis of Variance

Source	DF	SS	MS	F	P
Factor	5	49076	9815	32.69	0.000
Error	36	10809	300		
Total	41	59885			

Level	N	Mean	StDev
C1	5	186.00	3.81
C2	5	186.00	3.81
C3	5	186.00	3.81
C4	7	185.71	4.27
C5	10	187.00	3.09



```
C6          10    106.00     34.06   (--*---)
                                    ---------+---------+---------+-------
Pooled StDev =    17.33                    120       150       180
```

TSH

```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      5   0.29258   0.05852     7.48    0.000
Error      36   0.28146   0.00782
Total      41   0.57405
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev   ---+---------+---------+---------+---
C1          5   0.15400   0.09476   (-------*-------)
C2          5   0.15400   0.09476   (-------*-------)
C3          5   0.15400   0.09476   (-------*-------)
C4          7   0.16714   0.08558      (------*-----)
C5         10   0.18200   0.08217         (----*-----)
C6         10   0.36000   0.08756                          (-----*-----)
                                    ---+---------+---------+---------+---
Pooled StDev =  0.08842               0.10      0.20      0.30      0.40
```

Male hypo analysis appendix
C1, C2, C3, C4, C5 represent age groups 30 – 40, 41 – 50, 51 – 60, 61 – 70, and normal respectively.

T3

```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      4   22.1993    5.5498    80.11    0.000
Error      41    2.8404    0.0693
Total      45   25.0398
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev   -------+---------+---------+---------
C1         10    0.4550    0.1691     (--*-)
C2         12    0.4250    0.1545     (-*--)
C3          8    0.4187    0.1731    (--*--)
C4          6    0.4500    0.1871    (---*--)
C5         10    2.1200    0.4638                                (-*--)
                                    -------+---------+---------+---------
Pooled StDev =   0.2632                   0.60      1.20      1.80
```

T4

```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      4     54969     13742    39.61    0.000
```

```
Error       41     14224       347
Total       45     69193
                                     Individual 95% CIs For Mean
                                     Based on Pooled StDev
Level        N      Mean     StDev   -----+---------+---------+---------+-
C1          10     27.50     12.08   (---*---)
C2          12     30.83     13.46     (--*---)
C3           8     31.87     13.35    (----*---)
C4           6     31.67     15.71   (-----*----)
C5          10    114.00     30.62                              (---*---
)
                                     -----+---------+---------+---------+-
Pooled StDev =    18.63                  30        60        90       120
```

TSH

```
One-Way Analysis of Variance

Analysis of Variance
Source      DF        SS        MS        F        P
Factor       4    0.5104    0.1276     5.16    0.002
Error       41    1.0129    0.0247
Total       45    1.5233
                                     Individual 95% CIs For Mean
                                     Based on Pooled StDev
Level        N      Mean     StDev   --+---------+---------+---------+----
C1          10    0.6250    0.1620                  (------*-----)
C2          12    0.6542    0.1602                     (-----*-----)
C3           8    0.6250    0.1732                 (-------*------)
C4           6    0.6167    0.2137               (---------*--------)
C5          10    0.3800    0.0789    (-----*------)
                                     --+---------+---------+---------+----
Pooled StDev =    0.1572             0.30      0.45      0.60      0.75
```

Female hypo analysis appendix
C1, C2, C3, C4, C5, C6 represent age groups 20 – 30, 31 – 40, 41 – 50, 51 – 60, 61 – 70, and normal respectively.

T3

```
One-Way Analysis of Variance

Analysis of Variance
Source      DF        SS        MS        F        P
Factor       5     12.41      2.48     1.50    0.203
Error       68    112.75      1.66
Total       73    125.15
                                     Individual 95% CIs For Mean
                                     Based on Pooled StDev
Level        N      Mean     StDev   ---+---------+---------+---------+---
C1          10     0.630     0.437    (---------*---------)
C2           9     0.644     0.482   (----------*----------)
C3          20     1.130     2.316             (------*------)
C4          10     0.660     0.448    (---------*---------)
C5          15     0.653     0.484      (-------*-------)
```

```
C6          10     1.830     0.490                   (---------*---------)
                                     ---+---------+---------+---------+---
Pooled StDev =     1.288              0.00      0.80      1.60      2.40
```

T4

```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      5     55150     11030    28.35    0.000
Error      68     26458       389
Total      73     81608
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  -------+---------+---------+---------
C1         10     28.75     15.60    (----*---)
C2          9     29.17     17.23    (----*---)
C3         20     26.80     16.54     (--*--)
C4         10     24.35     16.35   (---*---)
C5         15     23.77     16.58    (--*--)
C6         10    106.00     34.06                            (---*---)
                                   -------+---------+---------+---------
Pooled StDev =    19.73                  30        60        90
```

TSH

```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      5    1.5123    0.3025    11.18    0.000
Error      68    1.8398    0.0271
Total      73    3.3522
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  --------+---------+---------+--------
C1         10    0.7800    0.1751                      (----*----)
C2          9    0.7667    0.1581                     (----*-----)
C3         20    0.7450    0.1791                      (--*---)
C4         10    0.7800    0.1687                      (----*----)
C5         15    0.8067    0.1751                        (---*----)
C6         10    0.3600    0.0876   (----*----)
                                   --------+---------+---------+--------
Pooled StDev =   0.1645                  0.40      0.60      0.80
```

Appendix 7 effect of thyroid dysfunction on fertility

Male hyper fertility

Testosterone

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
30-40	10	5	6	7	0.5	0.4	9	3	8	0.7	1															
41-50	13	3	5	0.4	6	10	7	2	9	5	0.4	4	8	0.5												
51-60	25	0.5	3	2	0.4	1	2	0.9	1	0.6	0.4	0.5	10	6	3	0.8	7	5	7	4	0.4	8	0.4	10	8	9
61-70	20	0.5	8	6	0.4	0.6	0.7	0.8	0.4	5	1	1	10	0.4	7	2	8	3	9	0.4	10					

LH

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
30-40	10	4	2	4	3	5	2	5	3	5	3															
41-50	13	3	2	2	5	2	5	4	2	5	3	3	4	4												
51-60	25	2	3	4	3	2	4	2	5	3	2	3	5	4	3	5	5	2	3	5	2	2	3	5	5	5
61-70	20	5	4	2	4	2	5	4	3	4	4	3	2	2	5	2	3	2	5	4	3					

FSH

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	2
30-40	10	4	4.5	4	5.5	5	4	5	6	6	4															
41-50	13	4	5	5.5	6	4	5.5		5	6	4.5	4	5	6												
51-60	25	4	5	4.5	6	4	4	6	5.5	5	4.5	6	4	6	5	6	5.5	4	4.5	5	5	4	4	5	6	
61-70	20	4	4.5	5	6	4	4.5	6	5.5	5	6	4	5	5.5	6	4	6	6	4	5	5					

Progesterone

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	2
30-40	10	0.2	0.6	0.3	0.5	0.4	0.6	0.2	0.5	0.7	0.8															

41-50	13	0.2	0.5	0.6	0.5	0.7	0.4	0.7	0.4	0.8	0.2	0.3	0.3	0.2												
51-60	25	0.4	0.2	0.3	0.5	0.7	0.3	0.6	0.2	0.3	0.3	0.7	0.8	0.2	0.2	0.5	0.5	0.2	0.3	0.8	0.4	0.2	0.8	0.7	0.6	0.
61-70	20	0.8	0.2	0.3	0.6	0.8	0.5	0.4	0.2	0.3	0.2	0.8	0.2	0.7	0.8	0.8	0.8	0.7	0.6	0.8	0.2					

Female Hyper fertility

Progesterone

age	no. of cases	1	2	3	4	5	6	7	8	9	10
20-30	5	1.5	2.5	2	1.5	3					
31-40	5	2	1.5	3	1.5	3					
41-50	5	3	1.5	3	2	2					
51-60	7	1.5	1.5	2.5	2	3	3	2.5			
61-70	10	3	1.5	3	1.5	2	1.5	3	2	3	1.5

FSH

age	no. of cases	1	2	3	4	5	6	7	8	9	10
20-30	5	8	3	4	5	7					
31-40	5	3	8	4	5	9					
41-50	5	3	4	7	5	6					
51-60	7	5	10	8	6	7	9	3			
61-70	10	3	9	8	4	7	10	5	5	3	6

LH

age	no. of cases	1	2	3	4	5	6	7	8	9	10
20-30	5	3	6	24	10	5					
31-40	5	10	8	3	7	24					
41-50	5	10	24	12	15	3					
51-60	7	3	5	24	6	20	15	7			
61-70	10	5	24	6	8	3	10	15	11	13	14

Testosterone

age	no. of cases	1	2	3	4	5	6	7	8	9	10
20-30	5	0.9	2	1	2	1					
31-40	5	2	0.9	2	1	1					
41-50	5	0.9	1	1	0.9	2					

51-60	7	0.9	2	2	1	0.9	1	1					
61-70	10	1	0.9	1	2	0.9	2	1	0.9	0.9	1		

Male hypo fertility

Testosterone

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12
30-40	10	5	9	5	5	9	9	7	5	7	6		
41-50	12	8	9	9		9	5	6	7	8	9	5	6
51-60	8	5	6	7	8	9	5	5	6				
61-70	6	7	9	5	7	6	5						

LH

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12
30-40	10	6	8	4	7	5	7	4	8	8	8		
41-50	12	8	5	8	4	7	4	6	8	4	8	7	8
51-60	8	4	4	7	4	5	6	8	4				
61-70	6	4	4	8	4	6	4						

FSH

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12
30-40	10	1	8	6	8	7	5	5	8	4	1		
41-50	12	4	2	3	5	8	1	1	6	7	1	8	8
51-60	8	4	6	8	1	2	1	1	5				
61-70	6	2	1	8	4	1							

Progesterone

age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12
30-40	10	0.2	0.4	0.4	0.2	0.3	0.4	0.3	0.2	0.2	0.4		
41-50	12	0.3	0.4	0.3	0.2	0.4	0.3	0.4	0.4	0.3	0.2	0.4	0.2

51-60	8	0.2	0.4	0.3	0.3	0.4	0.3	0.2	0.4
61-70	6	0.3	0.3	0.4	0.2	0.2	0.4		

Female Hypo fertility

		Progesterone concentration																			
age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
20-30	10	0.5	15	2	6	0.7	5	1	0.9	0.6	3										
31-40	9	0.5	0.7	0.8	2	5	1	7	0.6	0.7											
41-50	20	0.5	1	0.9	3	0.8	3	0.7	4	5	2	10	0.6	6	4	2	7	3	8	9	15
51-60	10	0.5	0.7	0.6	0.9	1	0.5	2	3	0.5	1										
61-70	15	0.5	2	1	0.9	9	8	0.6	3	10	0.7	6	7	0.8	4	5					

		FSH																			
age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
20-30	10	8	8	10	5	7	6	6	5	5	7										
31-40	9	5	20	9	10	8	5	10	6	7											
41-50	20	5	40	9	15	6	8	50	6	7	25	30	40	8	35	5	9	45	10	10	20
51-60	10	5	50	9	40	8	6	30	7	10	20										
61-70	15	5	5	50		10	40	30	5	50	10	20	30	40	20	50					

		LH																			
age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
20-30	10	0.09	6	5	2	3	4	3	5	4	6										
31-40	9	0.09	4	5	1	2	6	1	2	3			4	0.6	2	1	0.7	0.09	0.9	0.8	
41-50	20	0.1	5	0.2	0.09	6	0.3	6	5	0.4	0.09	3									
51-60	10	0.09	4	3	0.09	0.1	2	0.09	5	0.2	0.09	2	0.09	0.7	5	0.09					
61-70	15	0.09	3	0.3	0.09	2	0.4	0.8	4	0.9	0.1										

		Testosterone																			
age	no. of cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
20-30	10	0.2	0.3	0.2	0.3	0.5	0.2	0.4	0.4	0.2	0.5										

31-40	9	0.2	0.5	0.2	0.3	0.4	0.3	0.5	0.3	0.2											
41-50	20	0.2	0.3	0.3	0.3	0.5	0.2	0.5	0.2	0.5	0.4	0.4	0.2	0.5	0.2	0.3	0.4	0.2	0.5	0.5	0.2
51-60	10	0.3	0.2	0.4	0.3	0.4	0.5	0.2	0.3	0.4	0.2										
61-70	15	0.2	0.4	0.5	0.4	0.2	0.3	0.5	0.4	0.2	0.3	0.4	0.5	0.2	0.3	0.2					

Appendix 8 effect of thyroid dysfunction on fertility: Statistical analysis

Male hyper fertility appendix
C1, C2, C3, C4, C5 represent age groups 30 – 40, 41 – 50, 51 – 60, 61 – 70, and normal respectively.

Testosterone

```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      4     109.9      27.5     2.37    0.060
Error      73     844.6      11.6
Total      77     954.5
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  ---+---------+---------+---------+---
C1         10     4.060     3.351   (-------*--------)
C2         13     4.638     3.290      (-------*------)
C3         25     3.636     3.443    (-----*----)
C4         20     3.710     3.707    (-----*-----)
C5         10     7.300     2.751                (-------*--------)
                                   ---+---------+---------+---------+---
Pooled StDev =    3.401             2.5       5.0       7.5      10.0
```

LH

```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      4     15.64      3.91     1.07    0.380
Error      73    267.67      3.67
Total      77    283.31
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  -------+---------+---------+---------
C1         10     3.600     1.174   (-----------*-----------)
C2         13     3.385     1.193  (----------*---------)
C3         25     3.480     1.229      (-------*------)
C4         20     3.400     1.142    (--------*--------)
C5         10     4.780     4.437               (-----------*-----------)
                                   -------+---------+---------+---------
Pooled StDev =    1.915                  3.0       4.0       5.0
```

FSH

```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      4      5.99      1.50     1.09    0.367
```

```
Error       73    100.23       1.37
Total       77    106.22
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level        N      Mean     StDev  -+---------+---------+---------+-----
C1          10     4.800     0.823           (----------*---------)
C2          13     5.115     0.795                 (--------*--------)
C3          25     4.900     0.791                (------*------)
C4          20     5.050     0.793                  (------*-------)
C5          10     4.200     2.573  (----------*----------)
                                    -+---------+---------+---------+-----
Pooled StDev =     1.172           3.50      4.20      4.90      5.60
```

Progesterone

```
One-Way Analysis of Variance

Analysis of Variance
Source      DF        SS        MS        F        P
Factor       4    0.1153    0.0288     0.38    0.820
Error       73    5.4914    0.0752
Total       77    5.6067
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level        N      Mean     StDev  -+---------+---------+---------+-----
C1          10    0.4800    0.2044   (-----------*-----------)
C2          13    0.4462    0.2066   (---------*---------)
C3          25    0.4920    0.2499         (------*------)
C4          20    0.4650    0.2300      (-------*-------)
C5          10    0.5780    0.4829          (-----------*----------)
                                    -+---------+---------+---------+-----
Pooled StDev =    0.2743           0.30      0.45      0.60      0.75
```

Female hyper fertility appendix

C1, C2, C3, C4, C5, C6 represent age groups 20 – 30, 31 – 40, 41 – 50, 51 – 60, 61 – 70, and normal respectively.

Testosterone

```
One-Way Analysis of Variance

Analysis of Variance
Source      DF        SS        MS        F        P
Factor       5     5.046     1.009     4.87    0.002
Error       36     7.453     0.207
Total       41    12.499
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level        N      Mean     StDev  -------+---------+---------+---------
C1           5    1.3800    0.5675                  (--------*-------)
C2           5    1.3800    0.5675                  (--------*-------)
```

```
C3            5    1.1600    0.4722              (-------*-------)
C4            7    1.2571    0.5094                 (------*------)
C5           10    1.1600    0.4452                (-----*-----)
C6           10    0.4600    0.2675    (-----*-----)
                                       -------+---------+---------+---------
Pooled StDev =    0.4550                    0.50      1.00      1.50
```

LH

```
One-Way Analysis of Variance

Analysis of Variance
Source      DF        SS        MS        F        P
Factor       5     374.4      74.9     1.77    0.144
Error       36    1523.9      42.3
Total       41    1898.3
                                       Individual 95% CIs For Mean
                                       Based on Pooled StDev
Level        N      Mean     StDev     ----------+---------+---------+------
C1           5     9.600     8.444           (---------*---------)
C2           5    10.400     8.019            (---------*---------)
C3           5    12.800     7.662                (---------*---------)
C4           7    11.429     8.223                (-------*-------)
C5          10    10.900     6.082                (------*------)
C6          10     4.300     0.949     (------*------)
                                       ----------+---------+---------+------
Pooled StDev =     6.506                       6.0      12.0      18.0
```

FSH

```
One-Way Analysis of Variance

Analysis of Variance
Source      DF        SS        MS        F        P
Factor       5     14.66      2.93     0.60    0.698
Error       36    174.96      4.86
Total       41    189.62
                                       Individual 95% CIs For Mean
                                       Based on Pooled StDev
Level        N      Mean     StDev     ----------+---------+---------+------
C1           5     5.400     2.074        (------------*------------)
C2           5     5.800     2.588          (-------------*------------)
C3           5     5.000     1.581     (------------*-------------)
C4           7     6.857     2.410                   (-----------*----------)
C5          10     6.000     2.449                (--------*--------)
C6          10     5.300     1.889           (--------*---------)
                                       ----------+---------+---------+------
Pooled StDev =     2.205                       4.5       6.0       7.5
```

Progesterone

```
One-Way Analysis of Variance

Analysis of Variance
Source      DF        SS        MS        F        P
Factor       5    1384.9     277.0    17.40    0.000
```

```
Error       36     572.9       15.9
Total       41    1957.8
                                     Individual 95% CIs For Mean
                                     Based on Pooled StDev
Level        N      Mean     StDev   ---+---------+---------+---------+---
C1           5     2.100     0.652   (------*-----)
C2           5     2.200     0.758    (-----*-----)
C3           5     2.300     0.671    (-----*-----)
C4           7     2.286     0.636     (----*----)
C5          10     2.200     0.715     (----*---)
C6          10    15.700     7.889                             (---*---)
                                     ---+---------+---------+---------+---
Pooled StDev =     3.989              0.0       6.0      12.0      18.0
```

Male hypo fertility appendix

C1, C2, C3, C4, C5 represent age groups 30 – 40, 41 – 50, 51 – 60, 61 – 70, and normal respectively.

Testosterone

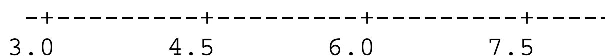
```
One-Way Analysis of Variance

Analysis of Variance
Source      DF        SS         MS        F        P
Factor       4      8.31       2.08     0.57    0.689
Error       41    150.49       3.67
Total       45    158.80
                                     Individual 95% CIs For Mean
                                     Based on Pooled StDev
Level        N      Mean     StDev   -+---------+---------+---------+-----
C1          10     6.700     1.767         (-----------*-----------)
C2          12     7.417     1.564                 (----------*----------)
C3           8     6.375     1.506    (-------------*------------)
C4           6     6.500     1.517   (---------------*---------------)
C5          10     7.300     2.751               (-----------*-----------)
                                     -+---------+---------+---------+-----
Pooled StDev =     1.916            5.0       6.0       7.0       8.0
```

LH

```
One-Way Analysis of Variance

Analysis of Variance
Source      DF        SS         MS        F        P
Factor       4     25.58       6.40     0.99    0.426
Error       41    266.07       6.49
Total       45    291.66
                                     Individual 95% CIs For Mean
                                     Based on Pooled StDev
Level        N      Mean     StDev   -+---------+---------+---------+-----
C1          10     6.500     1.650                (----------*----------)
C2          12     6.417     1.730                 (---------*---------)
C3           8     5.250     1.581       (-----------*-----------)
C4           6     5.000     1.673   (-------------*-------------)
C5          10     4.780     4.437     (----------*----------)
```




```
Pooled StDev =    2.547
```

One-Way Analysis of Variance

FSH

```
Analysis of Variance
Source     DF        SS        MS        F        P
Factor      4     28.49      7.12     0.92    0.461
Error      41    316.83      7.73
Total      45    345.33
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  ----+---------+---------+---------+--
C1         10     5.600     2.797               (--------*--------)
C2         12     4.500     2.876          (-------*--------)
C3          8     3.500     2.673    (---------*--------)
C4          6     3.667     2.875   (-----------*-----------)
C5         10     3.700     2.669      (--------*-------)
```

```
Pooled StDev =    2.780
```

Progesterone

One-Way Analysis of Variance

```
Analysis of Variance
Source     DF        SS        MS        F        P
Factor      4    0.5712    0.1428     2.50    0.057
Error      41    2.3446    0.0572
Total      45    2.9158
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
C1         10    0.3000    0.0943    (-------*-------)
C2         12    0.3167    0.0835      (------*------)
C3          8    0.3125    0.0835    (--------*-------)
C4          6    0.3000    0.0894  (---------*---------)
C5         10    0.5780    0.4829                 (-------*-------)
```

```
Pooled StDev =   0.2391
```

Female hypo analysis appendix

C1, C2, C3, C4, C5, C6 represent age groups 20 – 30, 31 – 40, 41 – 50, 51 – 60, 61 – 70, and normal respectively.

T3

One-Way Analysis of Variance

```
Analysis of Variance
Source     DF        SS        MS        F        P
Factor      5     12.41      2.48     1.50    0.203
Error      68    112.75      1.66
```

```
Total       73    125.15
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level        N       Mean     StDev ---+---------+---------+---------+---
C1          10      0.630     0.437  (---------*---------)
C2           9      0.644     0.482 (----------*----------)
C3          20      1.130     2.316           (------*------)
C4          10      0.660     0.448  (---------*---------)
C5          15      0.653     0.484    (-------*-------)
C6          10      1.830     0.490                (---------*---------)
                                    ---+---------+---------+---------+---
Pooled StDev =      1.288             0.00      0.80      1.60      2.40
```

T4

```
One-Way Analysis of Variance

Analysis of Variance
Source      DF        SS        MS        F        P
Factor       5     55150     11030    28.35    0.000
Error       68     26458       389
Total       73     81608
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level        N       Mean     StDev -------+---------+---------+---------
C1          10      28.75     15.60   (----*---)
C2           9      29.17     17.23   (----*---)
C3          20      26.80     16.54    (--*--)
C4          10      24.35     16.35  (---*---)
C5          15      23.77     16.58   (--*--)
C6          10     106.00     34.06                             (---*---)
                                    -------+---------+---------+---------
Pooled StDev =      19.73                 30        60        90
```

TSH

```
One-Way Analysis of Variance

Analysis of Variance
Source      DF        SS        MS        F        P
Factor       5    1.5123    0.3025    11.18    0.000
Error       68    1.8398    0.0271
Total       73    3.3522
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level        N       Mean     StDev --------+---------+---------+--------
C1          10     0.7800    0.1751                       (----*----)
C2           9     0.7667    0.1581                      (----*-----)
C3          20     0.7450    0.1791                       (--*---)
C4          10     0.7800    0.1687                       (----*----)
C5          15     0.8067    0.1751                         (---*----)
C6          10     0.3600    0.0876  (----*----)
                                    --------+---------+---------+--------
Pooled StDev =     0.1645                0.40      0.60      0.80
```

Appendix 9: cancer patients TH hormones measurements

cancer in Female

T3

Age	no. of cases	1	2
40-50	1	4	
51-60	2	7	10
61-70	2	5	10

T4

age	no. of cases	1	2
40-50	1	500	
51-60	2	600	300
61-70	2	400	600

TSH

age	no. of cases	1	2
40-50	1	0.2	
51-60	2	0.3	0.1
61-70	2	0.2	0.3

Male cancer

T3

age	no. of cases	1	2	3	4	5
40-50	2	5	6			
51-60	3	7	5	7		
61-70	5	5	6	7	5	7

T4

age	no. of cases	1	2	3	4	5
40-50	2	400	600			
51-60	3	600	400	700		
61-70	5	500	700	600	400	700

TSH

age	no. of cases	1	2	3	4	5
40-50	2	0.01	0.25			
51-60	3	0.25	0.1	0.01		
61-70	5	0.07	0.25	0.2	0.01	0.1

Appendix10: cancer patients statistical analysis

Male cancer analysis appendix
C1, C2, C3, C4 represent age groups 40 – 50, 51 – 60, 61 – 70, and normal respectively.

T3

```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      3    76.105    25.368    44.59    0.000
Error      16     9.103     0.569
Total      19    85.208
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  ----------+---------+---------+------
C1          2    5.5000    0.7071                   (------*------)
C2          3    6.3333    1.1547                          (-----*----)
C3          5    6.0000    1.0000                        (---*----)
C4         10    2.1200    0.4638  (--*--)
                                   ----------+---------+---------+------
Pooled StDev =   0.7543                    3.2       4.8       6.4
```

T4

```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      3   1003913    334638    37.41    0.000
Error      16    143107      8944
Total      19   1147020
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  --------+---------+---------+--------
C1          2    500.00    141.42                  (------*------)
C2          3    566.67    152.75                       (----*-----)
C3          5    580.00    130.38                         (---*---)
C4         10    114.00     30.62   (--*--)
                                   --------+---------+---------+--------
Pooled StDev =    94.57                  200       400       600
```

```
TSH
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      3   0.32526   0.10842    11.39    0.000
Error      16   0.15232   0.00952
Total      19   0.47757
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
```

```
Level        N       Mean     StDev  --+---------+---------+---------+----
C1           2    0.13000   0.16971   (---------*--------)
C2           3    0.12000   0.12124    (-------*-------)
C3           5    0.12600   0.09762      (-----*------)
C4          10    0.38000   0.07888                        (---*----)
                                     --+---------+---------+---------+----
Pooled StDev =    0.09757            0.00      0.15      0.30      0.45
```

aCancer analysis appendix

C1, C2, C3, C4 represent age groups 40 – 50, 51 – 60, 61 – 70, and normal respectively.

Female T3

```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      3    110.18     36.73    22.56    0.000
Error      13     21.16      1.63
Total      16    131.34
                                     Individual 95% CIs For Mean
                                     Based on Pooled StDev
Level        N      Mean     StDev   -------+---------+---------+---------
C1           3     4.000     1.000        (----*-----)
C2           2     8.500     2.121                      (-----*------)
C3           2     7.500     3.536                   (-----*-----)
C4          10     1.830     0.490   (--*--)
                                     -------+---------+---------+---------
Pooled StDev =     1.276                  3.0       6.0       9.0
```

T4

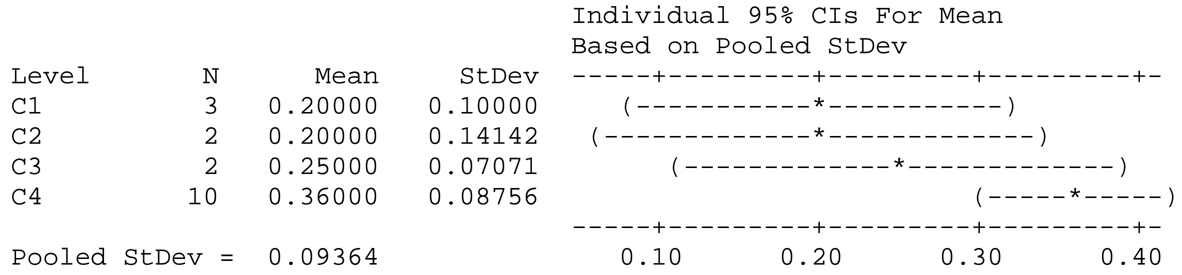
```
One-Way Analysis of Variance

Analysis of Variance
Source     DF        SS        MS        F        P
Factor      3    597266    199089    27.12    0.000
Error      13     95440      7342
Total      16    692706
                                     Individual 95% CIs For Mean
                                     Based on Pooled StDev
Level        N      Mean     StDev   --------+---------+---------+--------
C1           3    500.00    100.00                         (-----*------)
C2           2    450.00    212.13                    (-------*-------)
C3           2    500.00    141.42                       (-------*-------)
C4          10    106.00     34.06    (---*--)
                                     --------+---------+---------+--------
Pooled StDev =     85.68                   160       320       480
```

TSH

```
One-Way Analysis of Variance

Analysis of Variance
```



```
Source      DF        SS        MS        F        P
Factor       3   0.09100   0.03033     3.46    0.048
Error       13   0.11400   0.00877
Total       16   0.20500
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level        N      Mean     StDev -----+---------+---------+---------+-
C1           3   0.20000   0.10000    (-----------*-----------)
C2           2   0.20000   0.14142  (-------------*-------------)
C3           2   0.25000   0.07071       (-------------*-------------)
C4          10   0.36000   0.08756                         (-----*-----)
                                   -----+---------+---------+---------+-
Pooled StDev =   0.09364                0.10      0.20      0.30      0.40
```

الخلاصة

تضمنت هذه الدراسة 215 مريضا اظهروا أعراض خلل الغدة الدرقية وزعوا كما يأتي: 100 مريض ذوي أعراض انخفاض هرمونات الغدة الدرقية، و 100 مريض ذوي أعراض زيادة هرمونات الغدة الدرقية و 15 مريض ذوي أعراض سرطان الغدة الدرقية، فضلا عن 20 شخص من صحيحي الجسم كمجموعة سيطرة. تراوحت أعمار الأشخاص تحت الدراسة من 20 – 70 سنة. تم جمع عينات الدم من مستشفى اليرموك التعليمي في بغداد، و مستشفى الكاظمية التعليمي في بغداد، ومستشفى القديس رافاييل في بغداد، ومستشفى مرجان الجراحي في بابل، ومستشفى الزهراء التعليمي في النجف، ومستشفى البصرة العام في البصرة، ومستشفى الرمادي التعليمي في الانبار، ومستشفى الشفاء العام في الموصل، إضافة إلى مستشفى كركوك العام في كركوك.

كان الهدف الأول في البحث هو إيجاد العلاقة بين جنس وعمر المريض وخلل الغدة الدرقية. أظهرت النتائج إن مرض زيادة هرمونات الغدة الدرقية هو أعلى في الرجال منه في النساء بينما كان مرض انخفاض هرمونات الغدة الدرقية اظهر زيادة معنوية في النساء مقارنة بالرجال. ووجد أيضا إن حالات ظهور المرض متعلقة بالعمر أيضا حيث أظهرت النتائج إن ظهور المرض أعلى ضمن الفئات العمرية 41 – 50 والفئة العمرية 51 – 60 في كلا الجنسين. أما الهدف الثاني هو دراسة تأثير خلل الغدة الدرقية على الخصوبة. حيث أظهرت النتائج وجود تأثيرا معنويا بين المرض والخصوبة في كلا الجنسين حيث سجل التعداد الكلي للسائل المنوي في الرجال انخفاضا معنويا ($P<0.05$) مقارنة بالأشخاص الطبيعيين بينما لوحظ انخفاض مستوى البروجيستيرون وبفرق معنوي ($P<0.05$) في النساء اللواتي اظهرن أعراض المرض بنوعيه عند مقارنته بالقيمة الطبيعية. الهدف الثالث من الدراسة هو تشخيص الخلل النسيجي في أنسجة الغدة الدرقية. اظهر الفحص النسيجي للعينات من

المرضى وجود أدمة حلمية في حالات تضخم الغدة الدرقية ووجود تليف وأورام حميدة ضمن أنسجتها كانت أكثرها في النساء واقل في الرجال. دراسة حالات السرطان في مرضى الغدة الدرقية أظهرت وجود حالات سرطان الحلم بنسبة أعلى من سرطان اللويحات في المرضى.

الهدف الرابع هو التحليل الجزيئي لحالات خلل الغدة الدرقية باستخدام تقنية التضاعف العشوائي المتسلسل المعكوس للدم المأخوذ من الأشخاص تحت الدراسة. أظهرت النتائج عدم وجود خلل وراثي على مستوى الجينات في حالات زيادة أو نقصان هرمونات الغدة الدرقية بل إن الخلل قد يعزى لأسباب فسلجية. أما في حالات السرطان فقد اظهر التحليل الجزيئي وجود طفرة من النوع النقطي أو الحذف في حالات سرطان الحلم بينما كان الخلل في حالة سرطان اللويحات سببه وجود تضاعف أو إعادة ترتيب الجينات الغدة الدرقية.