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Genetic Association of *TSHR*, *TSHβ*, and *TPO* Genes Variants with Thyroid Disorders among Women in Misan Provinc

A Thesis-

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿يُؤْتِي الْحِكْمَةَ مَن يَشَاءُ وَمَن يُؤْتَ الْحِكْمَةَ فَقَدْ أُوتِيَ خَيْرًا كَثِيرًا وَمَا يَذَّكَّرُ إِلَّا أُولُو الْأَلْبَابِ﴾

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Dedication

First and foremost, I offer my deepest gratitude to God Almighty for His constant care, especially during the study and completion of this research.

I extend my heartfelt thanks to my twelve revered Imams, peace be upon them, and to Imam Hussein, Ali ibn Hussein, the children of Hussein, and the companions of Hussein.

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,With sincere appreciation.

Yamama Ahmed

Summary

Summary

Thyroid disorders are common endocrine conditions with significant clinical and metabolic implications, this study aims to shed light on some of the molecular and genetic basis of thyroid dysfunction in women by found the relationship between Single Nucleotide Variants SNVs in the Thyroid Stimulating Hormone Receptor (*TSHR*), Thyroid Stimulating Hormone Beta (*TSHβ*), and Thyroid Peroxidase (*TPO*) genes, and changes in Thyroid Hormones THs level and hyperthyroidism, hypothyroidism of the disease, this study was conducted in Misan Governorate, Iraq, for the period 17/10/ 2024 to 25 /5/ 2025 and involved (45) women, for age (18-46) years who were divided into three groups: group 1 (15) women with hyperthyroidism, group 2 (15) women with hypothyroidism, and group 3 (15) control women, samples were collected from the Diabetes and Endocrine Center, some private laboratories, and some districts and sub-districts, such as from (Kumait, Al-Mejar, Almaimouna and Al-Kahla), some demographic and clinical parameters were evaluated, including age, Body Mass Index BMI, family history, and hormonal parameters.

Anthropometric analysis reveales a significant increase in BMI ($P \leq 0.05$) among hypothyroid women, whereas a significant reduction in BMI ($P \leq 0.05$) was observed in hyperthyroid women compared with the control group.

There was a statistically significant increase in both mean age and family history $P \leq 0.05$ in both hyperthyroid and hypothyroid groups compared to the control group.

Hormonal results shows a significant increase in the levels of Free Triiodothyronine fT3 and Free Thyroxine fT4 in the serum of hyperthyroid women with a significant decrease $P \leq 0.05$ in the Thyroid Stimulating Hormone TSH compared to the control group, in hypothyroid women, a significant

decrease P≤0.05 in the levels of fT3 and fT4 was observed, with a significant increase P≤0.05 in TSH compared to the control group.

The molecular analysis involves DNA extraction from women with hyperthyroidism, hypothyroidism, and a control group, specific region of the gene (*TSHR*), gene (*TSHβ*), and the gene (*TPO*) were amplified using Polymerase Chain Reaction (PCR) and, the nucleotide sequence of the amplified products was then determined using DNA Sequencing Technology the obtained sequences were analyzed using bioinformatics tools to identify Single Nucleotide Variants (SNVs), with in the studied regions of the genes.

One missense genetic variation was detectes in the coding region of the *TSHR* gene at position c.477T>C, where Thymine (T) was replaced by Cytosine (C), the heterozygous (TC) genotype was found to be associated with an increased risk of developing thyroid disorders.

The present study reveales a missense transition mutation in the *TSHβ* gene at position c.113G>A, where Guanine (G) was replaced by Adenine (A), the heterozygous (GA) and (AA) genotype was found to be associated with an increased risk of thyroid disordersIn addition.

This study also identifies a missense mutation in the *TPO* gene at position c.361C>G, where Cytosine (C) was replaced by Guanine (G), the heterozygous (CG) genotype was found to be associated with an increased risk of developing thyroid disorders.

These findings suggest that genetic vairations in *TSHR*, *TSHβ*, *TPO* genes may contribute to the development of thyroid disorders in women, and potentially be used as molecular markers for early diagnosis and risk prediction.

All of these variants resultes in differences in the 3D Model of proteins in women with thyroid disorders compared to the control group.

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Abbreviations	Full Name
ATP	Adenosine Triphosphate
AITDs	Autoimmune Thyroid Diseases
AC	Adenylate Cyclase
ALB	Albumin
Aa	Amino Acid
ANS	Adenine Nucleoside Synthase
Bp	Base Pair
BMI	Body Mass Index
BMR	Basal Metabolic Rate
C	Cytosine
Ca^{2+}	Calcium Ion
CT	Calcitonin
CCP	Complement Control Protein
CH	Congenital Hypothyroidism
CI	Confidence Intervals
CAMP	Cyclic Adenosine Monophosphate
CNVs	Copy Number Variations
CCH	Congenital Central Hypothyroidism
DIO 1	Iodothyronine Deiodinase 1
DIO 2	Iodothyronine Deiodinase 2
DIO3	Iodothyronine Deiodinase 3
DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
DTC	Differentiated Thyroid Cancer
DUOX1	Dual Oxidase 1
D	Calciferol Vitamin
ELISA	Enzyme Linked Immuno Sorbent Assay
EDTA	Ethylene Diamine Tetra Acetic Acid
EGF	Epidermal Growth Factor
EF	Etiological Fraction
FSH	Follicle Stimulating Hormone
FT4	Free Thyroxine
FT3	Free Triiodothyronine
F	phenylalanine
GD	Graves' Disease
GWAS	Genome Wide Association Studies
GPCR	G Protein Coupled Receptor
Gsa-Camp	Gαs–Cyclic Adenosine Monophosphate Signaling
H2O2	Hydrogen Peroxide
HWE	Hardy Weinberg Equilibrium
HCG	Human Chorionic Gonadotropin
HPT	Hypothalamic Pituitary Thyroid
Indels	Insertion and Deletion
IP_3	Inositol Triphosphate
Ile	Isoleucine

Abbreviations

I	Iodide
Kb	Kilobase
KDa	Kilodalton
LH	Luteinizing Hormone
Lys	Lysine
MNVs	Multiple Nucleotide Variations
Mrna	Messenger Ribonucleic Acid
MCT8	Mono Carboxylate Transporter 8
MPO	Myeloperoxidase
MA	Milliamps
MSA	Multiple Sequence Alignment
NCBI	National Center for Biotechnology Information
NTIS	Non Thyroidal Disease Syndrome
NIS	Symporter of Sodium / Iodide
NGS	Next Generation Sequencing
NS	No Significant
Nt	Nucleotides
ORs	Odd Ratios
PF	Preventive Fraction
PCR	Polymerase Chain Reaction
PTH	Parathyroid Hormone
PLC	Phospholipase C
Pro	Proline
P	Phosphorus
Phe or F	Phenylalanine
PKA	Protein Kinase A
Phyre2	Protein Homology Analogy Recognition Engine V.2.0
P	Arm
Q	Arm
RNA	Ribonucleic Acid
Rs	Reference Single Nucleotide Variations
rT3	Reverse Triiodothyronine
SNVs	Single Nucleotide Variations
Ser	Serine
T or Thy	Thymine
Tyr	Tyrosine
TG	Thyroglobulin
TBG	Thyroxine Binding Globulin
TRα	Thyroid Hormone Alpha Receptor
TRβ	Thyroid Hormone Beta Receptor
TPO	Thyroid Peroxidase
TSH	Thyroid Stimulating Hormone
TSHR	Thyroid Stimulating Hormone Receptor
TRH	Thyrotrophin Releasing Hormone
T4	Thyroxine
TT4	Total Thyroxine
TT3	Total Triiodothyronine
TRs	Thyroid Hormone Receptors

Abbreviations

TREs	Thyroid Hormone Reaction Elements
TSHβ	Thyroid Stimulating Hormone Beta
TSHR	Thyroid Stimulating Hormone Receptor
TPO	Thyroid Peroxidase
TPOAb	TPO Antibodies
TgAbs	Antithyroglobulin Antibodies
TPOAbs	Antithyroid Peroxidase Antibodies
THs	Thyroid Hormones
Thr	Threonine
TTR	Transthyretin
THRA	Thyroid Hormone Receptor Alpha
THRB	Thyroid Hormone Receptor Beta
UCPs	Uncoupling Proteins
UTRs	Un Translated Regions
UV	Ultra Violet Radiation
Val	Valine
V	Volts
WB	Wash Buffer
WHO	World Health Organization
7TM	Seven Trans Membrane
3D	Model of Structure of the Protein
α	Alpha
β	Beta

Chapter one
Introduction

1. Introduction

Thyroid disorders are a significant global health problem, they have been classified into functional disorders, which are divided into hyperthyroidism (overproduction of Thyroid Hormones THs), which accelerates key metabolic processes (Muñoz-Ortiz *et al*., 2020). And hypothyroidism (deficiency in THs synthesis), which leads to a slowdown in metabolic processes (Nogueira *et al*., 2024). While structural abnormalities include goiter (An abnormal swelling of the thyroid gland) and thyroid cancer (that begins in the cells of the thyroid gland) (Huang and Gabe, 2015).

Thyroid disorders are known to be significantly more prevalent in women than in men, this difference is mainly attributed to hormonal fluctuations pregnancy, autoimmune susceptibility, and differences in thyroid gland physiology, women are therefore considered a high risk group for developing both hyperthyroidism and hypothyroidism (Al-Shahrani *et al*., 2016).

Genetic predisposition plays an important role in increasing the incidence of thyroid dysfunction, family history of thyroid disease, leads to an increased incidence of genetically transmitted diseases (Yu *et al*., 2021). Single Nucleotide Variations (SNVs) are a major genetic factor, particularly because they indicate the basis of interindividual variation in susceptibility to many diseases and, consequently, their response to treatment (Akgün *et al.*, 2015).

These variations affect many genes in different ways, particularly those responsible for and associated with thyroid function, they can alter the biological activity of the hormones they secrete, thus contributing to the occurrence of endocrine diseases, including thyroid disease (Fan and Zhou, 2024). Recent study was suggest that genes within chromosome (14) or regulatory elements have a significant impact on the incidence of thyroid diseases, extending beyond the coding region of the gene (Kaur *et al.*, 2025).

Several mutations associated with thyroid disease have been diagnosed, most of these mutations occur in the genes responsible for the synthesis of THs (Triiodothyronine T3/ Thyroxine T4) and in the Thyroid Stimulating Hormone (TSH), among these genes are the Thyroid Stimulating Hormone Receptor (*TSHR*) gene and Thyroid Stimulating Hormone Beta (*TSHβ*) gene, and Thyroid Peroxidase (*TPO*) gene (Hebrant *et al*., 2011; Maleki *et al*., 2020).

The (*TSHR*) gene, located on chromosome (14), this gene has been associated with several thyroid diseases, including hypothyroidism and Autoimmune Thyroid Diseases (AITDs) (Naghibi *et al.,* 2022).

The (*TSHβ*) gene located on chromosome (1), is also a contributing factor; it plays a vital role in thyroid function, variations in it can lead to disruption of hormonal signaling, or sometimes, this gene is involved in the development of thyroid tumors (Muthukrishnan *et al.*, 2010).

There is also the (*TPO*) gene located on chromosome (3), which is important and essential for the synthesis of THs, any change in it, such as a variations, can lead to a loss of its function, leading to thyroid diseases such as Congenital Hypothyroidism (CH), demonstrating its effective role in early endocrine development (Ris-Stalpers and Bikker, 2010; Xu *et al*., 2025).

However, to the best of our knowledge, no previous molecular studies have investigated the association of SNVs in *TSHR*, *TSHβ* and *TPO* genes with thyroid disorders among women in Misan province.

To the best of our knowledge, no prior molecular studies have examined the association between (SNVs) in the *TSHR*, *TSHβ*, and *TPO* genes and link it to physiological studies and thyroid disorders among women in Misan Province, given the limited availability of genetic research in this region particularly studies addressing thyroid dysfunction this investigation focuses on analyzing sequence variations in these three genes, such an approach provides valuable

insights into the molecular mechanisms underlying thyroid disorders and enhances our understanding of their pathophysiology.

1.1The Aims of the study

The aim of this study is to investigate the association between genetic variants in the *TSHR*, *TSHβ*, and *TPO* genes and the susceptibility to thyroid disorders among women in Misan Province, Iraq, the study focuses on identifying specific single (SNVs) within these genes and evaluating their potential impact on thyroid hormone levels and clinical presentation in affected individuals compared to healthy controls through:

1.To measure the serum levels of fT3, fT4 and TSH in women with hyperthyroidism and hyporthyroidism and compare them with the control group.

2.To amplify selected regions of the *TSHR*, *TSHβ*, and *TPO* genes using Polymerase Chain Reaction (PCR).

3.To determine the nucleotide sequences of the amplified regions using DNA sequencing techniques.

4.To identify Single Nucleotide Variations (SNVs) in the studied gene regions and compare their frequencies among the groups.

5.To analyze the association between the detected SNVs and the risk of developing thyroid disoeders.

Chapter Two
Literature-Review

2.Literature-Review

2.1 The Thyroid Gland

The thyroid gland is one of the largest endocrine glands in the human body (Anandkumar *et al.*, 2020). It has a butterfly like shape, its name comes from the Greek word (Thureos), meaning shield (D'Arbo Alves and Cintra Gabarra, 2019; Luaibi *et al*., 2021). It occupies a distinctive position in the front of the neck, specifically below its cartilage, known as (Adam's Apple), it arises from two distinct lobes, right and left, connected by an isthmus, in addition, there is a median structure, which is a pyramidal lobe that sometimes protrudes from this isthmus (Khan and Farhana, 2019).

Histologically, the thyroid gland is divided into lobules, and each lobule contains approximately (20-40) spherical follicles that vary markedly in size, with a diameter ranging from (45) to (250) μm, in newborns, the follicles are initially small and gradually enlarge with age, each follicle is lined by a single layer of cuboidal epithelial cells, most follicles are bordered by cuboidal thyrocytes, whereas some may display a multilayered follicular epithelium under specific physiological conditions, upon stimulation, these cells assume a columnar shape, resting on a thin basement membrane surrounding a central acidophilic colloid, thyrocytes exhibit distinct polarity, with their apical surfaces facing the follicular lumen and their basal surfaces attached to the basement membrane, the apical membrane contains numerous microvilli that extend toward the colloid, supporting hormone synthesis and secretion, the nuclei are typically spherical and located near the basal region of the cells (Bergman *et al*., 1989; Nilsson and Fagman, 2017).

The thyroid gland is predominantly composed of these spherical follicles, which synthesize and store colloid a viscous protein rich substance filling the follicular lumen (Dauod, 2017).

The thyroid gland tissue consists of spherical thyroid follicles, which are lining up by a layer of follicular epithelial cells filled with a sticky substance comprised of a glycoprotein called (Colloid), its main and basic component is the protein Thyroglobin Tg, which works as a basic precursor for the manufacture of THs, as it depends on iodine, which is the main component of these hormones (Basolo *et al*., 2022).

There are also cells called Parafollicular cells or C cells, which are located in the gaps in the follicles and release the hormone Calcitonin CT, which is a vital hormone responsible for Ion Calcium Ca^{2+}, stabilization (Bobyreva *et al*., 2021) (Figure 2-1).

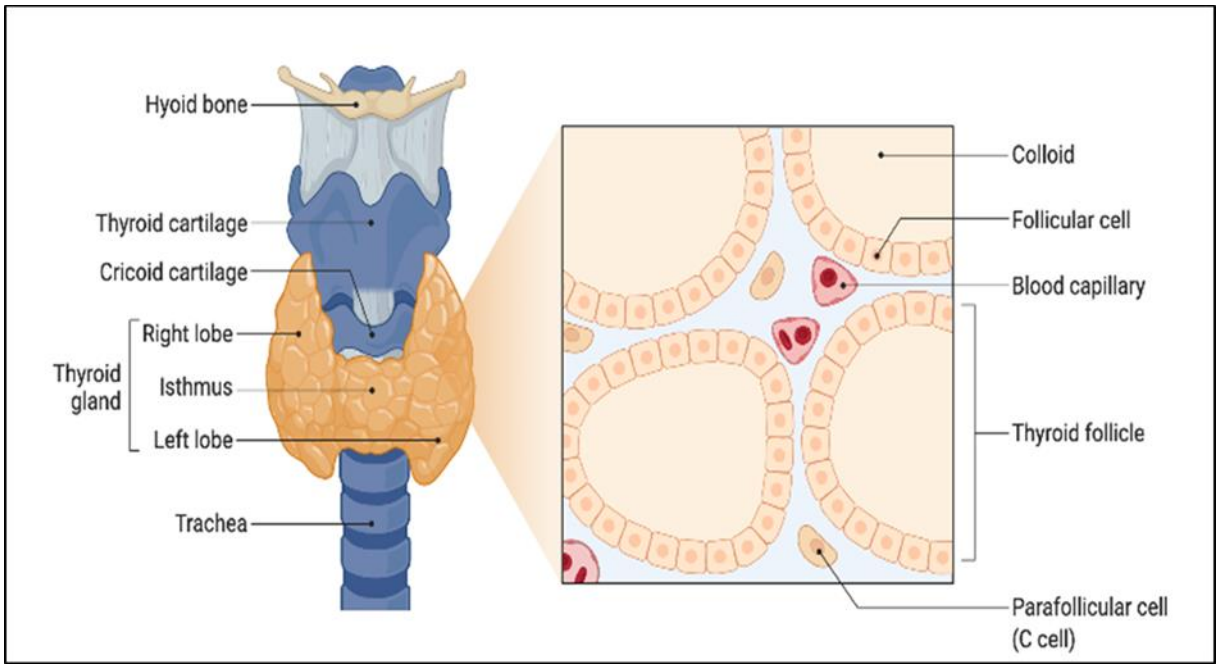


Figure (2-1): The Position and structure of thyroid gland (Capuzzo, 2021).

The thyroid gland is supplied with a dense network of blood vessels, characterized by an abundant blood supply from the superior and inferior thyroid arteries, which is of utmost importance in supporting the functions of the endocrine glands in general and the thyroid gland in particular (Branca *et al.*, 2022). The thyroid gland receives its arterial blood supply primarily from the superior and inferior thyroid arteries, the superior thyroid artery originates from

the external carotid artery, whereas the inferior thyroid artery arises from the subclavian artery (Waugh and Grant, 2014). In rare cases, an additional arteryreferred to as the thyroid ima artery (Neubauer's artery) may be present, branching from the common carotid artery or directly from the brachiocephalic trunk (Mohebati and Shaha, 2012). Venous drainage occurs through the superior thyroid veins, which empty into the internal jugular vein, and the inferior thyroid veins, which drain into the left brachiocephalic vein via the plexus thyroideus impar, lymphatic drainage predominantly passes through the lateral deep cervical lymph nodes and the pre and paratracheal lymph nodes, the gland receives sympathetic innervation from the superior cervical and cervicothoracic ganglia of the sympathetic trunk, while parasympathetic fibers are supplied by the superior and recurrent laryngeal nerves (Yalçin *et al*., 2007).

It is necessary to distinguish between the thyroid gland and the adjacent Parathyroid glands PTH, despite their anatomical proximity, they are two independent entities, each with its own distinct functions, the parathyroid glands are responsible for regulating Ca^{2+}, through the secretion of PTH, which increases Ca^{2+}, and reduces Phosphorus P, in the blood, they also have a role for Vitamin D by enhancing its synthesis (Liu *et al*., 2020).

2.1.1 Thyroid Hormones (THs)

The thyroid gland is primarily responsible for producing T3 and T4 from its follicular cells, in addition, the parafollicular cells produce CT, and these hormones are subject to close control by the Hypothalamic Pituitary Thyroid axis HPT, the negative feedback mechanism is the center of action of this axis, where the high level of T3 and T4 hormones in the bloodstream prevents the release of either Thyrotrophin Releasing Hormone TRH and TSH (Akhtamovna and Mustafoevich, 2024).

THR is secreted from the hypothalamus, the anterior pituitary gland releases TSH, this hormone binds to its receptor, called the (TSHR), specifically on the follicular cells (Colella *et al.*, 2020; Ertek, 2021; Wenzek *et al*., 2022; Sadiq and Tadi, 2023) (Figure 2-2).

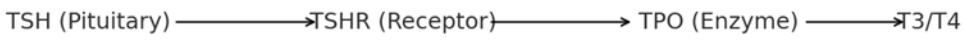


Figure (2-2): Overview of the TSH–TSHR–TPO pathway leading to thyroid hormone synthesis.

THs play an effective role in regulating metabolic processes, temperature generation, and growth, they work to modify the Basal Metabolic Rate (BMR) by affecting the increased activity of mitochondria, and also the extent of oxygen consumption in the various areas of the body's tissues (Anandkumar *et al.*, 2020). Any defect in the regulation of TH secretion leads to many systemic diseases, if there is an overproduction or increase in the production of hormones, it leads to hyperthyroidism however, if hormone production is deficient, this leads to hypothyroidism (Ozolek, 2021).

Also, through these hormones, especially T3, as they work to increase the body's heat production, through mitochondrial Uncoupling Proteins (UCPs) and increase the rate of ATP turnover, thus contributing to maintaining the body at a balanced temperature, thus, people with hypothyroidism suffer from an inability to tolerate cold, while people with hyperthyroidism suffer from an inability to tolerate heat (Romero-Ibarguengoitia *et al*., 2024).

2.1.1.1 Triiodothyronine (T3)

T3 is the biologically active form of THs (Salih and Yenzeel, 2020). Its playing a central role in oxygen consumption and regulating cellular metabolism and growth (Gumaah and Hussein, 2024). The amount of this hormone is small because the majority that circulates in the body is the result of the terminal conversion of T4, this conversion occurs in many tissues in the body, including the kidneys, liver, and skeletal muscles, and is catalyzed by deiodinase enzymes (Kabak and Kendüzler, 2024).

The effect of this hormone is when it binds to the Thyroid Hormone Receptors THR, which is one of the nuclear transcription factors that depend on the ligand, when T3 binds, the resulting T3-TR complex interacts with specific Thyroid Hormone Reaction Elements TREs located in the promoter regions of target genes, this interaction leads to the active transcription of these genes, thus affecting many different cellular functions, including those related to basic processes, including protein synthesis, carbohydrate and fat metabolism, and mitochondrial respiration (Gumaah and Hussein, 2024).

Low levels of T3 in the serum of people cause many diseases, including Shock (a medical emergency resulting from insufficient blood flow throughout the body, affecting the function of tissues and organs) or Sepsis, (life threatening condition that occurs when the body's response to an infection injures its own tissues and organs), T3 concentrations frequently decrease without an increase in TSH until they are replaced, this phenomenon is known as Low T3 Syndrome or Non Thyroidal Disease Syndrome (NTIS), and it is still an active area of research (Takahashi *et al*., 2021).

2.1.1.2 Thyroxine (T4)

T4 is the main hormone secreted by the thyroid gland, and its function is to prepare for T3, the more metabolically active hormone, unlike T3, which has

a direct effect (Salih and Yenzeel, 2020). Approximately 80% of the T4 secreted each day is metabolized by deiodination, with approximately 40% being changed to T3 and 40% to Reverse Triiodothyronine rT3 the remaining 20% is used by sulfate, and glucuronide conjugation and oxidative deamination to form tetraiodothyroacetic acid, and ether connect cleavage (Brassard, 2024; Salman *et al.*, 2024).

The primary effect of the hormone is when it is converted to T3 and binds to TRs within the nuclei of target cells, resulting in either the initiation or inhibition of gene transcription, in contrast, T4 has an affinity and activity for T4 receptors (Gunjača *et al.*, 2024).

2.1.2 Biochemical Pathways and Chemical Structures Underlying Thyroid Hormone (THs) Formation

THs are characterized by a phenolic ring linked to tyrosine through an ether bond and incorporate one to four iodine atoms, (T4), also called 3,5,3’,5’-tetraiodothyronine, contains four iodine atoms, whereas (T3) exists primarily as 3,5,3’-triiodothyronine, with the alternative isomer 3,3’,5’-triiodothyronine (reverse T3; rT3) being biologically inactive (Lum *et al*., 1984). T4 is synthesized in thyroid follicular cells as a component of Tg, while T3 differs by having one less I atom per molecule (Ishihara *et al*., 2003). Iodination of tyrosine residues within Tg generates Monoiodotyrosine (MIT) and Diiodotyrosine (DIT), the coupling of two DIT molecules produces T4, whereas coupling of one MIT with one DIT yields T3, these reactions are catalyzed by (TPO), impairment of TPO activity whether congenital or acquired disrupts organification of I and leads to elevated intrathyroidal non organified I (Roti *et al*., 1994; Ogasawara *et al*., 2001).

T4 is generally considered a prohormone and serves as a circulating reservoir for the biologically active form, T3, conversion of T4 to T3 occurs in

peripheral tissues through enzymatic deiodination mediated by deiodinases, defects in deiodinase activity can mimic iodine deficiency due to impaired production of active T3, despite its lower concentration, T3 is the primary biologically active TH (Boelaert and Franklyn, 2005).

2.1.2.1 Endocrine Regulation and Molecular Biosynthesis of Thyroid Hormones (THs)

HPT axis plays a fundamental role in regulating metabolic processes across all body cells, thus maintaining normal physiological function, secretion of (TRH) from the hypothalamus activates the HPT axis (Hiller-Sturmhofel and Bartke, 1998). TH production is regulated through two major mechanisms. First, secretion of (T4) and (T3) from the thyroid gland is stimulated by (TSH), circulating T3 and T4 exert negative feedback on TSH production, while TRH stimulates its release, TRH secretion increases under conditions such as exposure to cold, enhancing metabolism and heat generation, this first regulatory mechanism provides a finely tuned control system to maintain stable thyroid function, the second regulatory mechanism involves peripheral conversion of T4 to T3, which occurs primarily outside the thyroid gland, this process is modulated by multiple physiological factors that differ among tissues, enabling rapid metabolic adaptation in conditions such as non thyroidal illness (Santini *et al*., 1996).

Within the HPT axis, TRH released from the hypothalamus stimulates thyrotrophs in the anterior pituitary to produce and release TSH, TSH then acts on thyroid follicular cells, promoting the secretion of T4 (approximately 80%) and T3 (approximately 20%), once released into circulation, T4 is converted to T3 via deiodinase enzymes, circulating T3 and T4 subsequently suppress TSH secretion through negative feedback. T3 is considered the predominant inhibitor of TSH release, due to the high sensitivity of TSH to small fluctuations in free

T4 levels, changes in TSH are often detected earlier than alterations in free TH concentrations in both hypothyroidism and hyperthyroidism, the relationship between TSH and THs is log linear, whereby minimal changes in T3/T4 levels result in significant shifts in TSH concentrations (Mariotti and Beck-Peccoz, 2016). TSH production can also be suppressed by somatostatin, elevated glucocorticoid levels, sex hormones (such as estrogen and testosterone), and excessive iodide concentrations in the bloodstream (De *et al*., 2004).

THs synthesis begins with the active uptake of I "Iodide Trap," from the bloodstream into thyrocytes, this constitutes, the first stage and is mediated by the sodium/iodide symporter (NIS), which transports iodide across the basolateral membrane into the cell cytoplasm (Zhang *et al*., 2024). In the second stage, iodide (I^-) is transported across the apical membrane into the follicular lumen by pendrin and possibly by an additional human apical iodide transporter (Chakraborty, 2024). The third stage consists of three major biochemical reactions catalyzed by (TPO), first, iodide is oxidized to molecular iodine (I_2) within the colloid, second I binds to tyrosyl residues on (Tg), forming (MIT) and (DIT), third, coupling reactions occur: two DIT residues combine to form (T4), while one MIT and one DIT combine to generate (T3) (Miranda *et al*., 2023).

During the fourth stage, following stimulation of the thyroid gland by (TSH), iodinated thyroglobulin is endocytosed back into the follicular cells (Berlińska and Świątkowska-Stodulska, 2024). In the fifth stage, vesicles containing iodinated Tg fuse with lysosomes, where proteolytic enzymes cleave Tg and release T4, T3, as well as MIT and DIT residues (Bolton and Panciera, 2023). Finally, in the sixth stage, THs are secreted into the bloodstream through Monocarboxylate Transporter 8 (*MCT8*), the I released from MIT and DIT is efficiently recycled for new organification processes, thus maintaining hormone synthesis (Giwi, 2024) (Figure 2-3).

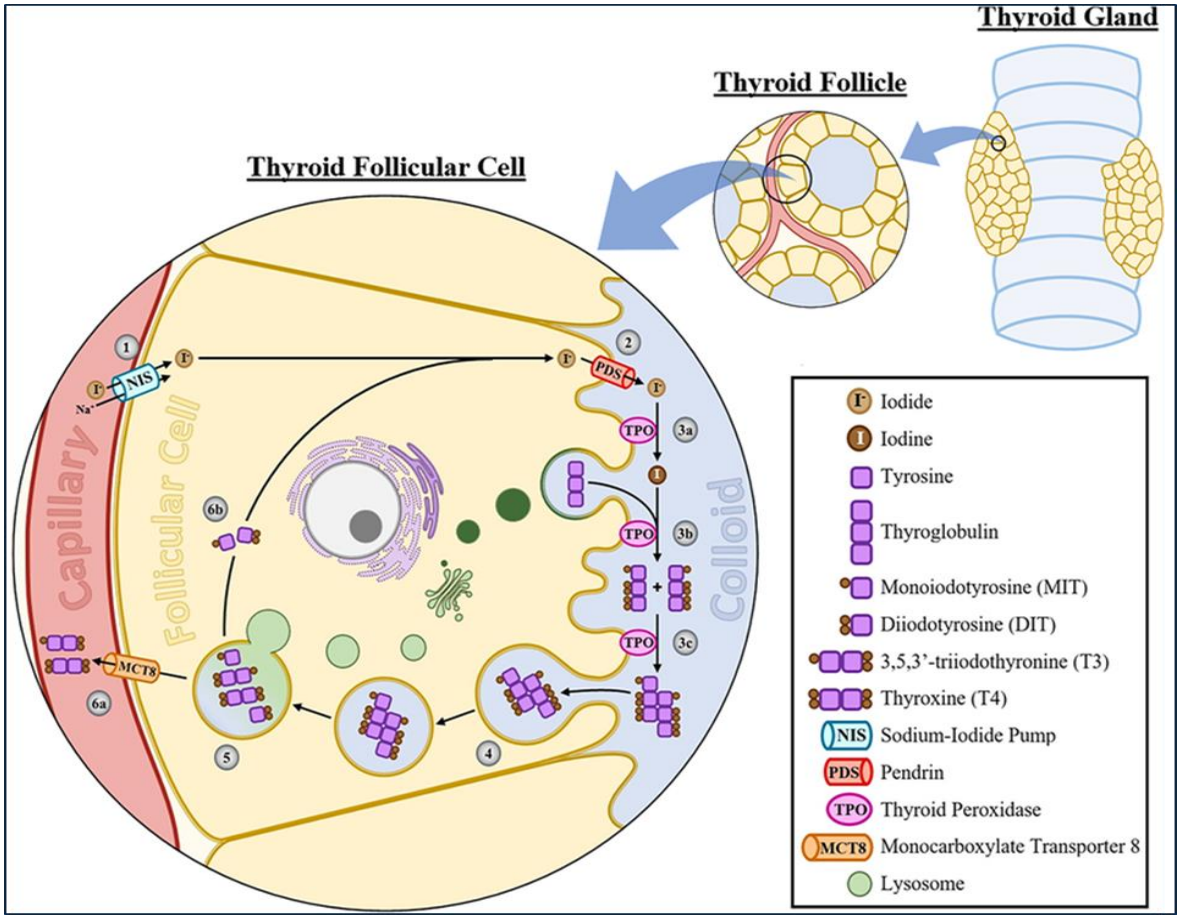


Figure (2-3): Thyroid hormone biosynthesis (Bolton and Panciera, 2023).

2.1.2.2 Genomic Actions of Thyroid Hormones THs: Molecular Pathways Linking Hormone Binding to Gene Expression

The biological effects of THs are primarily exerted through genomic mechanisms, where they regulate gene expression, however, there are also non genomic pathways that also influence cellular functions, when T3 and T4, the two main hormones, are secreted into the bloodstream, they bind to plasma proteins, primarily Thyroxine Binding Globulin TBG, Albumin ALB, and Transthyretin TTR (Bertolini, 2024).

Only a small portion remains in the free form, called Free fT3, Free fT4 and represents the active portion that enters target cells, upon entering cells, T4 is converted to T3 by deiodinase enzymes, of which there are three distinct types (Mohammed *et al*., 2020).

1.Type I Deiodinase D1: Which is found mainly in the kidneys and liver (Giwi, 2024).

2.Type II Deiodinase D2: Which is expressed in many major tissues, including the pituitary gland and brown adipose tissue (Modder *et al*., 2024).

3.Type III Deiodinase D3: Which has a role in converting THs from, for example, T4 to T3 (Salvatore *et al*., 2011).

When T3 enters the target cell, T3 will bind to the TRs, which are known as nuclear transcription factors activated by the ligand (Brtko, 2021).

These receptors are encoded by the Thyroid Hormone Receptor Alpha (*THRA)* gene which is located on chromosome (17), and Thyroid Hormone Receptor Beta (*THRβ)* gene, which is located on chromosome (3) (Torabinejad *et al*., 2023). There are two isoforms types of Thyroid receptors TRs, Thyroid Receptor Alpha TRα which are mainly found in the brain, heart, and skeletal muscles, and Thyroid Receptor Beta TRβ which is found in abundance in the kidneys, liver, and pituitary gland (Gnocchi *et al*., 2016).

When T3 interacts with TR, T3-TR the receptor hormone complex is formed , this complex binds to specific DNA sequences known as TREs in the promoter region of the target genes (Yao *et al*., 2022).

This interaction stimulates either gene transcription or gene inhibition, depending on the recruitment of co-activators or late inhibitors, thus, this precise genomic regulation is what governs thyroid hormones, thus exerting their influence on the main biological processes that occur in the body, humans, including metabolism, growth, and heat generation (Shi, 2021) (Figure 2-4).

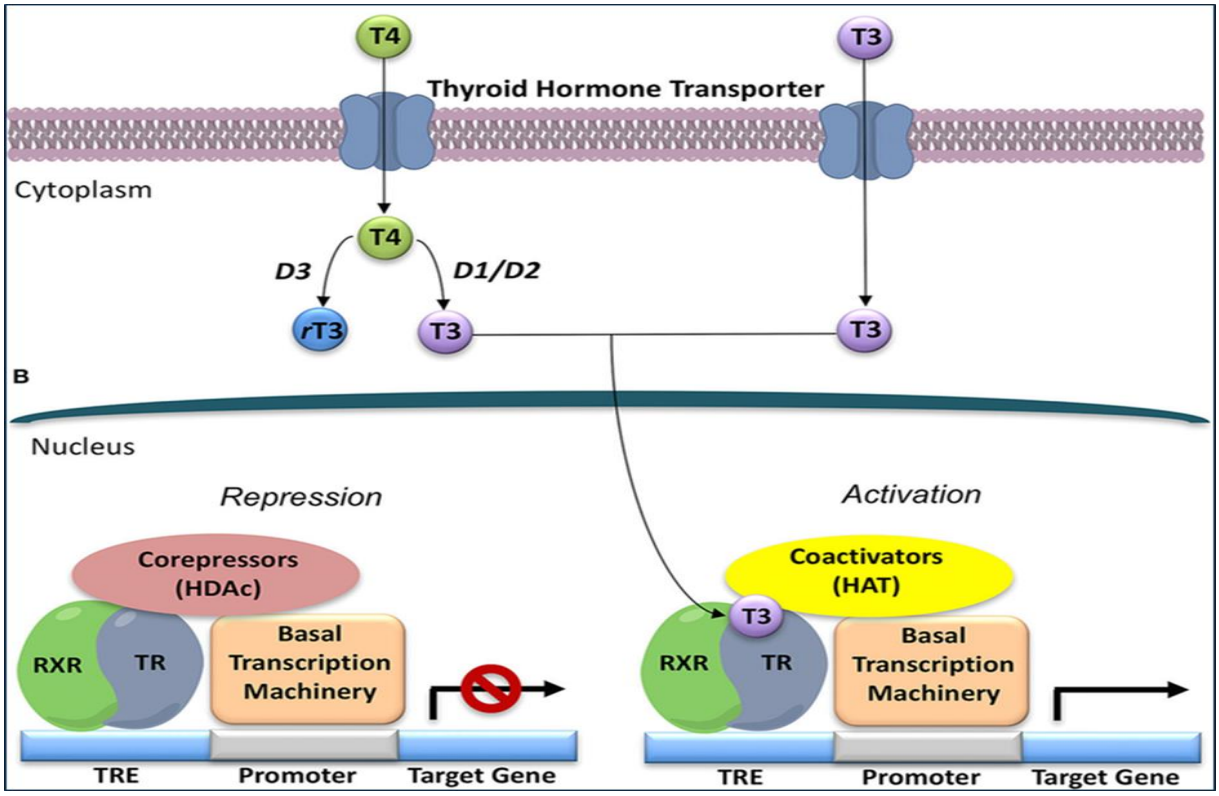


Figure (2-4): Mechanism of Thyroid Hormone Action (Saponaro *et al*., 2020).

2.2 Disorders of The Thyroid Gland

Thyroid disorders encompass a range of conditions associated with THs T3 and T4, it is usually divided into two main categories, hypothyroidism and hyperthyroidism, depending on whether TH levels are raised or reduced (Shabsigh *et al.*, 2008). Thyroid disorders represent one of the most common endocrine diseases worldwide, and their prevalence is increasing in many regions, including the Middle East and Iraq, recent epidemiological studies have reported that thyroid dysfunction is particularly widespread among women in Arab countries, with hypothyroidism and hyperthyroidism constituting the most frequently diagnosed endocrine conditions in clinical practice (Al-Shahrani *et al*., 2016; Ahmed *et al*., 2020; Al-Jubouri *et al*., 2022).

Women are disproportionately affected by thyroid disorders due to hormonal and immunological factors, and thus represent a high risk group (Al-Shahrani *et al*., 2016).

In the US National Health and Nutrition Examination Survey, the prevalence of hypothyroidism was 4.6%, and the prevalence of hyperthyroidism was 1.3% (Saran *et al.*, 2016).

The measurement of serum TSH concentration is used to identify thyroid dysfunction, for the diagnosis of thyroid gland diseases, serum TSH remains the most effective and dependable test to date (Ladenson *et al*., 2000). When TSH levels are higher than the reference range, hypothyroidism is diagnosed when they are lower than the reference range, hyperthyroidism is identified (Molina *et al.*, 2007).

TH levels in the blood are used to categorize thyroid disorders (Vanderpump, 2011). Thyroid disorders can be caused by a goiter, one or more cell nodules, low or excessive TH output, or any combination of these, Grave's Disease (GD), Hashimoto's thyroiditis, adenoma, thyroid cancer, hyperthyroidism, and hypothyroidism are among the other common thyroid problems (Schultz *et al*., 2011; Grais and Sowers, 2014).

2.2.1 Hyperthyroidism

Hyperthyroidism is a clinical condition characterized by the excessive production and secretion of thyroid hormones (T3 and T4), which leads to an increased basal metabolic rate and hyperactivity of multiple organ systems. The most common causes include Graves' disease, toxic multinodular goiter, and solitary toxic adenoma, recent epidemiological data indicate that hyperthyroidism affects approximately (0.8–2.0%) of the global population. Women are (4–10) times more likely to develop hyperthyroidism than men, mainly due to hormonal fluctuations and increased susceptibility to autoimmune conditions, in Middle Eastern countries, including Iraq, hyperthyroidism remains among the most frequently diagnosed endocrine disorders in clinical practice (Chaker *et al*., 2017; Taylor *et al*., 2018; Al-Jubouri *et al.*, 2022).

Patients with this disorder suffer from excessive production of THs, which leads to an acceleration of major metabolic processes, its symptoms include weight loss, tremor in the hand, increased appetite, difficulty sleeping, increased heartbeats, increased sweating, and severe eye problems, known as Grave's ophthalmopathy (Muñoz-Ortiz *et al*., 2020).

Hyperthyroidism can be Overt Hyperthyroidism or Subclinical Hyperthyroidism, Overt Hyperthyroidism is characteincreased by low serum TSH levels and raised serum levels of thyroid hormones T3, T4, or both, but Subclinical Hyperthyroidism is characteincreased by low serum TSH, but normal serum T3 and T4 levels (Tsai and Leung, 2021).

From a genetic perspective, many mutations have been identified in thyroid genes, including somatic activating mutations that occur in the (*TSHR)* gene, especially in autonomic thyroid adenomas, where they lead to constitutive activation of the thyroid receptor and thus sustained production, independent of TSH stimulation, these mutations enhance congenital Cyclic Adenosine Monophosphate (cAMP) signals, thus enhancing the proliferation of thyroid cells (Castro *et al*., 2009).

The diseases that affect the thyroid gland and cause excessive production of its hormones, such as toxic multinodular goiter and toxic adenoma, where the thyroid nodules overproduce hormones independently of regulating the work of TSH, as the toxic tumor represents a single independent nodule, there is also multinodular goiter, both of which contribute to excessive production of THs (Davies *et al*., 2020).

In addition, there is acute thyroiditis or postpartum thyroiditis, which causes temporary hyperthyroidism due to the release of hormones formed by damaged follicles (Bouloux, 2022). There is I induced hyperthyroidism, known as the Goodpassed Phenomenon, where exposure to I affects individuals

exposed to it, there are rare diseases that lead to pituitary tumors that secrete TSH, causing them to develop secondary hyperthyroidism, in fewer cases (Muñoz-Ortiz *et al*., 2020).

2.2.2 Hypothyroidism

Hypothyroidism is a condition in which the thyroid gland fails to produce sufficient amounts of THs, resulting in a reduced metabolic rate and generalized slowing of physiological processes, the disorder may be classified into overt hypothyroidism, characterized by elevated TSH and reduced fT4 levels, and subclinical hypothyroidism, where TSH is elevated while fT4 remains within normal range, recent epidemiological studies report that overt hypothyroidism affects (1–2%) of adults, while subclinical hypothyroidism may occur in (5–8%), particularly among women and older individuals, the higher prevalence in women is attributed to pregnancy related hormonal shifts and increased risk of autoimmune thyroiditis (Chaker *et al*., 2017; American Thyroid Association, 2022; Kahapola-Arachchige *et al*., 2020).

Hypothyroidism is one of the most common thyroid disorders worldwide, occurring when there is a deficiency in the production of hormones secreted by the thyroid gland, and thus, a slowdown in the main metabolic processes occurs, among the symptoms associated with it are weight gain, intolerance to cold, bradycardia, and other symptoms (Chaker and Peeters, 2022; Nogueira *et al*., 2024; Rad *et al*., 2024).

There are two types of Primary Hypothyroidism: Subclinical Hypothyroidism SCH, when T3 and T4 is normal and TSH is increased, and Clinical Hypothyroidism, where T4 is decreased and TSH is elevated (Ross, 2022). In Secondary Hypothyroidism, T4 and T3 is dropped yet TSH is either reduced or normal (Koyyada and Orsu, 2020).

I deficiency is the main factor in the occurrence of hypothyroidism, especially in areas where I intake is low and below the required level in newborns and pregnant women, when the I level is severely low, this leads to a defect in neurodevelopment and also the appearance of cretinism, especially if it is not treated (Männistö *et al.*, 2010).

In terms of genetic factors that are the cause of the appearance of hypothyroidism, many mutations occur in genes encoding enzymes that work to synthesize THs, such as (TPO) and (Tg), some of them can cause CH when there is a defect in the formation of hormones (Chaker *et al.*, 2022). As (TPO) is a membrane bound enzyme that is very necessary for the oxidation of I and its addition to tyrosine residues on Tg, this is one of the important and key steps in the synthesis of THs, autoantibodies that target this (TPO) enzyme can also activate complement pathways, and also stimulate antibody dependent cytotoxicity, thus ending in the destruction of the thyroid follicular cells, Hashimoto's eventually develops into overt hypothyroidism, this is regarding the immune mechanisms, autoimmune (Kopp, 1998).

Also, if SNVs occur in the (*TSHβ)* gene, which is the gene that encodes (TSHB), it causes isolated central hypothyroidism, in cases of rarely, when I levels are sufficient, SNVs is the most common cause of primary hypothyroidism, which is Hashimoto's thyroiditis, which is known as a chronic autoimmune disorder characterized by destroying the follicular cells and eventually causing fibrosis, thyroid autoantibodies, especially Antithyroglobulin Antibodies (TgAbs), and Antithyroid Peroxidase Antibodies (TPOAbs), are used in diagnostic matters that give a result of the ongoing immune injury (Chaker *et al*., 2022).

In addition, when there are mutations in TH transport defects, this leads to a defect in the hormone’s bioavailability in tissues, it has been linked to the

Monocarboxylate Transporter 8 (*MCT8*) gene, which mediates the entry of thyroid hormones into cells, it is characterized by severe neurodevelopmental disorders and symptoms similar to hypothyroidism (Park and Chatterjee, 2005).

2.3 Genetic Variation: Definition and Forms

Genetic variation is defined as the differences that occur in the DNA sequence between individuals within a population, these differences are considered a molecular basis for phenotypic diversity in many traits, including behavior, fingerprint, shape, and individual susceptibility to disease, thus, this genetic variation is considered an essential pillar of evolutionary adaptation and natural selection, as it provides an effective response to many environmental pressures for population groups (Charlesworth *et al*., 2017).

There are many mechanisms in which genetic variation is involved, including mutations that are either spontaneous or occur when, environmental factors result in new nucleotide changes in the genome, ranging from a slight change of a Single Nucleotide Variations (SNVs) or Multiple Nucleotide Variations (MNVs), alleles can also be rearranged between homologous chromosomes during genetic recombination during meiosis, thus producing different alleles in offspring, in addition, genetic material is exchanged between population groups during mating or migration of individuals, as this gene flow enhances allelic diversity, there are many types of genetic variation that have multiple forms that have functional or structural effects, which distinguish them, including (Johnston, 2024).

1.Single Nucleotide Variations (SNVs): This type of genetic variation represents the most common and widespread form within the human genome, and it serves as an essential tool for investigating genetic diversity among individuals and across different population groups (Morgil *et al*., 2020).

2.Deletion and Insertion Processes Indels: Their action is either by adding one or more nucleotides or losing them, in coding regions, these deletion and insertion processes lead to frameshift mutations, thus causing a change in the protein structure or disrupting the gene function Tay-Sachs disease (Savino *et al*., 2022).

3.Copy Number Variations (CNVs): Are a difference in the deletion or duplication of large parts of the genome, thus affecting the gene dosage, as they may contribute to a developmental disorder or cause cancer (Pös *et al*., 2021).

4.Structural Differences Include: Rearrangement processes and are widespread, including segmental duplications, translocations, and inversions, the results of these differences are a change in gene expression by modifying the chromatin structure or changing regulatory interactions (Wang *et al*., 2020).

5.Microsatellites: Are they are short, tandemly repeated DNA sequences characterized by a large number of polymorphisms and are used in forensic analysis (Keerti and Ninave, 2022).

6.Regulatory Polymorphisms: Are found in variations in non concentrated regions such as enhancers or promoters and regulate the level of gene expression and transcriptional activity, thus affecting an individual's susceptibility to disease (Wu *et al*.,2024).

7.Haplotype Polymorphisms: These represent specific combinations of alleles inherited together due to a linkage imbalance, they affect the haplotype structure and interactions between genes and are relevant to linkage studies (Bhat *et al*., 2021).

8.Epigenetic Polymorphisms: In this case, no change occurs in the DNA sequences, as the response is often from environmental factors such as toxins, stress, dietary pattern, or lifestyle, including DNA methylation and histone modification (Abdul *et al*., 2017).

2.3.1 Single Nucleotide Variations (SNVs):

SNVs is most frequently seen when a nucleotide shifts from one purine base to another (adenine to guanine, for example), known as a transition, or from a purine base to a pyrimidine base (guanine to cytosine, or vice versa), known as a transversion (Hailemariam and Yadeta, 2020). The SNVs located in promoter regions typically influence gene expression through a variety of mechanisms, including changes in the activity of the promoter, methylation of DNA, modification of histone proteins, and effects on the binding of transcription factors (He *et al*., 2016).

They have been identified in many genes associated with various types of cancer and are found in diverse genomic regions, including promoter sequences, exons, introns, and translated regions, because they have a potential regulatory and structural impact, and their effects depend on their presence at the site, as they can either affect protein function, binding, or gene expression, and are mostly associated with many complex diseases (Sameer *et al*., 2021; Al-Shuhaib, 2024).

When a single nucleotide change occurs at a population frequency ≥1%, it is classified as a Single Nucleotide Polymorphism (SNP); if its frequency is <1%, it is considered a (SNV) (Brookes, 1999; Feuk *et al*., 2006; Cooper and Shendure, 2011).

2.3.2 The Relationship between Single Nucleotide Variations SNVs and Thyroid Disorders

Thyroid disorders are affected by many factors, including genetic factors and environmental factors, they are classified into two sections:

1.structural abnormalities, which change the size of the thyroid gland, causing many diseases, including goiter, tumors, and thyroid nodules.

2.functional abnormalities include changes in the increase or decrease in hormone secretion, causing many disorders, including hyperthyroidism and hypothyroidism.

Some diseases affect the thyroid gland and are monogenic, but the majority of them arise as a result of the cumulative effect of, especially (MNVs), which lead to modifications in the function of receptors, hormone synthesis and gene expression (Huang and Jap, 2015).

Many (SNVs) in genes have been linked to the extent of an individual's susceptibility to diseases, hormone synthesis, and modification of immune tolerance, among these genes are (*TSHR)* and (*TPO*) genes , such as if any change occurs in thyroid genes this leads to a significant impact on the process of synthesis and metabolism of TH levels, thus affecting the onset of any disease.

The response to treatment, transferring, the effect of variation in thyroid hormone receptor genes, which affects the calculation of receptors or on, the availability of TH T3 and its biological effect or the effect on deiodinase genes (Fan and Zhou, 2024).

It has been shown that familial genetic predisposition has an impact on thyroid diseases, including hypothyroidism and AITDs, and that genetic variants play a role in the dysfunction that affects the thyroid gland and thus causes its diseases, including Hashimoto's disease and (GD), which are among the most common AITDs and may coexist with other autoimmune diseases, thus indicating the presence of a common immune genetic mechanism (You *et al.*, 2019).

In addition, there are other mechanisms that cause thyroid diseases, including epigenetic mechanisms that contribute to gene expression independently of the primary DNA sequence (Bender, 2004). Including

regulation of DNA methylation, there is a note found for hypermethylation of genes related to the thyroid gland, including (*TSHR)* or (*TPO)*, which inhibits gene expression and thus disrupts thyroid function, this is what happens when hypothyroidism occurs, especially Hashimoto's disease thyroiditis (Jones and Takai, 2001).

2.4 Thyroid Genes

TSHR , *TSHβ*, and *TPO* constitute a core regulatory axis controlling THs synthesis and signaling, therefore, protein altering (SNVs) in these genes may modulate receptor activation, enzyme efficiency, and ultimately serum TH levels (Balmiki *et al*., 2014; Chaker *et al*., 2017; Jameson and De Groot, 2022; Kaplan *et al*., 2022; Zufry and Hariyanto, 2024) (Figure 2-5).

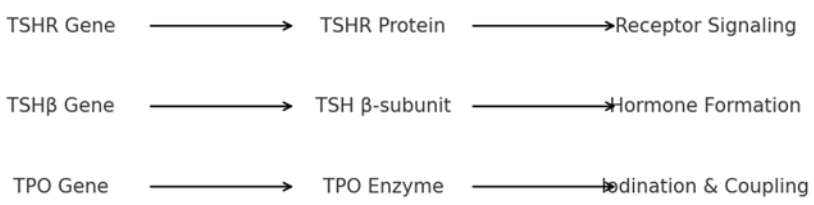


Figure (2-5): Conceptual Relationship between *TSHR*, *TSHβ*, and *TPO* genes, their Protein products, and their Functional roles.

2.4.1 Thyroid Stimulating Hormone Receptor (*TSHR)* Gene

The (*TSHR)* gene encodes the (TSHR), which is located on chromosome (14) in the bundle (31) (14q31) and consists of (10) exons, the length of this gene is (190778bp), its translated into a membrane glycoprotein consisting of (764) amino acids and functions as a G Protein Coupled Receptor GPCR, its molecular weight is approximately (84) kDa (Murat, 2021).

Structurally, this receptor consists of a signal peptide consisting of (21) amino acids, a large extracellular domain encoded by exons (1) to (9), a

transmembrane domain encoded by exon (10), and a transmembrane domain, which typically consists of seven alpha helical segments, these are characteristic of the GPCR family, this is followed by a cytoplasmic tail that mediates intracellular signaling (Tuncel, 2017) (Figure 2-6).

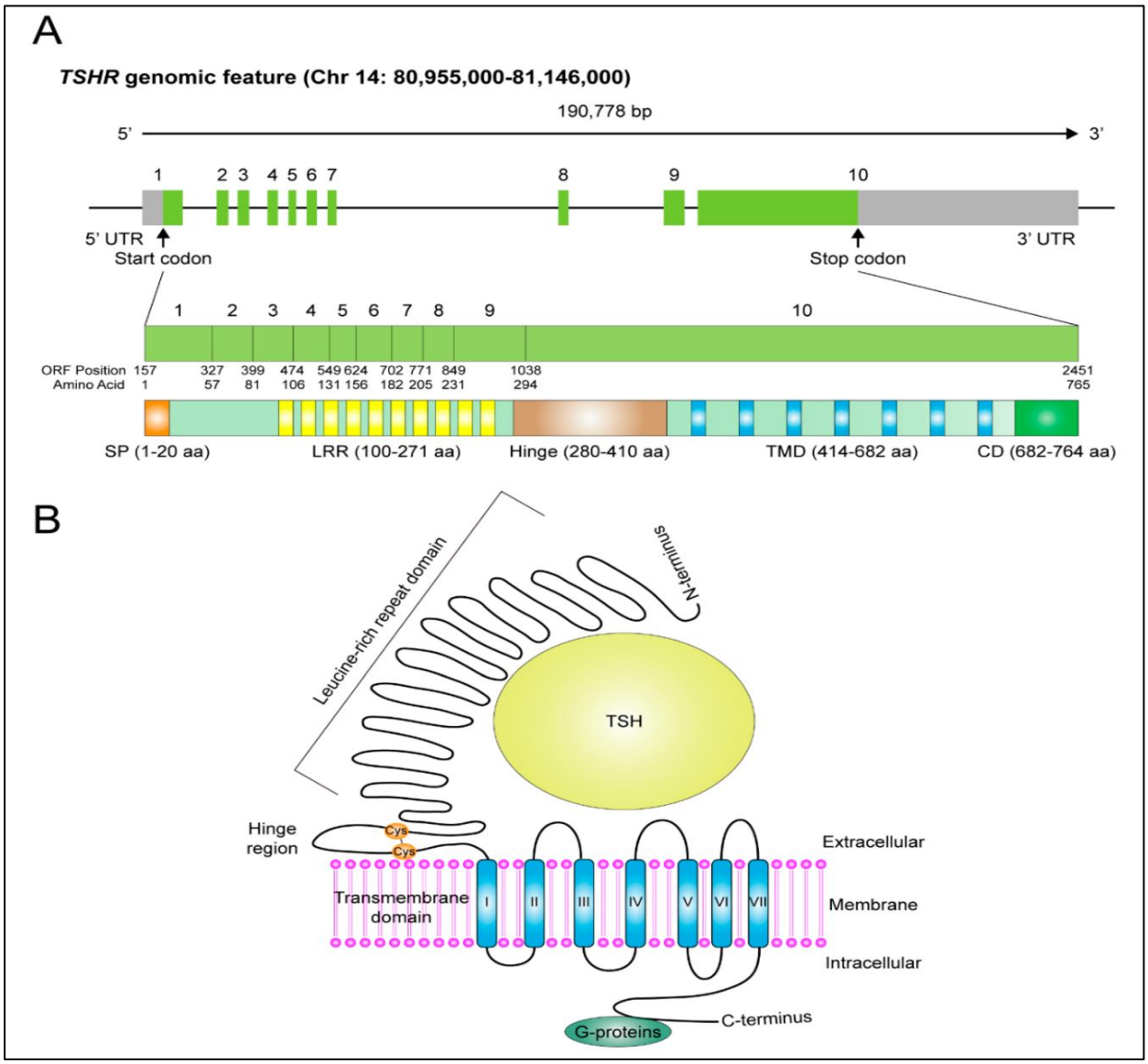


Figure (2-6): Protein Structure and Genetic Characteristics of Thyroid Stimulating Hormone Receptor. (A) Structure of the *TSHR* Gene (B) Structure of TSHR Protein (Chu and Ye, 2020).

In its basic form, the (TSHR) is expressed on the basolateral surface of folliular cells of the thyroid gland when the (TSHR) binds with TSH thyroid

gland activation, of the receptor occurs through two main intracellular signaling pathways by coupling two G protein coupling (Yeste *et al*., 2024).

1.Gsa-cAMP Gαs Cyclic Adenosine Monophosphate Signaling Pathway (Galpha Stimulatory Subunit): The enzyme Adenylate Cyclase AC stimulates this pathway, and thus this stimulation leads to an increase in intracellular cAMP levels, which subsequently activates the Protein Kinase A PKA, finally, this pathway enhances the synthesis of TG and also the uptake of I through the Symporter of Sodium / Iodide NIS, thus achieving complete production of THs (Latif *et al*., 2020).

2.Gq Protein Coupled Receptor Phospholipase C (PLC) IP_3/DAG Signaling Pathway: Stimulates this pathway, thus leading to the production of Inositol Triphosphate IP_3 and Diacylglycerol DAG, which is followed by Ca^{2+} mobilization, to control the synthesis of THs, this pathway contributes to IP_3/Ca^{2+} where it exerts either a regulatory or an inhibitory effect under certain physiological conditions (Yeste *et al*., 2024) (Figure 2-7).

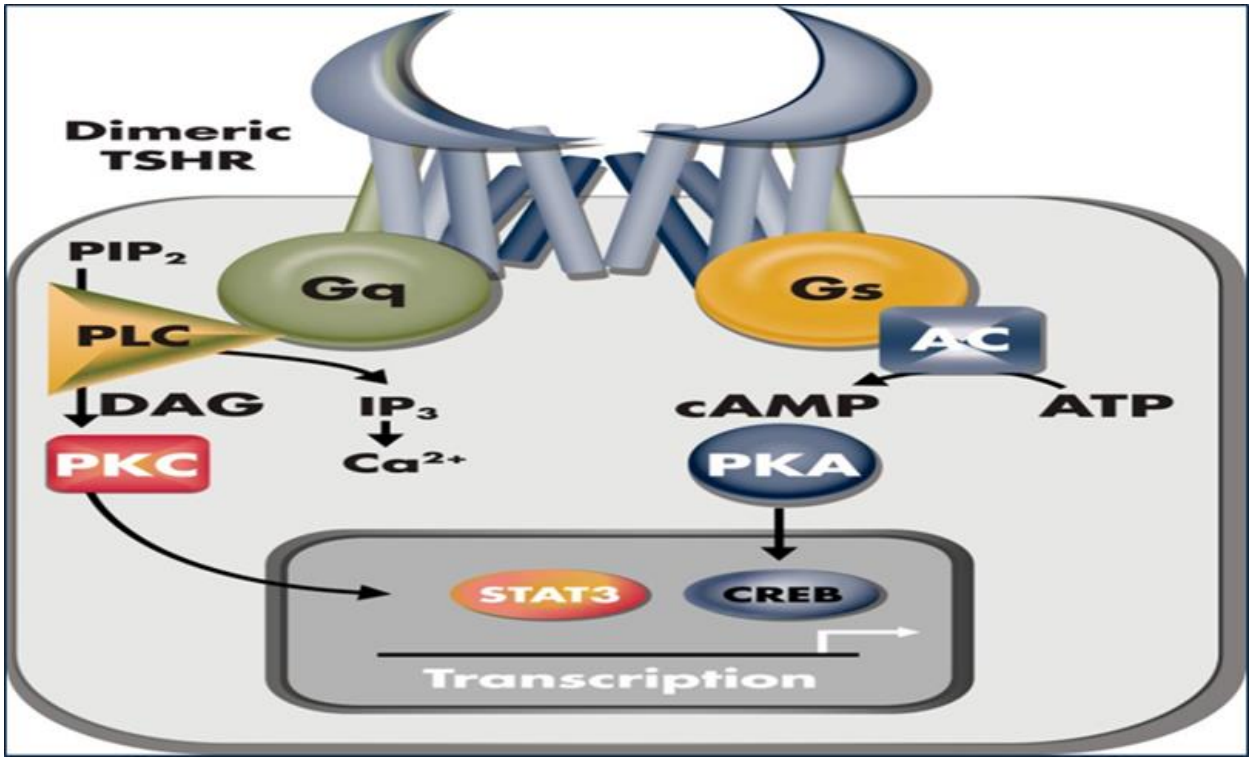


Figure (2-7): Mechanism Action of Thyroid Hormone Receptor (Bassett and Williams, 2016).

2.4.1.1 SNVs in the *TSHR* Gene and their Relationship to Thyroid Disorders

SNVs in TH metabolites have been associated with many thyroid disorders, especially functional and autoimmune ones (Castro *et al*., 2009). Including hypothyroidism, which causes Hashimoto's thyroiditis, hyperthyroidism, which causes (GD), toxic multinodular goiter, and autoimmune thyroid nodules (Stephenson *et al*., 2020). Common genetic variations in SNVs that occur may cause changes in the function or expression of genes within the (*TSHR)* gene, these changes can lead to either hyperactivity or inhibition of receptor activity, thus affecting the production of THs and its function, these polymorphisms may be present in non coding regions or may also be present in coding regions, thus affecting regulatory mechanisms or affecting the structure of receptors (Naghibi *et al*., 2022). Some variations cause gain of function mutations, which result in persistently active receptors, such as the (TSHR), where the production of THs is stimulated in excess, leading to hyperactivity (Stephenson *et al*., 2020).

In contrast, these variations may weaken receptor activity, causing loss of function and, consequently, hypothyroidism, differences in signaling efficiency, protein stability and receptor binding occur (Stephenson *et al*., 2020; Gnanavel, 2023). In addition, specifically in AITDs conditions such as (GD) and Hashimoto's thyroiditis, SNVs variations have been linked to thyroid disorders in (TSHR), which are associated with somatic mutations in (TSHR) that impair hormone production and, consequently, hyperthyroidism, these genetic changes contribute to the susceptibility and development of the disease (Kaur *et al*., 2024). Variations at the (*TSHR*) in Germ cells may affect immune tolerance and also contribute to the production of autoantibodies (Pujol-Borrell *et al*., 2015).

There are also somatic mutations in the (TSHR) that cause autoimmune thyroid nodules, where the receptors become activated and thus the production

of THs is unregulated (Stephenson *et al*., 2020). SNVs located in promoter regions or non coding regions can alter the expression of (TSHR) (Begum *et al*., 2023). Which play a role in disease by affecting their binding efficiency, mRNA stability, or the binding of transcription factors (TSHR) (Stefan and Faustino, 2017). Mutations within the (*TSHR*) can disrupt the receptor's function and cause many thyroid disorders, these mutations may impair signal transmission or cause loss of TSH binding function, causing CH or hypoplasia of the thyroid gland, these mutations are found in exon (10), where they often follow an autosomal recessive inheritance pattern (Fokina and Shpakov, 2022).

In contrast, there are mutations, such as gain of function mutations, that cause constitutive activation of the (TSHR) even in the absence of TSH, causing hyperthyroidism, the cause of which is excessive and uncontrolled production of hormones, and also due to the proliferation of follicular cells (Stephenson *et al.*, 2020). Given that the expression of (TSHR) in tissues outside the thyroid gland has an impact on genetic and physiological systems due to the influence of genetic variations in this receptor, which is a focus of attention and exploration within the framework of scientific research and due to its potential role in autoimmune disorders and the effect of SNVs on immune function in (TSHR) (Naghibi *et al*., 2022). Genetic testing has helped predict the risks of many diseases and thus assist in therapeutic intervention, while current research discusses mechanistic pathways for thyroid receptors affected by polymorphisms of nucleotides, especially when the disease appears (Gnanavel, 2023). Among these studies that identified the existence of associations between SNVs in the TRH and GD, a study conducted in Singapore reported the existence of an association between SNVs in the (*TSHR)* gene, specifically in the intron (1), and (GD) (Ho *et al.*, 2003).

A also another study that found an association between SNVs in the (*TSHR)* gene, specifically in intron (1) and intron (7), and a predisposition to

GD when studying the Japanese population and Caucasian populations (Dechairo *et al*., 2005). It is worth noting that there is rs12101255, it was found in sites close to the start codon or close to the promoter region in intron (1), which could indicate that its effect is either in post-translational modifications or on gene expression (Bogusławska *et al*., 2022). However, another study was conducted on the Chinese population, where no association was observed between three of the genetic variations SNVs rs179247, rs12101255, and rs2268458 and GD (Xu *et al*., 2011). There is also a study conducted on the Lebanese population, where they shown that there was no association between hypothyroidism and the number of nucleotide polymorphisms in (TSHR), specifically in rs2268458 (Al-Azzam *et al*., 2014).

Some genetic variations were also counted for the last (5) years, according to what was recorded on the National Center for Biotechnology Information NCBI database 2025, as shown in Table (2-1).

Table (2-1): Some Studies of the *TSHR* Gene Variation (NCBI, 2025).

SNVs	Location in Gene	Molecular Effect	Reference
rs2268458	Intron 1	Associated with Reduced TSHR Expression in the Thymus, Possibly Impairing Immune Tolerance.	(Tyagi *et al*., 2024).
rs1054708	Exone	Reported to Exert a Protective Effect Against Autoimmune Thyroiditis.	(Zaaber *et al*., 2020).
rs179247	Intron 1	Alters TSHR Expression in the Thymus and Influences Immune Regulation.	(Zufry and Hariyanto, 2024).

2.4.2 Thyroid Stimulating Hormone Beta (*TSHβ*) Gene

TSH belongs to the glycoprotein family of hormones, many hormones belong to this family, including Follicle Stimulating Hormone FSH, Luteinizing Hormone LH, and Human Chorionic Gonadotropin HCG, these hormones have a heterogeneous structure and consist of a common subunit called (Alpha) and a special subunit called (Beta), this subunit has biological properties (Yang *et al.*, 2024).

The (*TSHβ)* gene is located on the short arm of chromosome (1), specifically (1p13.2) at location (chr1:115,029,826-115,034,309), it is approximately (4.5) kb long and consists of three exons and two introns, the first exon of which is untranslated, the remaining two exons code for the functional protein, consisting of (138) aa (Nicholas *et al*., 2017) (Figure (2-8).

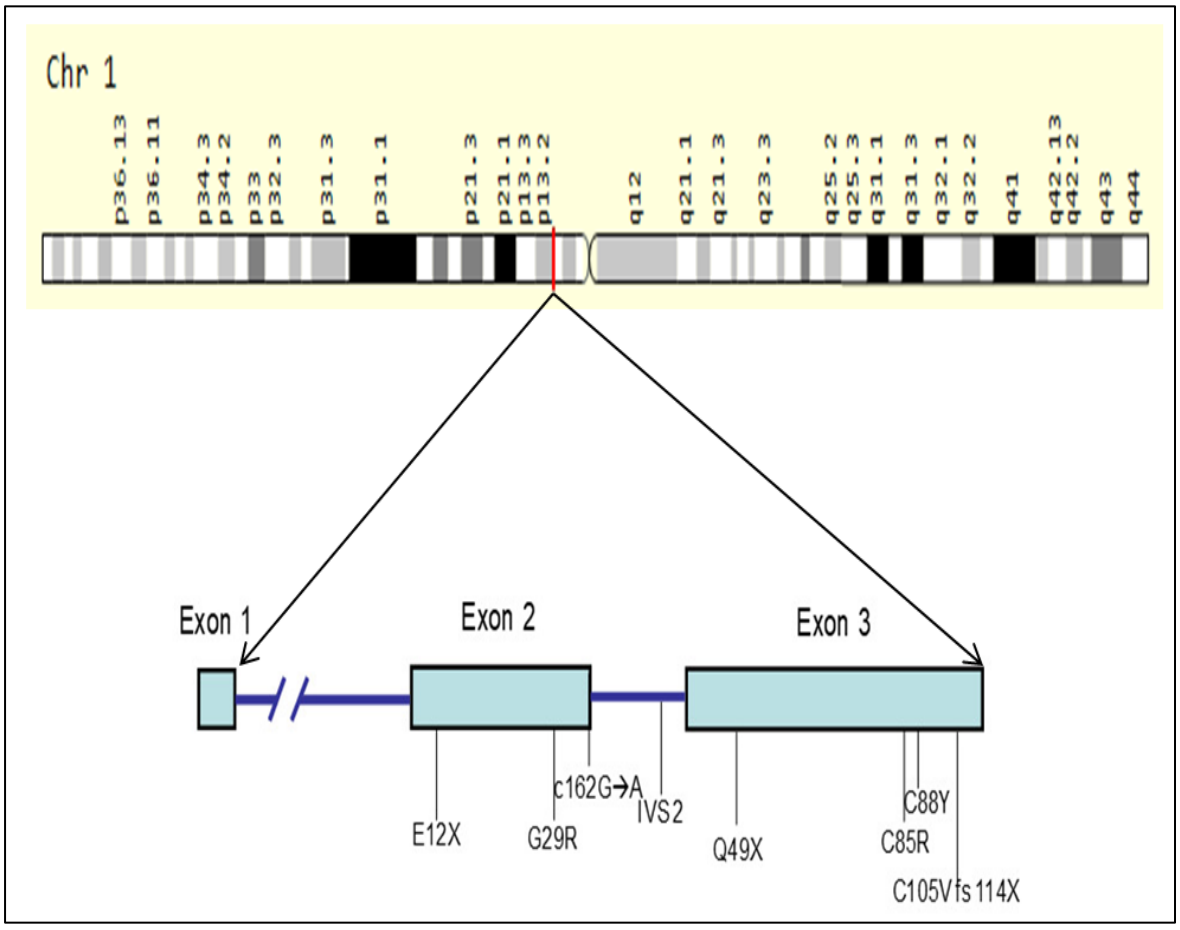


Figure (2-8): Structure of *TSHβ* Gene (Nicholas *et al*., 2017).

Glycosylation plays an important and critical role in the activity and stability of TSH, as many of its sites are linked to the nitrogen of the beta subunit, this linkage is important and necessary for the process of protein folding and secretion in the correct manner (Alrasheed, 2013).

When the beta subunit is active, it forms a non covalent complex with a subunit called the alpha subunit, this bond, which is often described as structurally as the beta subunit wraps around the alpha subunit, similar to a seatbelt, and is therefore necessary for high affinity binding to the thyroid hormone receptor (Kaplan *et al*., 2022).

When TSH is secreted from the pituitary gland, (TSHβ) binds to the (TSHR), which is located on the basolateral membrane of the follicular cells in the thyroid gland, this interaction activates a signaling cascade for protein receptors compared to Gs Protein Coupled Receptor GPCR, it stimulates the enzyme AC, which subsequently increases the level of congenital AMP (CAMP) inside the cells, the latter stimulates the intermediate pathway for transcription of genes involved in the synthesis of THs T3 and T4, it also stimulates I uptake and enhances the proliferation of thyroid cells, among all of this, TSH and, consequently, (TSHβ) play a fundamental role in regulating metabolic balance and physical growth, as well as in heat generation (Latif *et al*., 2020) (Figure 2-9).

The level of TSH is considered a good indicator in peripheral blood, as it works as a screening test to determine thyroid function, the main function of this hormone is to stimulate its receptor, the (TSHR), and thyroid cells ultimately, THs are produced (Ushakov, 2024).

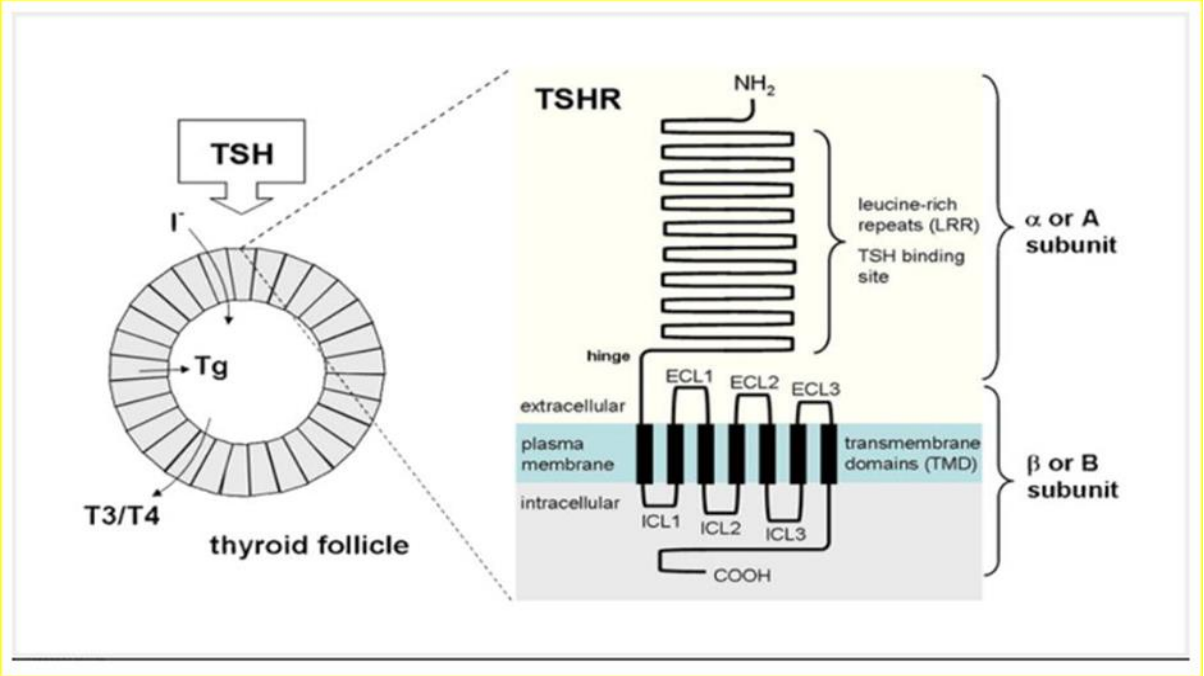


Figure (2-9): Thyroid Hormone Binding site with Receptor (Latif *et al*., 2020).

2.4.2.1 SNVs in the *TSHβ* Gene and their Relationship to Thyroid Disorders

Several (SNVs) have been identified within the *TSHβ* gene, among these is rs201857310, located specifically in the coding region of exon 2, in addition, rs7530810 has been reported within the promoter region of the gene, these genetic variations have been investigated for their potential influence on gene expression and their association with an increased susceptibility to thyroid related disorders (Hansen *et al*., 2004).

Despite the advances in this field, the precise functional consequences of these variants on the regulation of (TH) production remain an active area of research, some studies have suggested that polymorphisms in the *TSHB* gene may alter serum hormone concentrations in affected individuals and could potentially influence therapeutic dosing requirements (Samollow *et al*., 2004).

Study used Next Generation Sequencing NGS, through which new and recurring variations were identified between different population groups (Moon *et al*., 2021).

Other studies have shown that there are variants that may weaken TSH dimers or may activate receptors, which proves their pathogenic role, it was also discovered in the (*TSHβ)* gene, specifically in the regulatory elements of gene expression, including transcription factors and non coding RNA, that they have certain new mechanisms that may cause thyroid dysfunction, the (*TSHβ)* gene genotype also contributed to the development of a strategy for those affected by thyroid disorders (Bianco *et al*., 2019). Regarding mutations that occur in the (*TSHβ)* gene, they cause many diseases, including isolated central congenital hypothyroidism, which is an autosomal recessive disorder characterized by low levels of TSH in the serum of affected individuals, while the activity of TRH is high or normal, these mutations cause the beta subunit to be incomplete or truncated, thus resulting in impaired formation of heterodimers with the alpha subunit, or the secretion of biologically active TSH is blocked (Dacou-Voutetakis *et al*., 1990).

Genetic variants in the (*TSHβ)* gene also include missense, nonsense mutations and splice site mutations, each of which produces diverse phenotypes, for example, when disulfide or glycosylation bonds are disrupted when the mutation occurs, it leads to protein instability or an imbalance in receptor interaction, compound heterozygous mutations or homozygous mutations may also occur in individuals who suffer from persistently low T4 and low or normal TSH (Persani *et al*., 2019).

According to Al-Rasheed *et al*. (2015) nucleotide variations and mutations in the (*TSHβ)* gene have been linked, (*TSHβ)* is associated with thyroid dysfunction, including Differentiated Thyroid Cancer DTC and CH.

Some genetic variations were also counted for the last (5) years, according to what was recorded on the NCBI 2025, as shown in Table (2-2).

Table (2-2): Some Genetic Variation in *TSHβ* Gene (NCBI, 2025).

SNVs	Location in Gene	Molecular Effect	Reference
rs10776792	Exon 2	Missense Mutations Converts the Amino Acid Phenylalanine to Tyrosine	(Heidari *et al*., 2020).
rs7530810	Promoter	Deletion	(Al-Rasheed, 2022).
rs201857310	Exon 2	Potential Alteration in Protein Conformation	(Tiucă *et al*.,2023).

2.4.3 Thyroid Peroxidase (*TPO*): Gene and Protein Analysis

Thyroid peroxidase is an important essential enzyme encoded by the (TPO) body, located on the chromosome (2p25) at location (chr2:1,374,047-1,543,711) (Godlewska and Banga, 2019).

The size of the gene is (169,627nt), and encodes a protein consisting of (933) amino acids and consisting of (17) exons (Fernando *et al*., 2014) (Figure 2-10).

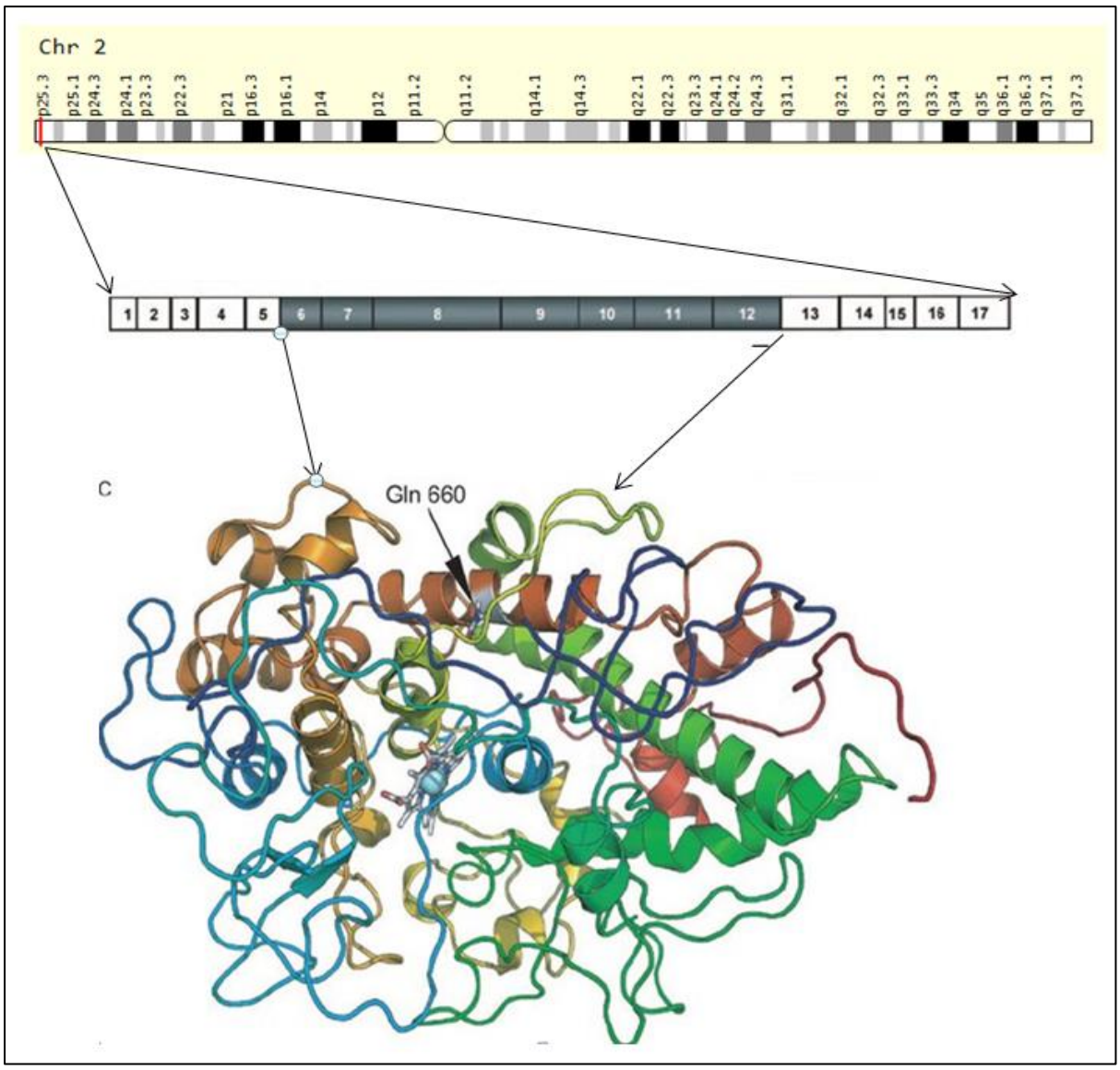


Figure (2-10): Structure of *TPO* Gene (Fernando *et al*., 2014).

The (TPO) protein is often alpha helical and has three distinct domains that, the Myeloperoxidase (MPO) like domain, the Complement Control Protein (CCP) like domain, the Epidermal Growth Factor (EGF) like domain (Muzza and Fugazzola, 2017) (Figure 2-11).

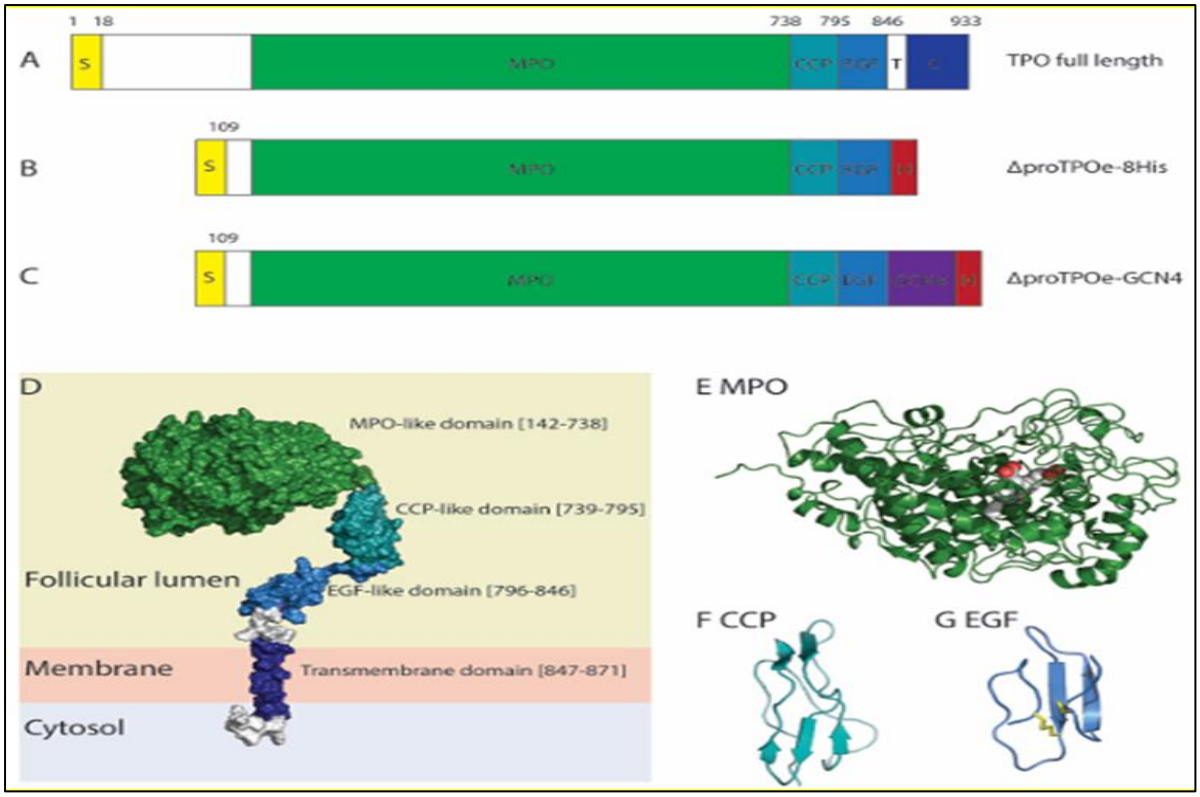


Figure (2-11): TPO Installation (Muzza and Fugazzola, 2017).

Domain contribute to its enzymatic activity and also participate in its binding to the substrate, these are essential in the production of THs (Muzza and Fugazzola, 2017). That (TPO) generates multiple similar forms through, alternative splicing occurs whereby the full length (TPO) forms constitute the functional enzyme, while the isoforms (TPO3) and (TPO4) retain enzymatic activity, while the isoforms (TPO2) and (TPO5) are enzymatically inactive and subject to rapid degradation (Ferrando *et al*., 2003).

However, the various isoforms are still poorly understood, requiring further research, gene expression of (TPO) is mainly restricted to thyroid follicular cells, but is also subject to sensitive regulation by key transcription factors (Grasberger *et al*., 2005).

TSH via the TSHR receptor, modulates gene expression primarily for (TPO) through the cAMP signaling pathway (Gerard *et al*., 1988).

Which promotes transcription of the (*TPO*) gene and the subsequent transport of the protein to the apical thyroid membrane, where THs are synthesized (Penel *et al*., 1998).

Since (TPO) is a membrane bound glycoprotein, it stimulates TH enzymes and thus regulates their function (Godlewska and Banga, 2019). The function of the (TPO) enzyme is to stimulate the important iodination of tyrosine residues within Tg, which results in the conjugation of iodotyrosines to form the THs active T3 and active T4 (Williams *et al*., 2018).

Given TPO's central catalytic role, coding SNVs in TPO could disrupt iodination and coupling, thereby influencing TH biosynthesis (Baloch *et al*., 2015).

This essentially depends on Hydrogen Peroxide H2O2, which is generated mainly through the enzymes of Dual Oxidase DUOX1 thus, a delicate enzyme network important for the production of THs emerges (Carvalho and Dupuy, 2017).

Since T3 and T4 are characterized by containing part of their special structure on I atoms, their manufacture takes place inside unique structures called thyroid follicles, when iodide reaches the thyroid cells through the bloodstream, it works to nourish the basolateral plasma membrane of the thyroid cells, so that it is actively absorbed by the NIS, there is another function of the thyroid cells, as it works to secrete Tg, which is a protein with a high molecular weight in the lumen of the follicles, during the process of manufacturing THs, I is added to the tyrosyl residues based on the reaction of I, Tg, H2O2, and TPO at the apical plasma membrane of the thyroid cells, where this process is subject to the control of the TSH (Carvalho and Dupuy, 2017; Ali *et al*., 2025).

2.4.3.1 SNVs in *TPO* Gene and their Relationship to Thyroid Disorders

Thyroid disorders occur due to interaction between genetic and environmental causes, these causes, such as the environment, affect the health of the thyroid gland (Cooper and Biondi, 2018).

Including a deficiency or scarcity of I, exposure to chemicals and heavy metals, or lifestyle factors such as stress and smoking, which cause a malfunction in the thyroid gland (Zimmermann and Boelaert, 2015). This interaction and combination occur between environmental stimuli, genetic predisposition, genes, and multiple signaling pathways, resulting in a predisposition to thyroid diseases (Ramos-Levi and Marazuela, 2019; Tyagi *et al*., 2024).

The (*TPO)* gene is one of the major autoantigens in thyroid diseases, the presence of (TPO) antibodies (TPOAb) in patients with autoimmune thyroiditis is one of the characteristics that distinguish them from this disease, it is one of the important diagnostic factors in the disease, these antibodies also play a pathological role in the development of this disease, several SNVs in the (*TPO)* gene have been investigated, which are associated with the infection with autoimmune thyroiditis, these include variants in the coding regions and also the promoter region of the gene, the presence of the SNV rs1126797 has been indicated and its association with thyroid diseases, including hypothyroidism and diseases related to autoimmunity (Lacka *et al*., 2025).

The person responsible for congenital goiter is a mutation in the (*TPO)* gene, environmental factors, for example, an increase in I, can have a significant impact on the expression and function of (TPO) (Ris-Stalpers and Bikker, 2010). And the Wolf Chaikoff effect, where high concentrations of I are associated with inhibiting the production of THs, perhaps by interfering with the production of H2O2 and stimulating (TPO) (Liu *et al*., 2017).

Genetic variations in the (*TPO)* gene affect I regulation, leading to a defect in it and in the production of THs (Abramowicz *et al*., 1992).

Which may result in many different effects, including a defect in protein folding, a defect in its transport to the cell membrane, or a weakness in enzyme activity (Carvalho *et al.,* 1996).

Several studies have found SNVs in the (*TPO)* gene that have been associated with AITDs, these variants included several regions, including the coding region and the promoter region of the gene (Brčić *et al*., 2016; Lacka *et al*., 2024). The rs1126797 polymorphism has been associated with hypothyroidism and AITDs (Balmiki *et al*., 2014).

In the (*TPO)* gene rs1126797, specifically in exon (11), a relationship was found between the development of clinical autoimmune diseases of the thyroid gland in the caucasian polish population it indicates a difference in the genetic patterns and size of the thyroid gland in people with thyroid diseases, which may play a role in the phenotype of the disease (Lacka *et al*., 2025).

Many studies have shown that multiple mutations in this (*TPO)* gene may cause a defect and thus affect the function of this enzyme, resulting in many diverse human diseases (Yu *et al*., 2021).

AITDs has been found to be associated with the (*TPO*) rs 2048722 polymorphism and has been reported in polish caucasian patients (Jendrzejewski *et al*., 2016).

A mutation in the (*TPO)* gene primarily causes CH, which occurs due to a defect in the synthesis of THs (Ris-Stalpers and Bikker, 2010).

some genetic variations were also counted for the last (5) years, according to what was recorded on the NCBI database 2025, as shown in Table (2-3).

Table (2-3): Some Genetic Variation of *TPO* Gene (NCBI, 2025).

SNVs	Location in gene	Molecular Effect	Reference
rs732609	intron 10	Associated with Regulatory Activity on TPO Expression.	(Al-Mofarji *et al*., 2023).
rs2048722	3′ UTR	Potentially Impacts mRNA Stability and Gene Regulation.	(Shen *et al*., 2023).
rs2071400	Exon	Associated with Enhanced Expression of Thyroid Peroxidase Antibodies (TPOAb).	(Radhi *et al*., 2023).

To illustrate how genetic variations can influence TH regulation, the functional consequences of coding SNVs within the *TSHR*, *TSHβ*, and *TPO* genes are summarized in Figure (2- 12), these genes represent key molecular checkpoints in the synthesis, signaling, and catalytic conversion processes that govern TH production, therefore, any alterations in their coding regions may affect receptor sensitivity, hormone assembly, or enzymatic activity, potentially contributing to thyroid dysfunction.

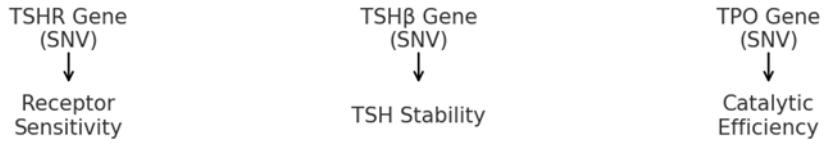


Figure (2 - 12): Representative functional impact points of coding SNVs in thyroid related genes.

To summarize the potential impact of coding SNVs in thyroid related genes, representative variants reported in the literature and their functional consequences are listed in Table (2-4).

Table (2 - 4): Representative SNVs reported in Thyroid Related Genes and their Potential Effects.

Gene	Example SNV (Coding Region)	Functional Effect	Potential Clinical Outcome	Reference
TSHR	rs179247 (A/G)	Alters receptor sensitivity and signaling pathway activation	May increase susceptibility to Graves' disease or hyperthyroidism	(Zufry and Hariyanto, 2024).
TSHβ	c.113G>A (missense)	Reduces β-subunit stability → impaired TSH synthesis	May contribute to central hypothyroidism	(Kaplan *et al*., 2022)
TPO	c.361C>G (missense)	Disrupts catalytic efficiency of iodination/coupling	Reduced T3/T4 production → hypothyroidism risk	(Balmiki *et al*., 2014; Baloch *et al*., 2015).

Chapter Three

Materials and Methods

3. Materials and Mothods

3.1 Materials

3.1.1 Apparatus and Equipment

The apparatus and instruments that were used in this study are listed in Table (3-1).

Table (3-1): The Apparatus and Equipment used in this Study.

No.	Apparatus and Equipment	Company	Country
1	Autoclave	Hirayama	Japan
2	Centrifuge	FANEM	Germany
3	CL-900i	Mindray	China
4	Cobas (e411)	Cobas	Germany
5	Cool Box	Unsef	Russia
6	Electrophoresis Unit	Labnet International	USA
7	Electrical Sensitive Balance	Denver	Germany
8	ELISA	Human	Germany
9	EDTA Test Tubes (2ml)	AFCO	Jordon
10	Eppendorf Tube	BDH	UK
11	Gel Test Tube	AFCO	Jordon
12	Gel Documentation	Biometra	Germany
13	Gloves	Broche	Malaysia
14	Incubator	Yamato	Japan

15	Microwave Oven	LG	Korea South
16	Micro Spin Centrifuge	My Fugene	China
17	Measuring Tape	China	China
18	Microcentrifuge Tubes	Bio Basic	Canada
19	Micropipette (10 - 100µl) (20 – 200µl) (100-1000µl)	Slamed	Japan
20	Nanodrop	Thermo Fisher Scientific	USA
21	Sterile Syringes	Sterile EO.	China
22	Rack	Sterellin Ltd.	UK.
23	Roller Mixer	Biobase	China
24	Water Bath	Memmert	Germany

3.1.2 Chemical and Biological Materials

The chemical and biological materials that were used in this study are listed in Table (3-2).

Table (3-2): Overview of Chemical and Biological Reagents Employed in DNA Extraction, PCR, and Gel Electrophoresis Procedures.

No.	Chemical materials	Company	Country
1	Agarose	TransGen Biotech	Chin
2	Absolute Ethanol Alcohol	SIGMA	USA
3	DNA and Gene Dye " Safe-Green abm"	TransGen Biotech	China

4	Loading Dye	TransGen Biotech	China
5	Nuclease Free Water	TransGen Biotech	China
6	Oligo Primers	Alpha DNA	South Korea
7	Primers	Jeiotech	Korea South
8	Proteinase K Enzyme	TransGen Biotech.	China
9	10X TBE (Tris Base -Boric Acid -EDTA)	Promega	USA
10	1X TBE (Tris Base -Boric Acid- EDTA)	Promega	USA

3.1.3 Kits

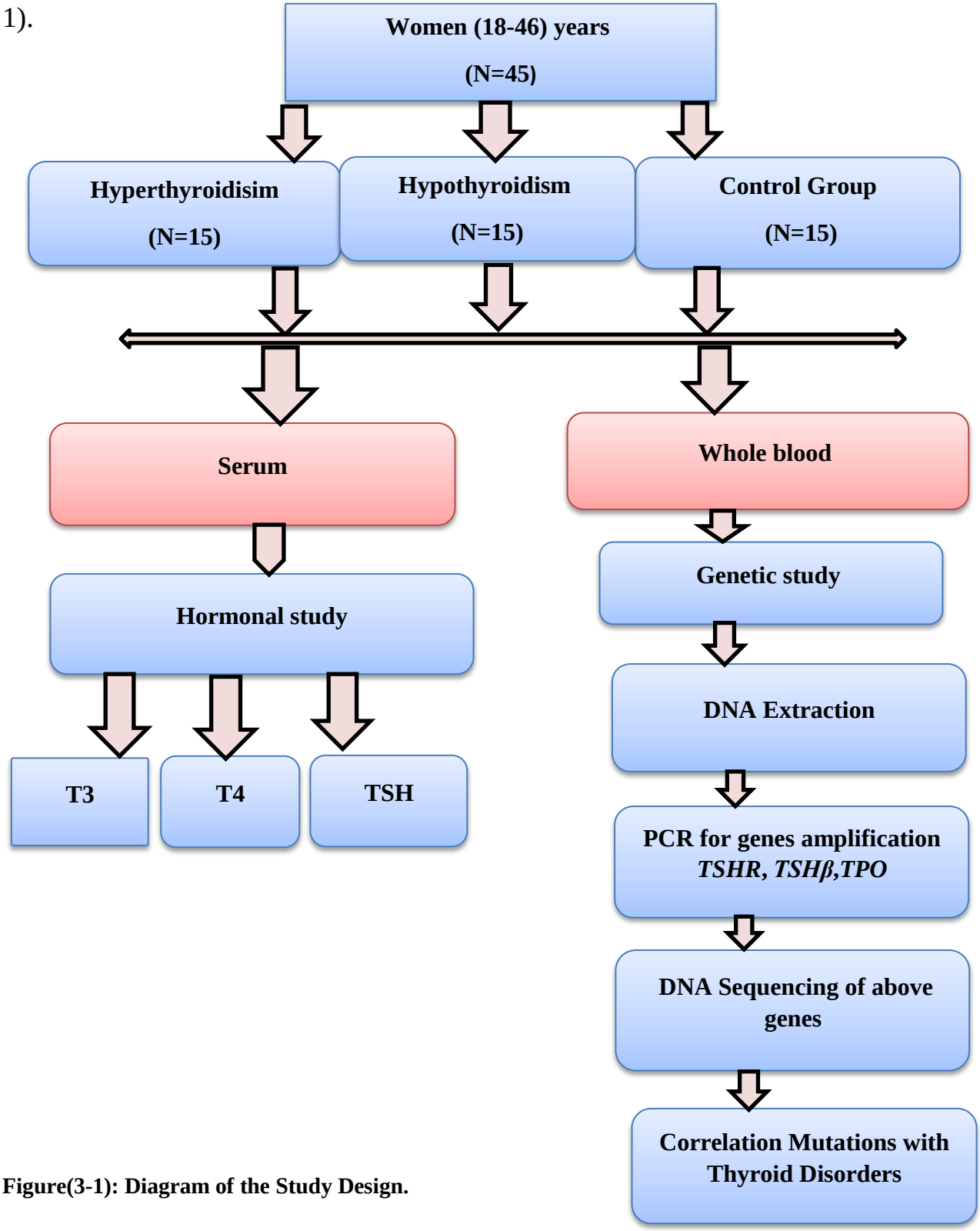
The kits that were used in this study are listed in table (3-3).

Table (3-3): The kits used in this study.

No.	Kits	Companies	Countries
1	gSYNC ™	Geneaid	Taiwan
2	Master mix	TransGen Biotech	China
3	Triiodothyronine (T3)	Roche	Germany
4	Thyroxine (T4)	Roche	Germany
5	Thyroid Stimulating Hormone (TSH)	Roche	Germany

3.1.4 Study Design

The present study adopted a case–control design consisting of two women groups (hyperthyroidism and hypothyroidism) and one healthy control group, the workflow of sample collection and analysis is illustrated in Figure (3-1).



Figure(3-1): Diagram of the Study Design.

3.1.5 Study Design and Location

This study was designed as a case–control study and was conducted in Misan Governorate, Iraq, a total of 45 women participated in the study according to the official approval from the Ministry of Health / Misan Health Directorate (No. 756, dated 18/11/2024) (Appendix A), all participants completed a structured questionnaire regarding health status and medical history (Appendix B), the subjects were divided into two main groups according to clinical diagnosis confirmed by endocrinologists and laboratory hormone measurements diagnosed with thyroid disorders, including

- Hyperthyroidism group: 15 women

- Hypothyroidism group: 15 women

- Control group : 15 women

Healthy women with no history of thyroid disease and normal T3, T4, and TSH levels, a case control design was selected because it allows comparison between affected and unaffected individuals to determine whether specific (SNVs) are associated with thyroid dysfunction.

3.1.5.1 Inclusion Criteria

Participants were selected according to the following criteria Women aged (18–46) years Patients were clinically diagnosed with either hyperthyroidism or diagnosis was supported by TH measurements, where :

- Hypothyroidism was indicated by low T3 and T4 levels with elevated TSH

- Hyperthyroidism was indicated by elevated T3 and T4 levels with reduced TSH.

- Control subjects were clinically healthy women with no history of thyroid disorders and with normal T3,T4 and TSH levels.

3.1.5.2 Exclusion Criteria

The following cases were excluded to avoid confounding effects:

1.Women diagnosed with chronic systemic diseases (e.g., hypertension, diabetes, cardiovascular diseases.
2. Individuals currently receiving TH therapy or anti-thyroid medications.
3. Patients with a history of thyroidectomy or thyroid cancer
4. Individuals taking medications known to affect thyroid function or hormone levels (e.g., corticosteroids, lithium).
5. Patients with autoimmune diseases (e.g., Hashimoto's thyroiditis, GD).
6. Pregnant or lactating women
7.Smokers

These criteria were applied to ensure that the observed hormonal and genetic variations are related to thyroid dysfunction itself, rather than to other medical or pharmacological factors.

3.1.6 Sample Collection and Handling

Venous blood samples (5 ml) were collected from each participant under aseptic conditions. A volume of (5) mL of whole blood was selected to ensure sufficient yield for both hormonal measurements and molecular genetic analysis, the blood was divided into two parts:

- Serum was separated for estimation of TSH, T3,T4 and TSH.

-Whole blood with EDTA anticoagulant was used for genomic DNA extraction.

3.2 Methods

3.2.1 Sterilization Methods

Initially, used several sterilization methods to avoid contamination, including

- Autoclave Sterilization: The tips of both micropipettes and eppendorf tubes were autoclaved at (121)°C for (15-20) minutes.

- Dry heat sterilization: The glassware such as conical flasks, volumetric flasks, and other tools were sterilized in an oven at (180)°C for (1:30-2) hrs.

3.2.1.1 Calculating Body Mass Index (BMI)

The mathematical equation was relied upon, which included body weight in kilograms divided by the height squared in meters, and the ratios were as follows: if the body mass index is (30) kg/m^2 or more, the person is considered obese, and if it is (25-29.9) kg/m^2 or more, the person is considered overweight, and if it is between (18.5-24.9) kg/m^2 , is considered normal weight, if it is between (18) kg/m^2 , is considered underweight, the weight and height of each individual participating in this study were relied upon, when it was winter, winter coats and shoes were removed, but in summer, shoes were removed as well (WHO, 2022).

$$BMI = Mass\ (Kg) / Height\ (m)^2$$

3.2.2 Hormonal Study

3.2.2.1 Chemical Test for T3, T4, TSH Hormone Competition Principle

The physiological testing for the hormones T3, T4, and TSH was performed according to the Kits in the Roche Germany (Tietz, 1995), (Sakai *et al*., 2009**).**

3.2.3.1 Estimation of Laboratory Solutions

3.2.3.2 Phosphate Buffered Saline (PBS) Solution

PBS this solution is prepared by dissolving (0.24, 1.44, 0,2, 8) g of the following solutions in succession Na2HPO4, Kcl, Nacl in (800) ml of distilled water,the medium is adjusted to pH (7.4) medium, after that, the rest of the volume was completed with distilled water to about a liter and sterilized using a sterilizer (Sambrook and Russell, 2001).

3.2.3.3 Preparation of 10X TBE Buffer Solution

A 10X TBE stock buffer was prepared by dissolving (108) g Tris base and (55) g Boric acid in approximately (900) ml of distilled water, followed by the addition of 40 ml of (0.5) M EDTA (pH 8.0), the pH was adjusted to (8.0), and the final volume was brought to (1) liter with distilled water.

This 10X stock solution is diluted to 1X working concentration by mixing (50) ml of 10X TBE with (450) ml distilled water to make a total of (500) ml of 1X TBE, used in agarose gel electrophoresis (Sambrook and Russell, 2001).

3.2.3.4 Proteinase K Enzyme

It is prepared by dissolving (10) g Proteinase K of it in (100) ml of distilled water that is free of enzymes nuclease water free nuclease (Sambrook and Russell, 2001).

3.2.3.5 Preparation of Master Mix

The components of the master mix were prepared by the company of Promega/USA.

3.2.3.6 Agarose Gel

A 1% agarose gel was prepared by dissolving (0.5) g of agarose powder in (50) ml of 1X TBE buffer, while a 2% agarose gel was prepared by dissolving (1) g of agarose in (50) ml of 1X TBE buffer, the mixture was heated in a microwave oven until the agarose was completely dissolved, the solution was then left to cool to approximately (55–60)°C, after which (3) µl of ethidium bromide (0.5) µg/ml was added, the molten gel was poured into the casting tray with the comb in position and allowed to solidify at room temperature before electrophoresis (Sambrook and Russell, 2001).

3.2.3 Hormonal Parameters Assessment

THs concentrations (TSH, fT3, and fT4) were measured using an automated chemiluminescent immunoassay system, this method is based on the binding between the antigen (hormone) and its specific antibody on the surface of streptavidin-coated magnetic microparticles, followed by a chemiluminescent reaction that produces light proportional to the hormone concentration, the samples were automatically processed by the analyzer, and the final results were displayed digitally on the screen (Burtis and Bruns, 2019; Riley *et al*.,2022).

3.2.4 Genetic Study

A genetic analysis was performed on 45 blood samples collected from women, including 30 patients with thyroid disorders and 15 healthy controls. Genomic DNA was extracted to determine the genotypes of the *TSHR*, *TSHB*, and *TPO* genes, the genetic data were analyzed to evaluate the association of these variants with clinical parameters such as age and (BMI), in addition, the predicted amino acid changes and allele frequencies of the detected SNVs were assessed, the potential structural impact of the missense variants on protein 3D conformation was also examined was performed according to the Kits in the book below gSYNC ™ DNA Extraction Kit functional test data according to

(Geneaid Taiwan), Samples taken from study participants were extracted after being placed in preservative tubes and the instructions for DNA extraction were applied, the instructions included these steps (Sambrook and Russell, 2001):

3.2.4.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed to verify the integrity of genomic DNA and to confirm PCR amplification, according to the method described by Sambrook and Russell (2001) with minor modifications, a 1% agarose gel was prepared by dissolving (0.5) g of agarose in 50 ml of 1X TBE buffer. The mixture was heated in a microwave oven until completely dissolved and then allowed to cool to approximately (55–60)°C, after cooling, (3) µL of Safe-Green DNA stain was added and gently mixed.

The molten gel was poured into the casting tray with the comb in place and left at room temperature until solidified, after solidification, the comb was removed, and the gel tray was placed into the electrophoresis tank, the tank was filled with 1X TBE buffer until the gel was fully submerged.

DNA samples were prepared by mixing (3) µL of loading dye with (3) µL of DNA sample and loaded into the wells, electrophoresis was carried out at (85) V for approximately (1) hour, after migration, the gel was visualized using a gel documentation system to assess DNA quality and confirm successful PCR amplification (Sambrook and Russell, 2001).

3.2.4.2 Primers

Primers specific for the *TSHR*, *TSHβ*, and *TPO* genes were synthesized by Bioneer Corporation (South Korea), the primer sequences, annealing temperatures, and expected PCR product sizes for each gene are listed in Table (3-4), the selection of these primers ensures amplification of exonic regions likely to contain functionally relevant SNVs , the primers used for amplification

of *TSHR*, *TSHβ*, and *TPO* genes in this study were custom designed and synthesized by Bioneer Corporation (South Korea), primer sequences were checked using NCBI Primer-BLAST to ensure specificity to the target regions, optimal GC content, and absence of secondary structures or primer dimer formation.

Table (3- 4): The Primers used in this study.

Gene	Sequences (5'-3')	Product size (bp)	Tm (°C)	References
TSHB	F:GGCTAAGCAATTCTTTCCCAGT	520	55	(Özhan *et al*.,2017).
	R: GCTCTCTAACGCCTGTGTAGG			
TSHR	F: CCTCCCTCTTTCCTCCCAGA	499	59	(Juma'a and Allami, 2021).
	R: TGTTTCCTCTGCATCCCACC			
TPO	F: CTATCCCCAGATTGCTCCTG	449	59	(Guria, *et al*., 2014).
	R: GCTCAGTGAGTGACCACAGC			

The primers used for amplification of *TSHR*, *TSHβ*, and *TPO* genes in this study were custom-designed and synthesized by Bioneer Corporation (South Korea), primer sequences were checked using NCBI Primer BLAST to ensure specificity to the target regions, optimal GC content, and absence of secondary structures or primer dimer formation.

3.2.4.3 Polymerase Chain Reaction (PCR)

PCR reactions were prepared inside a UV-sterilized PCR workstation to prevent contamination, all pipettes and surfaces were disinfected, and sterile filter tips were used, each reaction was prepared in a sterile (0.2) mL PCR tube with a final volume of (25) µL, as detailed in Table (3-5), the reaction tubes were briefly centrifuged to collect the reaction mixture at the bottom of the tube.

PCR amplification was performed in a programmable thermal cycler, using the cycling conditions shown in Table (3-6), which included initial denaturation, denaturation, annealing, extension, and final extension steps, an annealing temperature of (60)°C was used for all primer sets, based on their calculated melting temperatures (Tm), upon completion, PCR products were stored at (4)°C until electrophoresis (Green and Sambrook ,2012).

Table (3-5): Components of the PCR Reaction Mixture.

No.	Reagents	Volume (µl)
1	Master Mix	13
2	Primer forward	1
3	Primer reverse	1
4	DNA template	3.5
5	Free water	6.5
6	Total	25

Table (3-6): PCR Cycling Condtion for Amplification *TSHR, TSHβ* and *TPO* Genes.

No.	Step	Temp.	Time	Cycle
1	Initial Denaturation	95 °C	5.00 min	1
2	Denaturation	95 °C	30 sec.	35
3	Annealing	55-58 °C	40 sec.	
4	Extension	72 °C	45 sec.	
5	Final Extension	72 °C	10.00 min	1

The temperature of the PCR device required for each reaction was set, as was the annealing temperature and the number of cycles as in Tables (3-6), the optimum temperature for each pad was known and documented with the equation.

3.2.4.4 Detection of PCR Product

PCR products were analyzed by agarose gel electrophoresis. A 2% agarose gel was prepared in 1X TBE buffer, stained with Safe-Green DNA dye. A 3 µL DNA ladder was loaded into the first well to serve as a molecular size marker. Then, 5 µL of each PCR product was mixed with loading dye and loaded into the remaining wells, electrophoresis was carried out at 75 V and 65 MA for approximately (1) hour, following electrophoresis, the gel was visualized using a Gel Documentation System to confirm successful DNA amplification by observing bands of the expected sizes (Green and Sambrook ,2012).

3.2.4.5 DNA Sequencing and Bioinformatic Analysis

Genomic DNA samples from all 45 participants were subjected to sequencing after successful PCR amplification, the purity and concentration of the extracted DNA were assessed using a NanoDrop spectrophotometer, and samples with an A260/A280 ratio of 1.7–2.0 were considered suitable for sequencing.

Forward-strand Sanger sequencing was performed by Macrogen Inc. (Seoul, Korea), the resulting chromatogram files (.ab1) were examined and edited using Chromas and SnapGene Viewer software to ensure accurate base calling and removal of background noise. The edited sequences were then aligned against the corresponding reference sequences obtained from the NCBI GenBank database for the *TSHR* (NM_000369.2), *TSHβ* (NM_000549.4), and *TPO* (NM_175725.6) genes.

Variant detection and annotation were carried out using NCBI BLAST, Clustal Omega for multiple sequence alignment, and Ensembl Variant Effect Predictor (VEP) to determine allele frequency, amino acid substitution, and the potential functional impact of identified SNVs.

3.5 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 26. Differences in hormone levels and clinical characteristics among study groups were evaluated using the Chi-square test and one-way ANOVA, as appropriate. Statistical significance was considered at $P \leq 0.05$.

The Odds Ratios (ORs) and 95% Confidence Intervals (CIs) for different genotypes and alleles were calculated using MedCalc Statistical Software version 20.0111.

The Odds Ratio is used to determine whether a genotype increases the risk of disease. If OR > 1, the genotype is associated with higher risk; if OR < 1, it is protective; and if OR = 1, there is no association.

The genotype distribution of *TSHR*, *TSHβ*, and *TPO* variants was assessed for compliance with the Hardy Weinberg Equilibrium (HWE) using an online calculator (https://www.had2know.org/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html), departure from HWE was considered indicative of possible selection pressure or allelic association (Bland, 2015).

Chapter Four

Results and Discussion

4.Results and Discussion

4.1 Subjects Data

This study followed a case–control design and included a total of 45 women aged 18–46 years. The case group consisted of 30 patients diagnosed with thyroid disorders, subdivided into hyperthyroidism (n = 15) and hypothyroidism (n = 15). The control group (n = 15) included healthy women matched for age and with no history of thyroid or chronic diseases. The samples were collected from the city center and several districts and sub-districts of Misan Governorate, including (Kumait, Al-Mejar Al-Kabir, Al-Maimouna, and Al-Kahla), as shown in Figure (4-1).

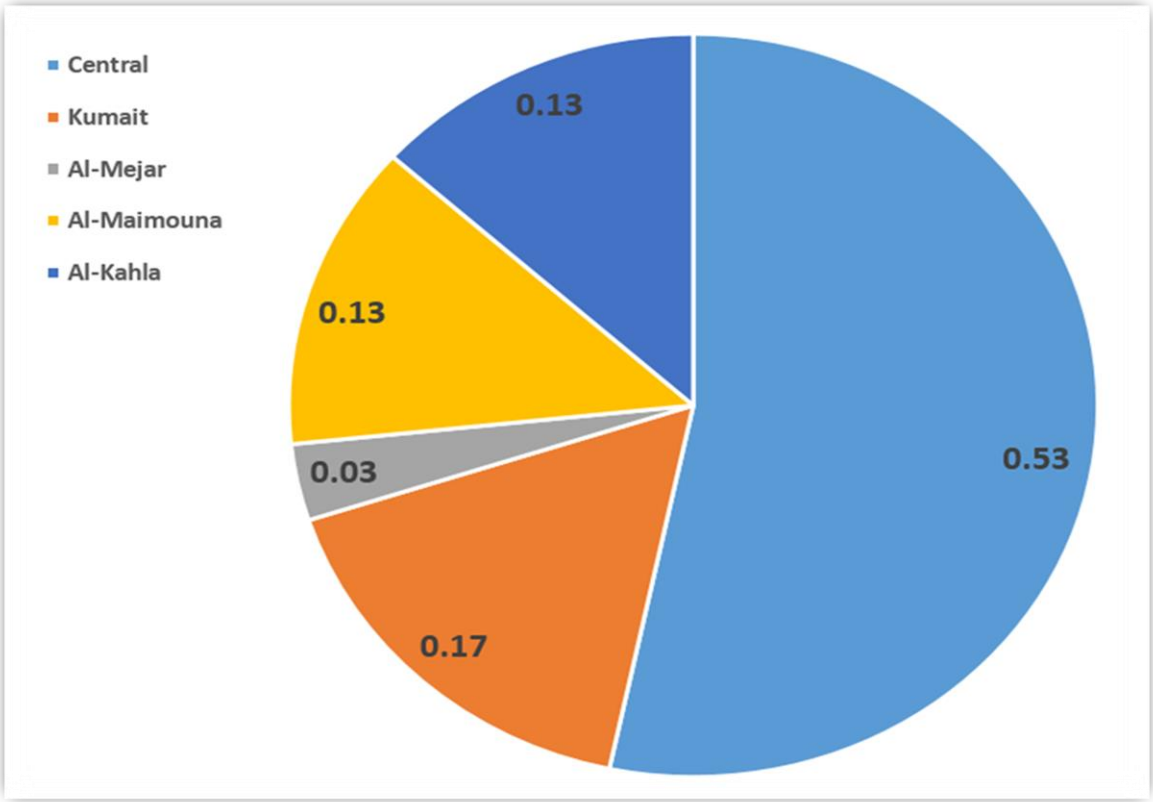


Figure (4-1): Geographic distribution of the study participants across regions of Misan Governorate

The highest proportion of participants were from the central urban area (53%), followed by Al-Mejar Al-Kabir (17%), Al-Maimouna and Kumait (13% each), while Al-Kahla represented the lowest proportion (3%). This distribution reflects the population density and accessibility of healthcare centers in these regions Table (4-1).

Table (4-1): Distribution of the Study Participants Across Misan Governorate.

Al-Mejar Al-Kabir	Al-Maimouna	Kumait	Al-Kahla	Center Misan
N=1Hyperthyroidism	N=3 Hyperthyroidism N=1 Hyporthyroidism	N=2 Hyporthyroidism N=3 Hyperthyroidism	N=2 Hyperthyroidism N=2 Hyporthyroidism	N=6 Hyperthyroidism N=10 Hyporthyroidism

4.2 Demographic Study

4.2.1 Distribution of Study Subjects According to Anthropometric Parameters

Anthropometric characteristics were assessed in the study population, which included women with hyperthyroidism, hypothyroidism, and a matched control group, the parameters examined were age, body mass index (BMI), and family history of thyroid disorders, in order to identify potential demographic and physiological factors associated with thyroid dysfunction. The distribution of these characteristics among the study groups is presented in Table (4-2).

Women with thyroid disorders (hyperthyroidism and hypothyroidism) did not differ significantly in age and BMI compared to the control group ($P > 0.05$), indicating that the groups were demographically comparable and that age and body mass index were not confounding factors in the association analysis. However, a greater proportion of affected women reported a positive family

history of thyroid disorders compared to controls ($P \leq 0.05$), suggesting a hereditary predisposition, which aligns with the well established genetic contribution to thyroid diseases (Helmy, 2020; Effraimidis and Wiersinga, 2018).

Table (4-2): Evaluation of Clinical Characteristics in Thyroid Disorder Versus Control Group.

Parameters		Hyperthyroidism (No. 15)	Hypothyroidism (No. 15)	Control (No. 15)	P-value
Age (years) (Mean±SD)		33.06±3.17	35.93±5.25	30.20±6.80	0.4 NS
BMI (kg/m^2) (Mean±SD)		27.23±4.22	29.31±5.75	26.50±4.92	0.2 NS
Family history	Yes	6 (40%)	5 (33.33%)		0.03*
	No	9 (60%)	10 (66.67%)		

*: Difference of Statistical Significance, P≤ 0.05, NS: Non-Significant (P > 0.05).

The results showed no significant differences in age among the hyperthyroid, hypothyroid, and control groups ($P > 0.05$), indicating that age was not a confounding factor in the present study. Similarly, there was no significant difference in BMI between the groups ($P > 0.05$), suggesting that body weight was not associated with thyroid dysfunction in the study population, in contrast, family history of thyroid disorders was significantly higher among patients with thyroid dysfunction compared to controls ($P = 0.03$). This finding indicates a genetic predisposition to thyroid disease and supports the well-established role of hereditary and autoimmune mechanisms in thyroid dysfunction, these findings are in agreement with (Effraimidis and Wiersinga, 2018), who reported that genetic susceptibility is a key factor in thyroid disease, and with (Taylor *et al*., 2013) who noted that familial clustering is common among women with thyroid disorders.

The results demonstrated no statistically significant difference in age among the hyperthyroid, hypothyroid, and control groups ($P > 0.05$), indicating that age was not a contributing factor in disease occurrence within this study population. This finding is consistent with (Korevaar *et al*., 2018), who reported that although age may influence thyroid function in the general population, its effect can be minimal when the comparison groups are age matched, similarly (Cikim *et al*., 2004) also found comparable age distributions among patients and control groups in studies of thyroid dysfunction, regarding BMI, the mean values were slightly higher in the hypothyroid group (29.31±5.75 kg/m²) compared to the hyperthyroid (27.23±4.22 kg/m²) and control groups (26.50±4.92 kg/m²). However, this difference did not reach statistical significance ($P = 0.2$). These results are in agreement with Santini *et al*. (2014), who noted that changes in body weight may depend on disease duration, treatment status, and metabolic adaptation, rather than simply the presence of hyper or hypothyroidism.

Nevertheless, the physiological interpretation remains consistent Hypothyroidism is associated with decreased basal metabolic rate, reduced thermogenesis, and increased fat accumulation, often resulting in weight gain, while hyperthyroidism leads to increased energy expenditure and weight loss (Mullur *et al*., 2014; Song *et al*., 2019; Tiagi *et al*., 2024). Thus, although the differences were not statistically significant in this study, the trend in BMI variation aligns with the well established metabolic effects of THs.

A statistically significant association was observed for family history ($P = 0.03$), where a higher proportion of thyroid disorder patients reported a positive family history compared to controls, this finding strongly supports the genetic predisposition in the development of thyroid disorders and aligns with previous research indicating that first degree relatives of affected individuals have a substantially increased risk of developing thyroid dysfunction due to shared

genetic and autoimmune susceptibility (Effraimidis and Wiersinga, 2018; Taylor *et al*., 2013).

The significant association between family history and thyroid disorder observed in this study is consistent with the well established genetic component of (AITDs). *Yu et al*. (2021) demonstrated that genetic susceptibility is a key contributor to the development of Hashimoto's thyroiditis and Graves' disease (GD), both of which are major causes of hypothyroidism and hyperthyroidism, respectively, similarly, (Tomer *et al*., 2003), reported that familial aggregation is common among patients with AITDs, where shared genetic variants and immune regulatory pathways contribute to disease onset.

However, not all patients in the present study reported a positive family history, as 60% of the hyperthyroid group and 66.67% of the hypothyroid group had no known affected relatives, this may indicate the role of non-genetic factors, such as environmental exposure, stress, iodine intake, smoking, or hormonal influences, which can trigger autoimmune or functional thyroid disorders even in the absence of hereditary predisposition.

4.3 Assessment of Thyroid Hormone Profiles

4.3.1 Free Triiodothyronine (fT3)

The results showed a significant decrease in serum fT3 levels in the hypothyroid group compared to the control group ($P \leq 0.05$). In contrast, the hyperthyroid group demonstrated a significant elevation in fT3 levels compared to controls ($P \leq 0.05$) (Table 4-3, Figure 4-2).

4.3.2 Free Thyroxine (fT4)

A similar pattern was observed for fT4, where hypothyroid patients exhibited significantly lower fT4 levels, whereas hyperthyroid patients showed significantly higher fT4 levels compared to the control group ($P \leq 0.05$) (Table 4-3, Figure 4-2).

4.3.3 Thyroid Stimulating Hormone (TSH)

Conversely, TSH levels were significantly increased in the hypothyroid group ($P \leq 0.05$), while hyperthyroid patients demonstrated a significant reduction in TSH ($P \leq 0.05$) when compared with the control group (Table 4-3, Figure 4-2).

Table (4-3): The Values of Serum Thyroid Hormones Levels among Hypothyroidism , Hyperthyroidism and Control (Mean ± SD).

Parameters	Hyper	Hypo	Control	*P*-value
fT3 pmoL/L	8.25	2.2	5.44	0.02*
fT4 pmoL/L	25.4	9.35	14.56	0.01*
TSH uIU/Ml	0.22	5.62	2.15	0.03*

***: Difference of Statistical Significance, P≤ 0.05.**

4.3.4 Interpretation of Thyroid Hormonal Profile

The results revealed a significant increase in fT3 and fT4 levels in the hyperthyroid group compared to both the hypothyroid and control groups ($P \leq 0.05$), this reflects the enhanced synthesis and release of thyroid hormones characteristic of hyperthyroidism, which leads to increased metabolic rate and

accelerated physiological activity. Conversely, the hypothyroid group exhibited significantly lower fT3 and fT4 levels, along with a marked elevation in TSH ($P \leq 0.05$). This pattern corresponds to the negative feedback mechanism in the (HPT) axis, where reduced THs levels stimulate the pituitary to secrete more TSH in an attempt to compensate for impaired thyroid function.

The control group demonstrated normal reference levels for fT3, fT4, and TSH, which confirms the accuracy of the hormonal measurement procedure and supports the reliability of the comparison.

These findings are consistent with the classical endocrine regulation pathway described by (Mullur *et al*.,2014) and are in agreement with clinical reports that hyperthyroidism increases circulating thyroid hormones and suppresses TSH, whereas hypothyroidism decreases thyroid hormones and increases TSH (Taylor *et al*., 2013; De Leo *et al*., 2016).

This hormonal pattern reflects the negative feedback regulation of the (HPT) axis. Reduced fT3 and fT4 levels in hypothyroidism stimulate the pituitary gland to increase TSH secretion, whereas elevated fT3 and fT4 in hyperthyroidism suppress TSH release, these findings are consistent with previous reports, which demonstrated decreased fT3 and fT4 and elevated TSH in hypothyroidism, and the opposite profile in hyperthyroidism (Hashim *et al*., 2018; Helmy, 2020; Juma'a and Allami, 2021). Similarly, Unnikrishnan *et al*. (2013) emphasized that changes in TH concentrations directly influence TSH secretion due to feedback control. Furthermore, studies in women with GD have shown elevated T3 and T4 and suppressed TSH, consistent with the results of the present study (Ren and Zhu, 2022).

4.4 Molecular Study

4.4.1 DNA Extraction and Amplification

Genomic DNA was extracted of all subjects according to the standard protocol recommended by the manufacturing company (Geneaid, Taiwan), which gave a perfect concentration (109-121) ng/µl and purity DNA (purity and concentration were measured using a NanoDrop spectrophotometer, and samples with A260/A280 ratios between 1.7–2.0 were considered acceptable). (1.8-1.9), which were measured using nanodrop, PCR was performed for the target gene fragments in the study genes, as follows: (*TSHR* 499 bp; *TSHβ* 520 bp; *TPO* 449 bp) with the expected amplicons for genotyping and subsequent analysis, the amplified results were confirmed using agarose gel electrophoresis, the product showed clear lines, indicating the quality of the DNA and its suitability for genotyping, as a high molecular weight DNA band was obtained, the results are shown in Figures (4-2) - (4-3) - (4-4):

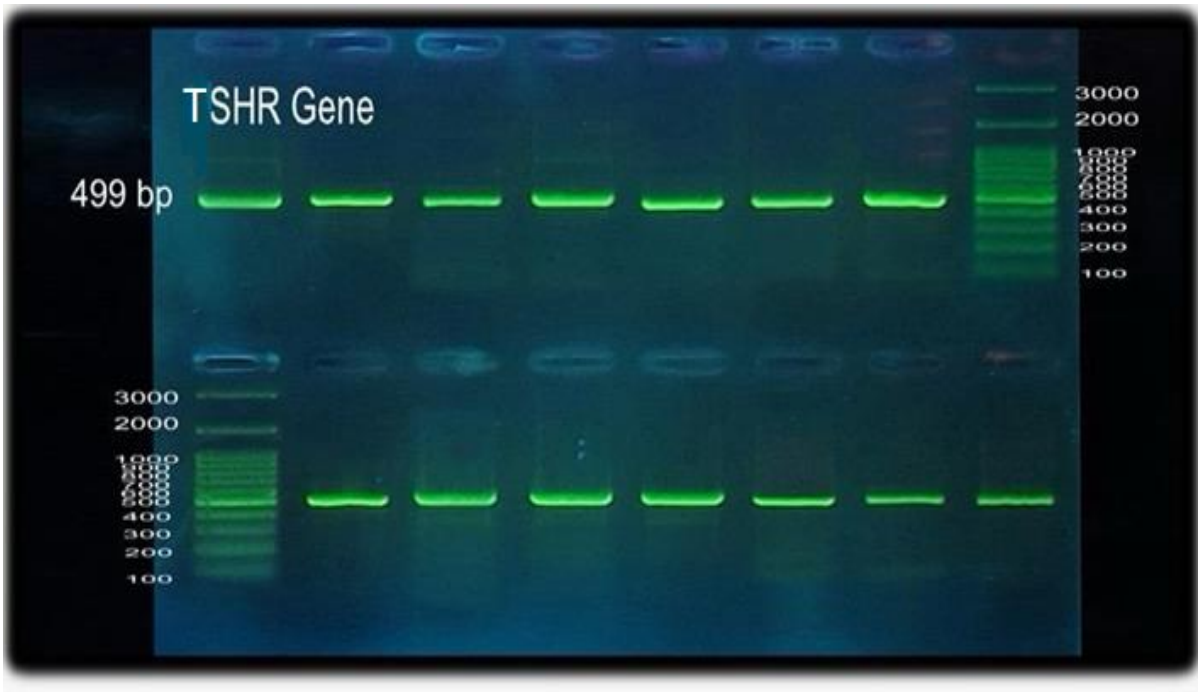


Figure (4-2): The Amplification product of the *TSHR* gene (499 bp).

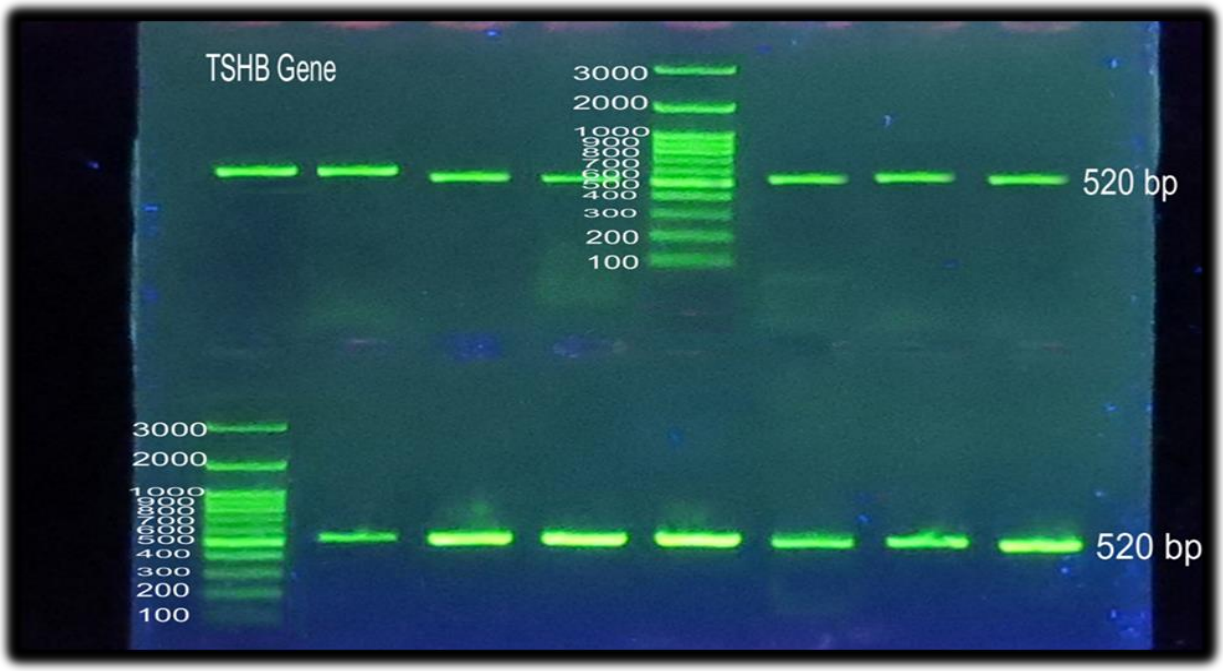


Figure (4-3): The Amplification product of the *TSHβ* gene (520 bp).

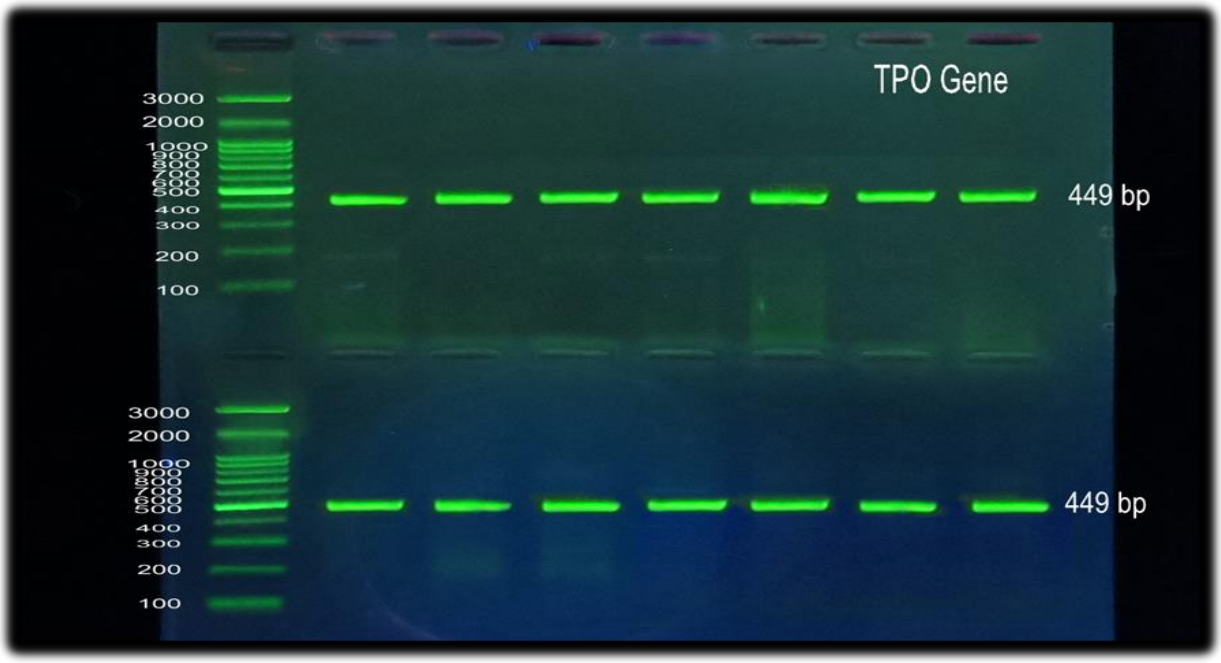


Figure (4-4): The Amplification product of the *TPO* gene (449 bp).

The presence of clear and sharp bands of the expected size confirms the successful amplification of the targeted gene regions, indicating that the extracted DNA was of sufficient purity and integrity for downstream sequencing analysis.

4.4.2 Multiple Sequence Alignment

The sequencing results were obtained from Macrogen in the form of chromatogram files (.ab1) for the amplified regions, high quality chromatogram peaks without overlapping background signal confirmed the accuracy of base calling and validated the identification of single nucleotide variants (SNVs), the obtained sequences were aligned against the reference gene sequences retrieved from the NCBI GenBank database using multiple sequence alignment tools in order to detect and confirm base substitutions.

4.4.2.1 *TSHR* Gene

The results of nucleotide sequence analysis using Multiple Sequence Alignment MSA revealed one SNV in (*TSHR)* gene in thyroid disorder patients, compared to the control group of healthy women and the reference sequence of the same gene registered in GenBank under Accession Number (KJ892323).

A point mutation c.477 T>C was identified at position 477 within exon (10) of the (*TSHR)* gene in both thyroid disorder samples, where the nucleotide (T) was substituted with (C), as shown in Figure (4-6).

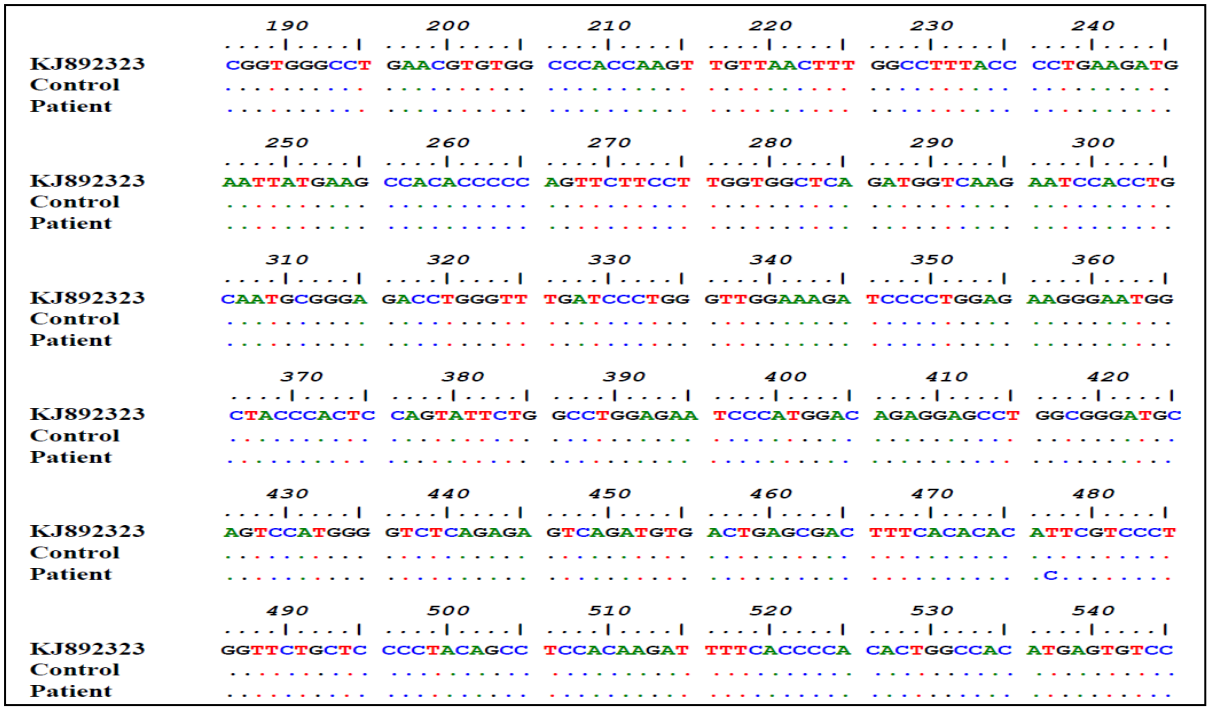


Figure (4-5): Alignment of *TSHR* nucleotide sequences.

Genotypes were identified using Geneious Software (version 10.1.3) by comparing the DNA sequences of control samples and those from patients and the reference sequence registered in the NCBI GenBank (Accession Number: KJ892323), the alignment of the DNA sequences revealed the presence of three genotypes at the studied site c.477 T>C within (*TSHR)* gene, the interpretation of the results is as follows in Figure (4-7).

This mutation occurs within the extracellular ligand binding domain of the TSH receptor, a region essential for TSH recognition and activation of the Gαs–cAMP signaling pathway, the substitution associated with the TC genotype may alter receptor sensitivity or signal transduction efficiency, potentially modifying thyroid hormone synthesis and release, this could predispose affected individuals to dysregulated thyroid function and increase susceptibility to thyroid disease (Pujol-Borrell *et al*., 2015).

1.The presence of a single blue peak indicates the wild type genotype (TT), meaning no mutation has occurred in either allele.

2.The presence of two peaks, one blue and one green, indicates a heterozygous genotype (Y=T/C), suggesting a mutation in one of the two alleles, regardless of whether the base change is visible above the peak.

3.A single green peak accompanied by a base change above the peak to (C) indicates a homozygous mutant genotype (CC), meaning a mutation has occurred in both alleles.

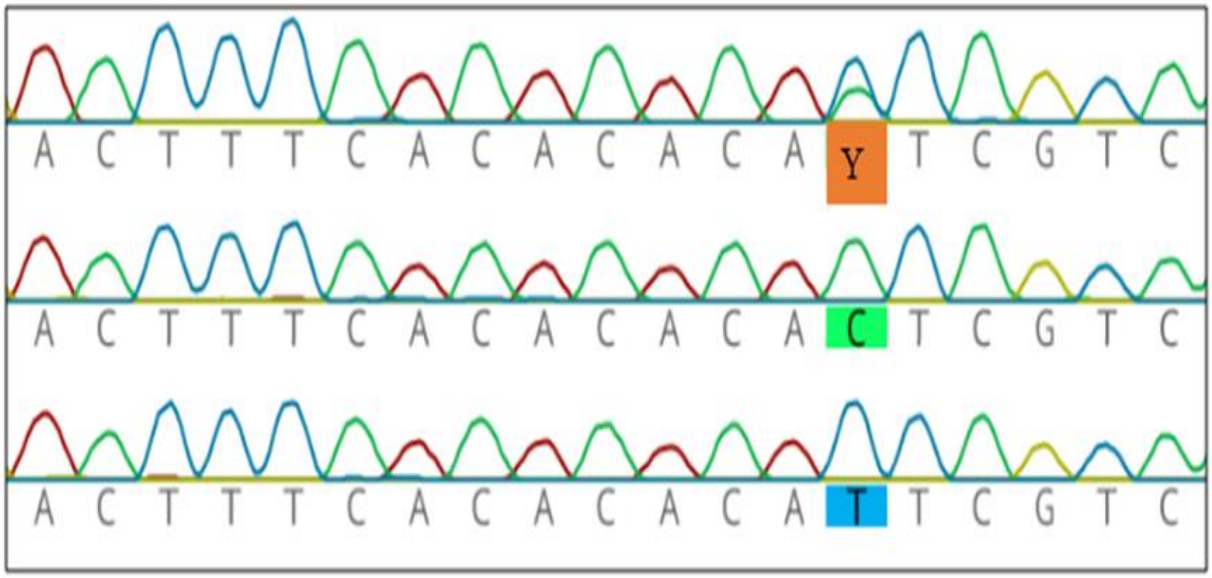


Figure (4-6): Sequencing chromatograms of *TSHR* gene polymorphisms (c.477T>C), Y: T/C .

4.4.2.2 *TSHβ* Gene

MSA analysis of the studied fragment of the (*TSHβ)* gene exon (3) region revealed a SNV at position 113 of the gene sequence in hyperthyroid women, this variation involved the substitution of (G) with (A) c.113G>A the mutation is a transition, involving a change from one purine to another purine base (Figure 4-8).

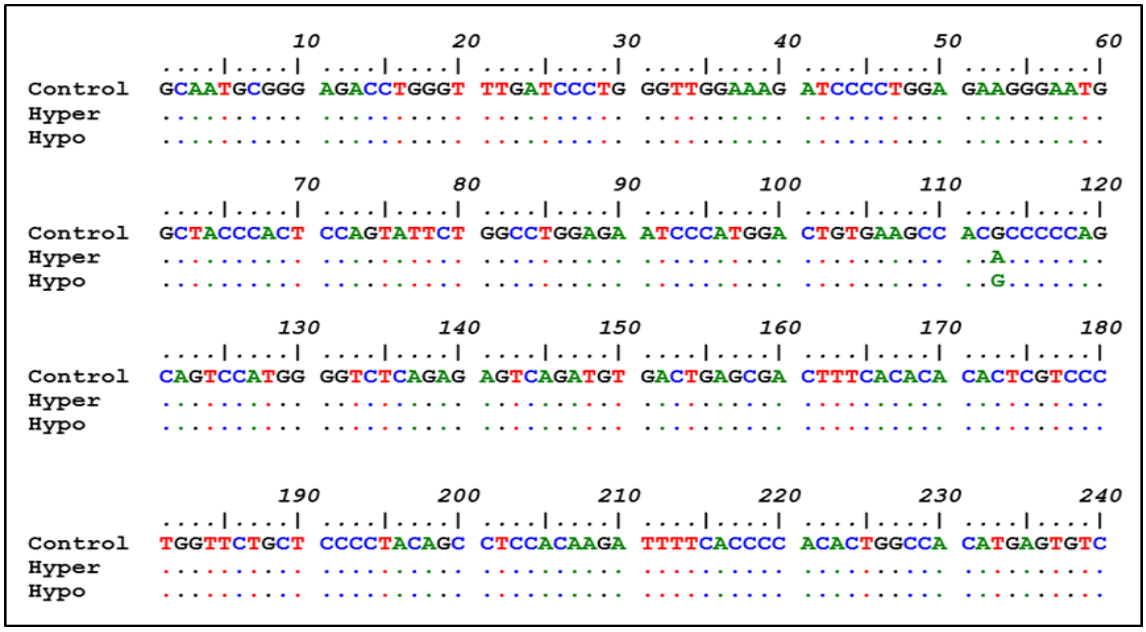


Figure (4-7): Alignment of *TSHβ* nucleotide sequences.

Sequence alignment of the samples revealed three genotypes at the studied site c.113G>A in the (*TSHB*) gene.

This substitution may affect hormone receptor interaction stability, altering TSH bioactivity and disrupting pituitary feedback regulation. Such changes can impair the physiologic signaling of the (HPT) axis and contribute to the development of thyroid dysfunction (Nicholas *et al*., 2017).

1.A single yellow peak in the chromatogram indicates the wild type genotype (GG), in which no mutation is present in either allele.

2.The presence of two peaks, red and yellow, indicates a heterozygous genotype (R), meaning a mutation occurred in one allele, regardless of whether the nucleotide above the red peak changed to A or remained unchanged.

3.A single red peak with the nucleotide above it changed to A indicates the homozygous mutant genotype (AA), in which both alleles carry the mutation (Figure 4-8).

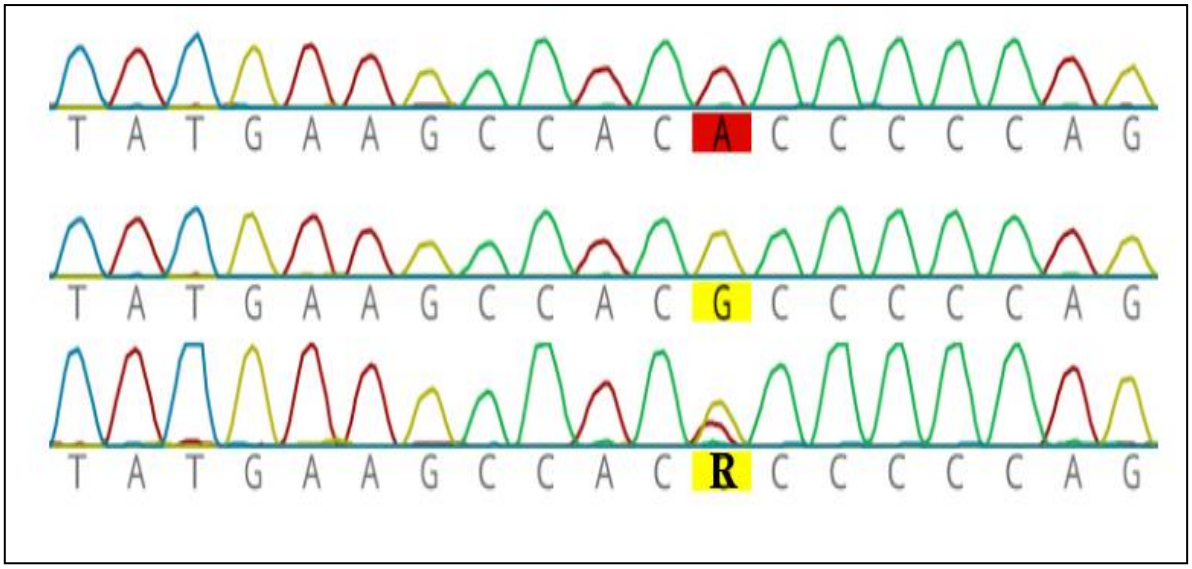


Figure (4-8): Sequencing chromatograms of *TSHβ* gene polymorphisms (c.113G>A), R: G/A.

4.4.2.3 *TPO* Gene

The results of the nucleotide sequence analysis using MSA in the promoter region revealed a single genetic variation at position 361 of the (*TPO)* gene in hyperthyroid and hypothyroid women, compared with the control group, this variation involved the substitution of the nucleotide Cytosine (C) with Guanine (G) c.361C>G, the mutation is classified as a transversion, in which one pyrimidine base is replaced by purine (C↔G), as shown in Figure (4-9).

This variant affects the heme-binding catalytic core of Thyroid Peroxidase, a region essential for iodine oxidation and thyroglobulin iodination. Such structural disruption can reduce the enzymatic efficiency of TPO, ultimately lowering thyroid hormone synthesis and contributing to hypothyroid tendencies in affected individuals (Ferrando *et al*., 2003).

Figure (4-9): Alignment of *TPO* nucleotide sequences.

The genotypes were determined using Geneious Software, the sequences of the control samples and those from patients with hyperthyroid and hypothyroid were compared with the reference sequence for women available in the NCBI GenBank database, sequence alignment revealed in Figure (4-10), three genotypes at the studied site.

1.A single blue peak in the chromatogram indicates the wild type genotype (CC), meaning no mutation is present in either allele.

2.The presence of two peaks, blue and yellow, indicates a heterozygous genotype (CG), where a mutation has occurred in one allele, regardless of whether the nucleotide above the peak changes to (C) or not.

3. A single yellow peak with the nucleotide above it changed to (G) represents the homozygous mutant genotype (GG).

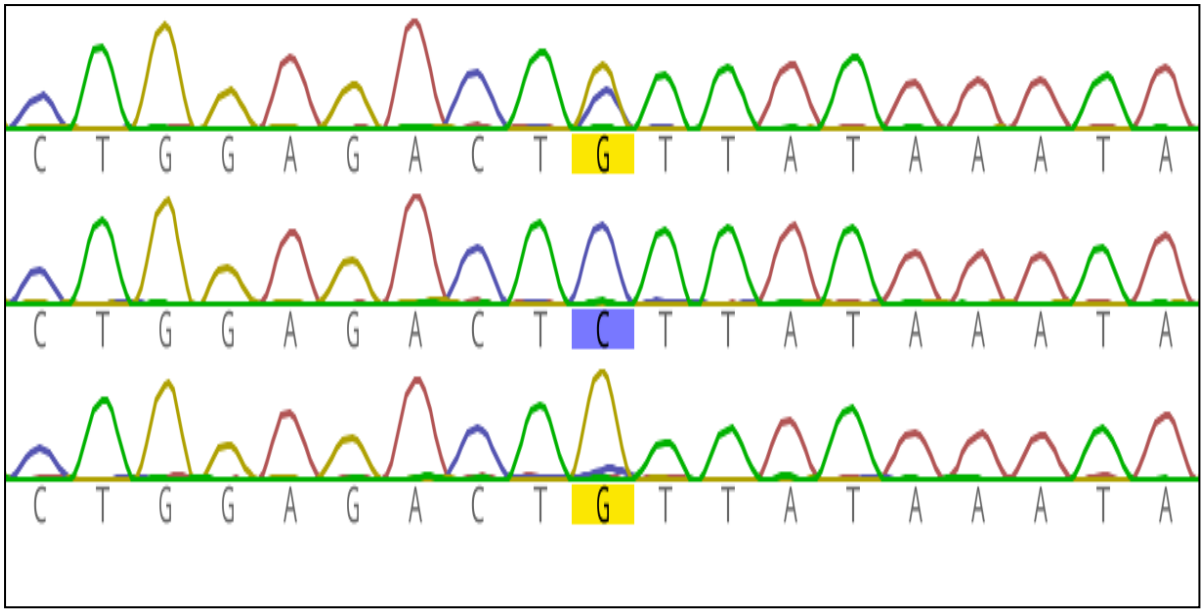


Figure (4-10): Sequencing chromatograms of *TPO* gene polymorphisms (c.361C>G), R: C/G.

4.4.3 Association Study of the *TSHR* Gene c.477T>C Polymorphism and Thyroid Disorders

This study addressed the SNV located within the exone (10) of the (*TSHR)* gene on chromosome (14q31) and its association in a group of patients with hyperthyroidism and hypothyroidism, it included: genotype analysis, Hardy Weinberg equilibrium (HWE) Test, Allele Frequencies, Odds Ratio (OR), Confidence Intervals (CI) and Amino Acid (aa) changes were also calculated to determine the risk of developing the disease.

4.4.3.1 c.477T>C and Hyperthyroidism

The analysis for c.477T>C SNV of the (*TSHR)* gene using, the HWE revealed no statistically significant difference between the hyperthyroidism group and the control group P= 0.218, P < 0.05 this finding indicates that the control group conformed to the, the observed genotype counts for (TT), (TC), and (CC) were (9), (4), and (2) respectively, while the expected counts were (8.07), (5.87), and (1.07) respectively, furthermore the calculated HWE values

for the genotypes were TT (53.78%), TC (39.11%), and CC (7.67%), as presented in Table (4-4).

Table (4-4): Observed and Expected Numbers and (HWE) of Genotypes *TSHR* Gene in Hyperthyroidism.

Case		Genotypes			*P*-value
	No.	TT	TC	CC	
Hyperthyroidism	Observed	9	4	2	0.218
	Expected	8.07	5.87	1.07	
	HWE %	53.78	39.11	7.67	

4.4.3.2 Analysis of Genotypes and Alleles Frequencies for c.477T>C in Hyperthyroidism

The results in Table (4-5), detecting the (*TSHR)* gene c.477T>C genotypes and allele frequencies in the hyperthyroidism group and control group, confirmed that there were no statistically significant (P>0.05) differences in the hyperthyroidism and control groups, the (TC) genotype were positively correlated with hyperthyroidism ORs of (5.09), these genotype were shown to be positively associated with the Etiological Fraction (EF) with a probability of (0.80), this indicates that individuals with these genotype is more likely to develop the disease than those with the (TT) and (CC) genotypes, as shown by ORs of (0.75) and (0.42) respectively, the (TT) and (CC) genotypes appeared to be linked to a decreased incidence of hyperthyroidism in this group.

The (C) allele lowers the risk of hyperthyroidism, whereas the (T) allele may raise the likelihood of hyperthyroidism, the (OR) for the (C) and (T) alleles were (0.84) and (1.17) respectively.

Table (4-5): Genotypes and Allele Frequencies of *TSHR* Gene c.477T>C SNV in Hyperthyroidism and Control Group.

Genotype	Study groups		OR	95% CI	*P*-value
	Hyper No. (%)	Control No. (%)			
TT	9 (60.00%)	10 (66.66%)	0.75	0.169 to 3.327	0.70
PF	0.25				
TC	4 (26.67%)	1 (6.67%)	5.09	0.495 to 52.287	0.17
EF	0.80				
CC	2 (13.33%)	4 (26.67%)	0.42	0.064 to 2.766	0.36
PF	0.58				
Alleles Frequency					
T	22 (73.33%)	21 (70.0%)	1.17	0.382 to 3.628	0.77
C	8 (26.67%)	9 (30.0%)	0.84	0.275 to 2.612	

OR: Odd Ratio; CI: Confidence Interval-PF: Preventive Fraction; EF: Etiological Fraction.

4.4.3.3 c.477T>C and Hypothyroidism

The analysis for c.477T>C SNV of the (*TSHR)* gene using the HWE revealed no statistically significant difference between the hypothyroidism group and the control group P= 0.782, P < 0.05 this finding indicates that the control group conformed to the, the observed genotype counts for (TT), (TC), and (CC) were (4), (8), and (3), respectively, while the expected counts were (4.27), (7.47), and (3.27) respectively, furthermore the calculated HWE values for the genotypes were TT (28.44%), TC (49.78%), and CC (21.78%), as presented in Table (4-6).

Table (4-6): Observed and Expected Numbers and (HWE) of Genotypes *TSHR* Gene in Hypothyroidism.

Case	Genotypes				*P*-value
	No.	TT	TC	CC	
Hypothyroidism	Observed	4	8	3	0.782
	Expected	4.27	7.47	3.27	
	HWE %	28.44	49.78	21.78	

4.4.3.4 Analysis of Genotypes and Alleles Frequencies for c.477T>C in Hypothyroidism

The results in Table (4-7), detecting the (*TSHR)* gene c.477T>C genotypes and allele frequencies in the hypothyroidism group and control group, confirmed that there were statistically significant P>0.05 differences in the hypothyroidism and control groups, the (TC) genotype were positively correlated with hypothyroidism (OR) of (16.00), these genotype were shown to be positively associated with the (EF) with a probability of (0.937), this indicates that individuals with these genotype is more likely to develop the disease than those with the (TT) and (CC) genotypes, as shown by ORs of (0.18) and (0.68) respectively, the (TT) and (CC) genotypes appeared to be linked to a decreased incidence of hypothyroidism in this group.

The (C) allele lowers the risk of hypothyroidism, whereas the (T) allele may raise the likelihood of hypothyroidism, the (OR) for the (C) and (T) alleles were (2.04) and (0.48) respectively.

Table (4-7): Genotypes and Allele Frequencies of *TSHR* Gene c.477T>C SNV in Hypothyroidism and Control Group.

Genotype	Study groups		OR	95% CI	*P*-value
	Hypo No. (%)	Control No. (%)			
TT	4 (26.67%)	10 (66.66%)	0.18	0.037 to 0.873	0.033*
PF	0.819				
TC	8 (53.33%)	1 (6.67%)	16.00	1.65 to 1.65	0.016*
EF	0.937				
CC	3 (20.00%)	4 (26.67%)	0.68	0.124 to 3.785	0.666
PF	0.313				
Alleles Frequency					
T	16 (53.33%)	21 (70.0%)	0.48	0.169 to 1.414	0.018*
C	14 (46.67%)	9 (30.0%)	2.04	0.707 to 5.894	

OR: Odd ratio; CI: Confidence Interval- PF: Preventive Fraction; EF: Etiological Fraction.

***: Difference of Statistical Significance, P≤ 0.05.**

The current study diagnosed one novel mutation in exon (10) in some patients, this mutation is of the transition type missense, this mutation has not been studied globally we haven't found any research examining the connection between this SNV and hypothyroidism and hyperthyroidism diseases.

According to Tenenbaum-Rakover *et al*.(2015), the (*TSHR)* gene's exon (10) is the largest and most significant coding region because it encodes the portion of the outer segment (α-subunit) that binds to the TSH and the entire beta-subunit, including the seven trans membrane 7TM, it is represented by the three outer and inner loops, and the carboxylic terminus is in charge of

delivering stimulus signals into the interior of the cell through its interaction with the G protein, several studies have shown that mutations that cause the increase or loss of the function of the gene encoding the TSH receptor protein are mainly located in exon (10) (Nakamura *et al.*, 2014; Rahmah, (2021).

According to a recent study, chromosome (14) is connected to thyroid related disorders like hyperthyroidism and hypothyroidism (Naghibi *et al.*, 2022). (*TSHR*) gene mutations and polymorphisms have been examined about the emergence of different thyroid disorders (Gnanavel, 2023; Zufry and Hariyanto, 2024).

Several possible genes, including the TSH receptor gene, may be responsible for the variation in TH levels (Babić Leko, *et al.,* 2021; Gunjača, *et al.*, 2024). Previous study have found that genetic variants in the (*TSHR*) gene are associated with an increased risk of thyroid diseases (Hussain *et al.*, 2018).

4.4.3.5 The Effect of Mutation in *TSHR* Gene of Amino Acid changes in Thyroid Disorders

SNVs are among the most common genetic variants, these mutations occur when two different types of bases are substituted, for example, from a pyrinidine to a purine or vice versa, transversions also occur when similar types of bases are substituted, such as a pyrinidine to a pyrimidine or a purine to a purine, transversions are among the most common mutations (Freitas, 2023). As for the SNV in the (TSHR) protein, it resulted in an aa change in this protein, several studies have examined polymorphisms in the (*TSHR)* gene, including those found in germ cells of (TSHR), where the aa proline was replaced by the aa threonine, and the aa glutamic acid was replaced by the aa aspartic acid (Fuhrer *et al*., 2000). Synonymous mutations have also been found in exon (8) (Shao *et al*., 2011).

In this current study, it was found that there was a change in the protein output due to a change in the aa, as a mutation was found in the (*TSHR)* gene that led to a difference and change in one of the aa in the (TSHR) protein, as the type of mutation (genetic transformation) showed the replacement of the aa thymine with the aa cytosine, and the aa threonine was replaced with the aa isoleucine, as shown in Table (4-8).

Table (4-8): The Effect of Mutation in the *TSHR* Gene on aa change and Translation.

Gene	Mutation	aa change	Effect on Translation
TSHR	**c.477 T>C**	**147Ile>Thr**	**Missense**

T: Thymine, C: Cytosine, Thr: Threonine, Ile: Isoleucine, aa: amino acid.

4.4.3.6 Functional Impact of the Detected Variants

The identified SNVs are missense mutations, meaning they result in the substitution of one amino acid for another in the protein structure. Such substitutions may alter protein folding, receptor binding affinity, or enzymatic stability, depending on the mutation's location within the functional domain of the protein, therefore, these variants have the potential to influence thyroid hormone synthesis, receptor signaling, and autoantigenic recognition, ultimately contributing to thyroid dysfunction.

4.4.3.7 Protein Modeling and Structural Prediction

We performed secondary and tertiary structure predictions of the TSHR protein using phyre2.2 the secondary structure analysis revealed that the mutant type variant slightly altered the alpha helices 6% and the beta strands 17% in the patient group (hyperthyroidism and hypothyroidism) and the alpha helices 6% and the beta strands 18% in the wild type, there was also a slight change in the random coils 2% in the patient group (hyperthyroidism and hypothyroidism) and 3% in the control group, this seemingly small change may affect the

receptor membrane conformation and its functional interactions, the three dimensional structural model further clarified these conformational changes between the wild type and variant forms of mutant type, indicating the potential effects on receptor binding to ligands and subsequent signaling, as shown in: Table (4-9) and Figures (4-11), (4-12), (4-13).

Table (4-9): Prediction of Secondary Structure of TSHR proteins by Phyre2.2.

Variant	Parameters		
	α-Helix	β-strand	Random coil
Mutant type	6%	17%	2%
Wild type	6%	18%	3%

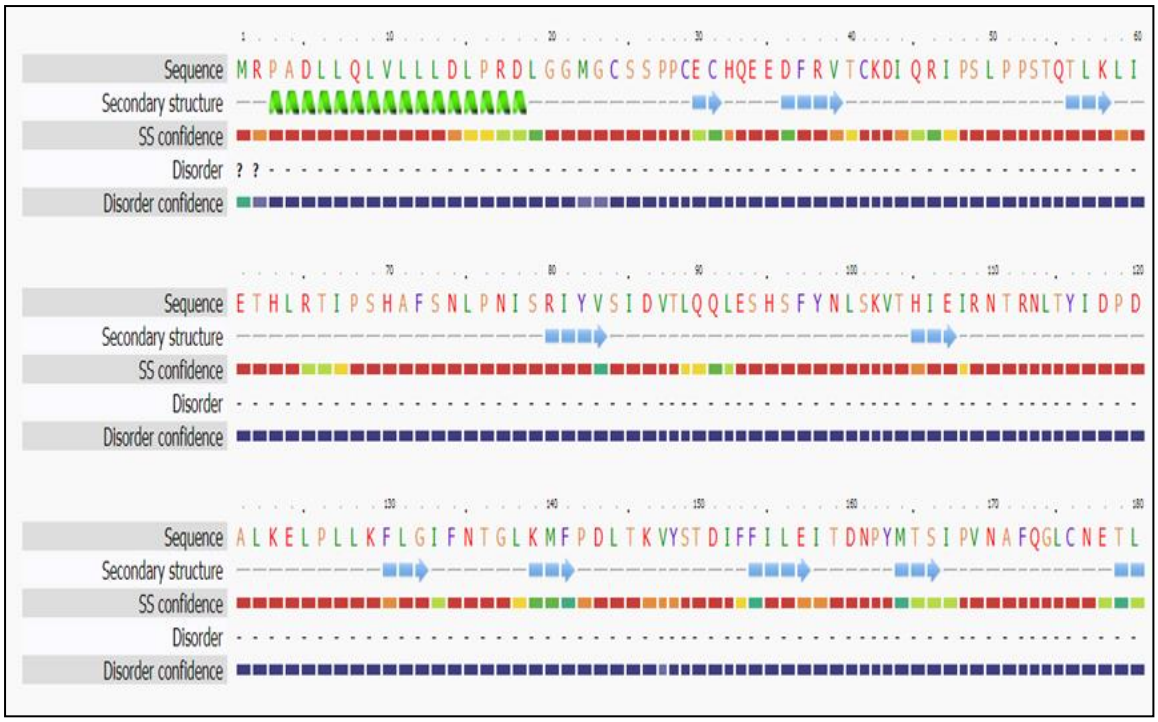


Figure (4-11): Analysis of TSHR's secondary structure in wild type.

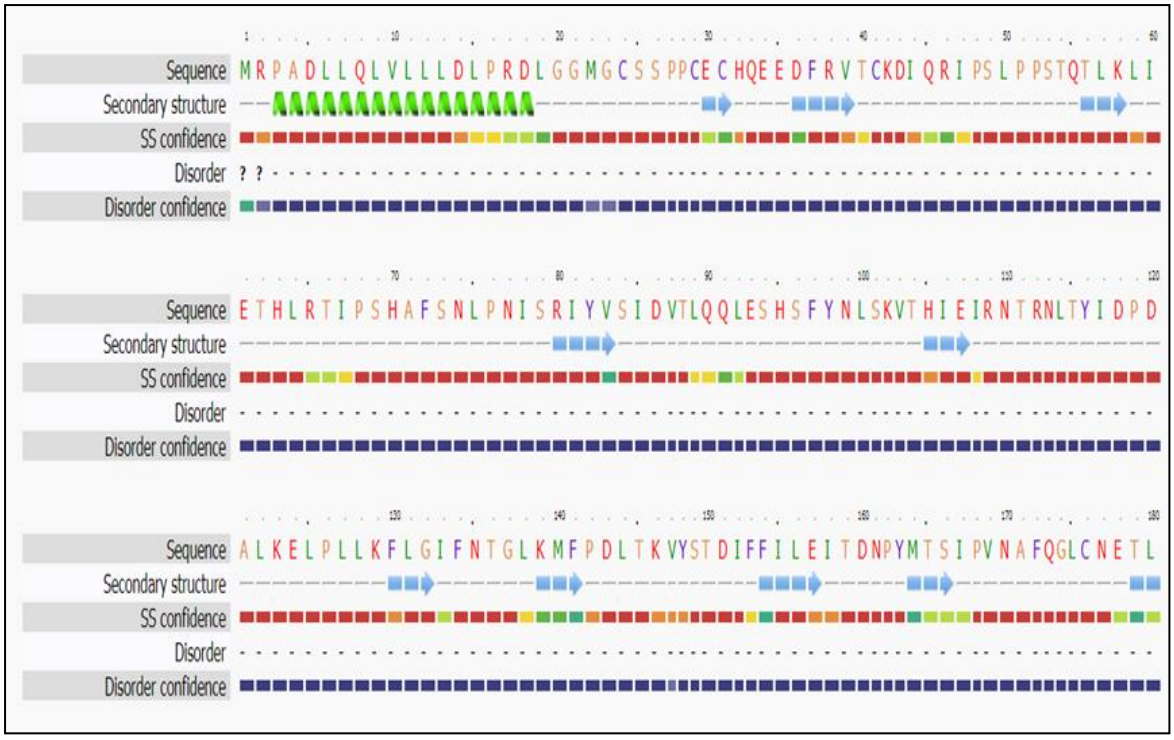


Figure (4-12): Analysis of TSHR's secondary structure in mutant type.

The mutation is in the chemical structure of the (TSHR) protein in patients having hyperthyroidism, Figure (4-14) includes A wild type and B represents the mutant type.

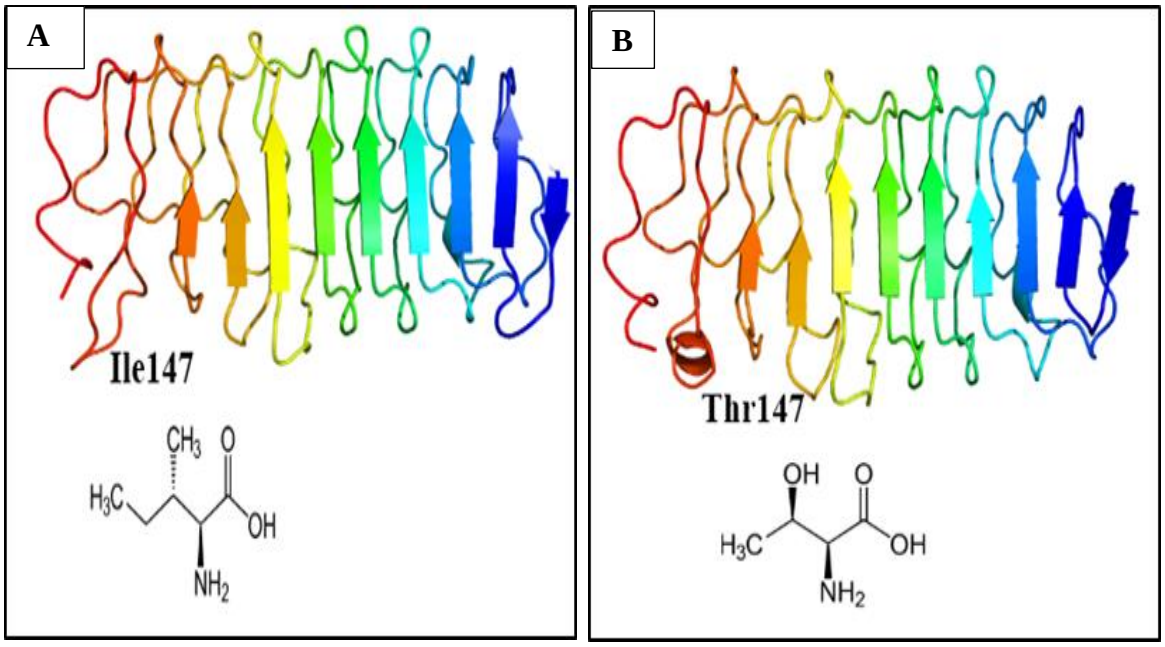


Figure (4-13): Model 3D structure of TSHR protein. A: wild type, B: mutant type.

(TSHR) mutations are mostly of the substitution type, which alters the amino acid, depending on the type of mutant base, these mutations can be either transitional or transformational, changing the receptor protein's structure, because of this, it interferes with its natural function, which results in either a gain-of-function that causes hyperthyroidism or a decrease in function that produces hypothyroidism, depending on where the mutation is located in the encoded area (Hussain *et al*., 2018).

Fuhrer *et al.* (2000) discovered that the conversion of guanine base to thymine causes the unique heterozygous germline mutation in hyperthyroidism patients, the aa valine (V) was changed to phenylalanine (F) at codon 597 as a result a shift in aa causes a malfunction in the production of the receptor protein, which impairs cell surface protein expression.

The mutations affect the biological function of the (TSHR), either or they cause an increase in the receptor function Gain of function, and it is either due to germinal or somatic mutations, both are located in the 7TM, and. inner and outer loops, but the mutations that cause the most effect occur in the three inner loops, which are a region of interaction with the G protein, which leads to a state of hyperthyroidism, or cause a loss of function of the receptor protein as a result of partial or complete resistance of the receptor protein to (TSH), and these mutations occur in the outer part and pieces across the membrane of the receptor protein, leading to hypothyroidism (Dumont *et al*.,1992 Derwahl, 1996).

The majority of research on hyperthyroidism claims that it is brought on by missense mutations (somatic or germinal) in seven transmembrane segments and the three inner and outer loops, which increase the activation of the enzyme AC and raise the intracellular cAMP concentration hence, promoting every

process that is in charge of producing thyroid hormones (Biebermann *et al.*, 2011).

4.4.4 A Genetic Association study of the *TSHβ* Gene c.113G>A SNV with Thyroid Disorders

This study addressed the SNV located within the (*TSHβ)* gene on chromosome (1) and its association in a group of patients with hyperthyroidism and hyporthyroidism, it included genotype, analysis, allele frequencies, (HWE), (OR), (CI) and genotyping of (aa) changes were also calculated to determine the risk of developing the disease.

4.4.4.1 c.113G>A and Hyperthyroidism

The analysis for c.113G>A SNV of the (*TSHβ)* gene using the HWE revealed no statistically significant difference between the hyperthyroidism group and the control group P= 0.101, P< 0.05 this finding indicates that the control group conformed to the, the observed genotype counts for (GG), (GA), and (AA) were (12), (2), and (1), respectively, while the expected counts were (11.27), (3.47), and (0.27) respectively, furthermore the calculated HWE values for the genotypes were GG (75.11%), GA (23.11%), and AA (1.78%), as presented in Table (4-10).

Table (4-10): Observed and Expected Numbers and (HWE) of Genotypes *TSHβ* Gene in Hyperthyroidism.

Case	**Genotypes**				***P*-value**
	No.	**GG**	**GA**	**AA**	
Hyperthyroidism	**Observed**	12	2	1	0.101
	Expected	11.27	3.47	0.27	
	HWE %	75.11	23.11	1.78	

4.4.4.2 Analysis of Genotypes and Alleles Frequencies of *TSHβ* Gene c.113G>A SNV in Hyperthyroidism

The results in Table (4-11), detecting the (*TSHβ)* gene c.113G>A genotypes and allele frequencies in the hyperthyroidism group and control group, confirmed that there were no statistically significant P>0.05 differences in the hyperthyroidism and control groups, the (AA) and (GA) genotypes were positively correlated with hyperthyroidism odds ratios of (3.20) and (2.15) respectively, these genotypes were shown to be positively associated with the (EF) with a probability of (0.687) and (0.534) respectively, this indicates that individuals with these genotypes are more likely to develop the disease than those with the (GG) genotype, as shown by ORs of (0.28), the (GG) genotype appeared to be linked to a decreased incidence of hyperthyroidism in this group.

The (G) allele lowers the risk of hypethyroidism, whereas the (A) allele may raise the likelihood of hyperthyroidism illness connection, the (OR) for the (G) and (A) alleles were (0.22) and (4.46) respectively.

Table (4-11): Genotypes and Allele Frequencies of *TSHβ* Gene c.113G>A SNV in Hyperthyroidism and Control Group.

Genotype	Study groups		OR	95% CI	*P*-value
	Hyper No. (%)	Control No. (%)			
GG	12 (80.00%)	14 (93.33%)	0.28	0.026 to 3.121	0.304
PF	0.72				
GA	2 (13.33%)	1 (6.67%)	2.15	0.173 to 26.673	0.550
EF	0.534				
AA	1 (6.67%)	0 (0.00%)	3.20	0.120 to 85.208	0.486

EF	0.687				
Alleles Frequency					
G	26 (86.67%)	29 (96.67%)	0.22	0.023 to 2.135	0.193
A	4 (13.33%)	1 (3.33%)	4.46	0.468 to 42.515	

OR: Odd Ratio; CI: Confidence Interval-PF: Preventive Fraction; EF: Etiological Fraction.

4.4.4.3 c.113G>A and Hypothyroidism

The analysis for c.113G>A SNV of the (*TSHB)* gene using the HWE revealed no statistically significant difference between the hypothyroidism and the control group P= 0.087, P< 0.05 this finding indicates that the control group conformed to the HWE, the observed genotype counts for (GG), (GA), and (AA) were (10, 3), and (2) respectively, while the expected counts were (8.82), (5.37), and (0.82) respectively furthermore, the calculated HWE values for the genotypes were GG (58.78%), GA (35.78%), and AA (5.44%), as presented in Table (4-12).

Table (4-12): Observed and Expected Numbers and (HWE) of Genotypes *TSHB* Gene in Hypothyroidism.

Case	**Genotypes**				***P*-value**
	No.	**GG**	**GA**	**AA**	
Hypothyroidism	**Observed**	10	3	2	0.087
	Expected	8.82	5.37	0.82	
	HWE %	58.78	35.78	5.44	

4.4.4.4 Analysis of Genotypes and Alleles Frequencies of *TSHβ* Gene c.113G>A SNV in Hypothyroidism

The results in Table (4-13), detecting the (*TSHβ)* gene c.113G>A genotypes and allele frequencies in the hypothyroidism group and control group, confirmed that there were no statistically significant P> 0.05 differences in the hypothyroidism and control groups, the (AA) and (GA) genotypes were positively correlated with hypothyroidism (OR) of (5.740) and (3.500) respectively, these genotypes were shown to be positively associated with the (EF) with a probability of (0.825) and (0.714) respectively, this indicates that individuals with these genotypes are more likely to develop the disease than those with the (GG) genotype, as shown by ORs of (0.142), the (GG) genotype appeared to be linked to a decreased incidence of hypothyroidism in this group.

The (A) allele may increase the probability of hypothyroidism disease association, while the (G) allele acts as a protective factor by reducing the chance of hypothyroidism, the (A) allele had an odds ratio of (8.826) whereas the (G) allele had an (0.113) OR.

Table (4-13): Genotypes and Allele Frequencies of *TSHβ* Gene c.113G>A SNV in Hypothyroidism and Control Group.

Genotype	Study groups		OR	95% CI	*P*-value
	Hypo No. (%)	Control No. (%)			
GG	10 (66.67%)	14 (93.33%)	0.142	0.014 to 1.418	0.096
PF	0.858				
GA	3 (20.00%)	1 (6.67%)	3.500	0.320 to 38.233	0.304
EF	0.714				

AA	2 (13.33%)	0 (0.00%)	5.740	0.252 to 130.379	0.272
EF	0.825				
Allele Frequency					
G	23 (76.67%)	29 (96.67%)	0.113	0.012 to 0.988	0.048*
A	7 (23.33%)	1 (3.33%)	8.826	1.012 to 76.963	

OR: Odd Ratio; CI: Confidence Interval-PF: Preventive Fraction; EF: Etiological Fraction.

***: Difference of Statistical Significance, P≤ 0.05.**

TSH hormone belongs to the glycoprotein hormones it is a heterogeneous protein, this hormone consists of two subunits, one of which is called the common alpha unit and the other the specific beta unit, thus giving the hormones an appropriate biological effect, therefore any defect in it causes multiple diseases (Nicholas *et al.*, 2017).

Many patients with mutations in the (*TSHβ)* gene have been identified worldwide, some cases may be due to autosomal recessive inheritance of defects in the (*TSHβ)* gene all patients with this defect in the (*TSHβ)* gene develop severe symptoms and problems of congenital hypothyroidism after birth, mutations have also appeared in the (*TSHβ)* subunit gene, especially in exons (2) and (3), and have shown cases of CCH, which led to a disruption in the biological functions of the protein (Heidari *et al.*, 2020).

More than (90) missense mutations have been reported in the (*TSHβ)* gene including, non-missense mutations, frameshift mutations (Hayashizaki *et al.*,1989; McDermott *et al.*, 2002). And splice site mutations c.162G>A (Pohlenz *et al*.,2002). A homozygous deletion has also been reported (Schmidts *et al.*, 2011). There is also a mutation that occurred in the (*TSHB)* gene, specifically in exon (3), where it changed the sequence of amino acids, especially in the seat belt region of the TSH diheterodimer, which is a very

important region in forming dimers with the a-subunit, and thus is important and necessary for the accurate secretion of the mature hormone TSH (Heidari *et al.,* 2020).

In contrast, not a single mutation has been found to be an aa change, but it has been found in the 5′ splice site of the donor of exon/intron (2), resulting in the skipping of exon (2), a homozygous deletion of the (*TSHβ)* gene has also been found. (Hermanns *et al.,* 2014). A heterozygous mutation in the second exon of the (*TSHβ)* gene were identified, where this mutation caused an aa change in the signal peptide of the (*TSHβ)* gene subunit, there is also a missense mutation in codon (11) of exon (2), where the aa phenylalanine was replaced by the aa tyrosine to the hydrophobic core of the signal peptide, there is also a change in the mutation in codon (14) of exon (2) in the aa sequence, where the aa threonine was changed to the aa alanine in the polar terminal region COOH of the signal peptide (Heidari *et al*., 2020).

4.4.4.5 The Effect of Mutation in *TSHβ* Gene on Amino Acid changes in Thyroid Disorders

The conversion of nucleotides from thymine to adenine at position (32) in exon (2) c.32T>A, the missense mutation occurs in the (*TSHβ*) gene, which leads to the change of the aa hydrophobic phenylalanine to the polar and hydrophilic tyrosine in position (11) localized in the especially near the cleavage site in the COOH terminal region of the signal peptide, this can cause changes in the (TSHβ) structure, that is, it will work on which can lead to a disruption of the efficiency of signal peptidase cleavage (Jiang *et al*., 2002; Heidari *et al*., 2020).

In current study, it was found that there is a change in the protein product due to a change in the aa, as a mutation was found in the (*TSHβ)* gene that led to a difference and change in one of the aa in the (TSHβ) protein, as the type of mutation, missense, changed the Guanine to Adenine at position (113) and the

aa Alanine was also replaced with the (aa) Threonine in position (32), as shown in: Table (4-14).

Table (4-14): The Effect of Mutation in the *TSHβ* Gene on aa change and Translation.

Gene	Mutation	aa change	Effect on Translation
TSHβ	**c.113 G>A**	**32Ala>Thr**	**Missense**

G: Guanine, A: Adenine, Thr:Threonine, Ala: Alanine, aa: amino acid.

4.4.4.6 Protein Modeling and Structural Prediction

We performed secondary and tertiary structure predictions of the (TSHβ) protein using phyre2.2 the secondary structure analysis revealed that the c.113G>A variant slightly altered the alpha helices 11% and the beta strands 41% and the random coils 1% in the wild type, and the alpha helices 11% and the beta strands 43% and the random coils 1% in the hypothyroidism, this seemingly small change may affect the protein conformation and its functional interactions, 3D Model of structure further clarified these conformational changes between the wild type and variant forms of c.113G>A indicating the potential effects on receptor binding to hormone, as shown in: Table (4-15), Figures (4-14), (4-15), (4-16).

Table (4-15): Prediction of Secondary Structure of TSHβ proteins by Phyre2.2.

Variant	Parameters		
	α-Helix	**β-strand**	**Random coil**
Wild type	**11%**	**41%**	**1%**
Mutant type	**11%**	**43%**	**1%**

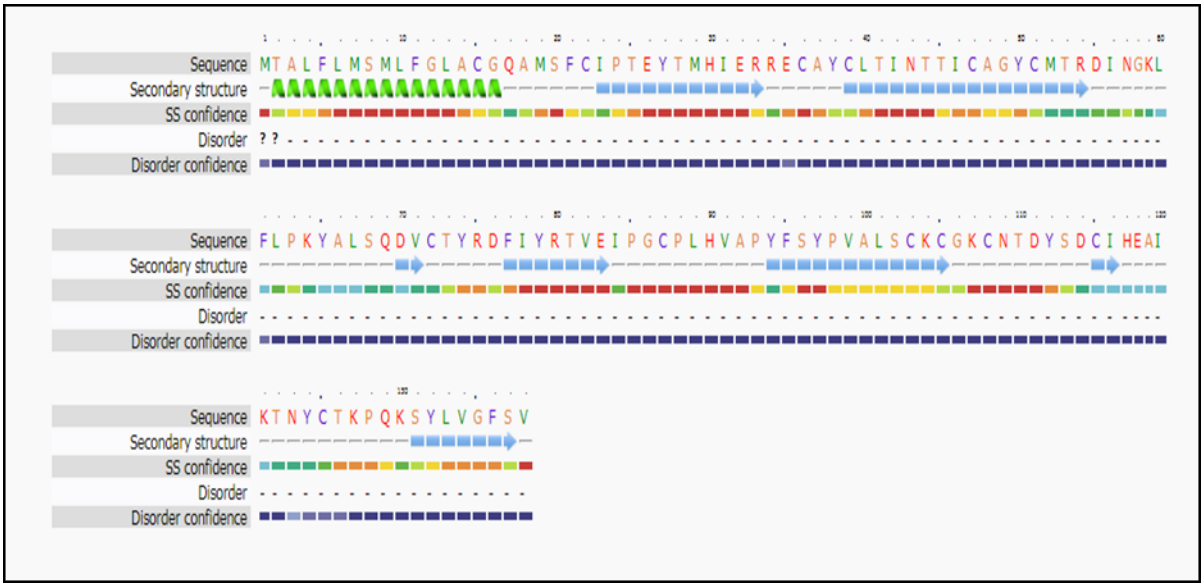


Figure (4-14): Analysis of TSHβ secondary structure in wild type.

Figure (4-15): Analysis of TSHβ secondary structure in mutant type.

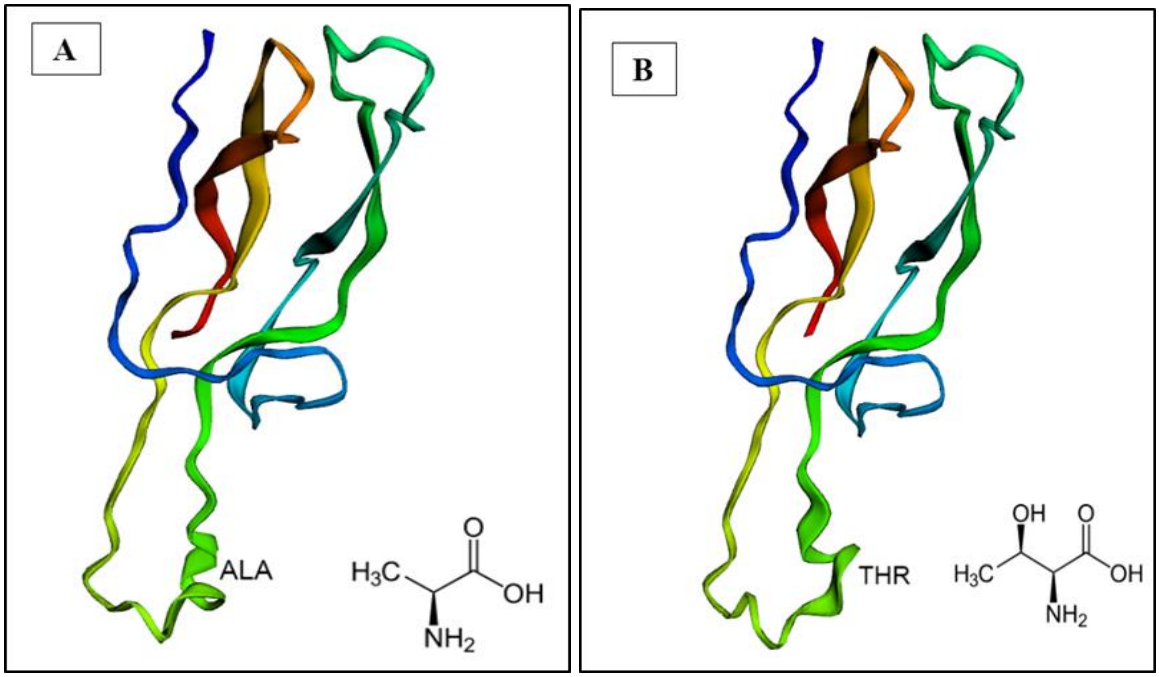


Figure (4-16): 3D Model of structure of TSHβ protein. A: wild type, B: mutant type.

SNVs are among the most common genetic variants, these mutations occur when two different types of bases are substituted, for example, from a pyrimidine to a purine or vice versa, transversions also occur when similar types of bases are substituted, such as a pyrimidine for a pyrimidine or a purine for a purine, transversions are among the most common mutations (Freitas, 2023).

Some research on hyperthyroidism suggests that missense mutations (somatic or germinal) at various locations in the 7TM, and the three inner and outer loops cause the illness, as a result, the AC enzyme is more activated, increasing the intracellular cAMP content, thus, encouraging every activity that generates thyroid hormones (Biebermann *et al.*, 2011). As for the change in aa in the (TSHβ) protein, a mutation was also found that changed the aa glycine to valine and cysteine at positions (71) and (72) respectively, these results indicate that the composition of the (TSHβ) subunit has changed due to these mutations, which led to these mutations being a cause of CCH disease (Heidari *et al.*, 2020).

Additionally, mutations in the trans membrane fragments result in a malfunction in the promoter signal's transmission, which leads to a weak expression of the receptor protein on the cell surface, alternatively, an aa may be substituted with another aa that alters its chemical characteristics, which could impact the proper folding of the protein at the cell surface (Tonacchera *et al*., 2000; Nagashima *et al.*, 2001).

A mutation was found c.316G>C in exon (3) with congenital central hypothyroidism, which was linked to this mutation in codon (106) in exon (3) resulting was a change in the protein product, i.e., a change in the aa here, a change occurred from a glycine aa to an arginine aa (Heidari *et al.,* 2020).

4.4.5 A Genetic Association study of the *TPO* Gene c.361C>G and its Relationship with Thyroid Disorders

This study addressed the SNV located within the (*TPO)* gene on chromosome (3) and its association in a group of patients with hyperthyroidism and hypothyroidism, it included genotype, analysis, allele frequencies, and HWE, OR, CI and genotyping of aa changes were also calculated to determine the risk of developing the disease.

4.4.5.1 c.361C>G and Hyperthyroidism

The analysis for c.361C>G SNV of the (*TPO)* gene using the HWE test revealed no statistically significant difference between the hyperthyroidism group and the control group P= 0.390, P< 0.05, this finding indicates that the control group conformed to the HWE, the observed genotype counts for (CC), (CG), and (GG) were (4), (9), and (2) respectively, while the expected counts were (4.82), (7.37), and (2.82) respectively furthermore, the calculated HWE values for the genotypes were CC (58.78%), GC (35.78%), and GG (5.44%), as presented in Table (4-16).

Table (4-16): Observed and Expected Numbers and (HWE) of Genotypes *TPO* Gene in Hyperthyroidism.

Case		Genotypes			*P*-value
	No.	CC	CG	GG	
Hyperthyroidism	Observed	4	9	2	0.390
	Expected	4.82	7.37	2.82	
	HWE %	58.78	35.78	5.44	

4.4.5.2 Analysis of Genotypes and Alleles Frequencies of *TPO* Gene c.361C>G SNV in Hyperthyroidism

The results in Table (4-17), detecting the (*TPO)* gene c.361C>G genotypes and allele frequencies in the hyperthyroidism group and control group, confirmed that there were no statistically significant P>0.05 differences in the hyperthyroidism and control groups, the (CG) genotype were positively correlated with hyperthyroidism ORs of (0.61), these genotype were shown to be positively associated with the (EF) with a probability of (0.66), this indicates that individuals with these genotype is more likely to develop the disease than those with the (CC) and (GG) genotypes, as shown by ORs of (0.41) and (3.00) respectively, the (CC) and (GG) genotypes appeared to be linked to a decreased incidence of hyperthyroidism in this group.

The (C) allele lowers the risk of hyperthyroidism, whereas the (G) allele may raise the likelihood of hyperthyroidism, the ORs for the (C) and (G) alleles were (0.75) and (1.32) respectively.

Table (4-17): Genotypes and Allele Frequencies of *TPO* Gene c.361C>G SNV in Hyperthyroidism and Control Group.

Genotype	Study groups		OR	95% CI	*P*-value
	Hyper No. (%)	Control No. (%)			
CC	4 (26.67%)	7 (46.67%)	0.41	0.090 to 1.917	0.260
PF	0.59				
CG	9 (60.00%)	5 (33.33%)	3.00	0.676 to 13.309	0.144
EF	0.66				
GG	2 (13.33%)	3 (20.00%)	0.61	0.087 to 4.341	0.486
PF	0.39				
Alleles Frequency					
C	17 (56.67%)	19 (63.33%)	0.75	0.268 to 2.133	**0.**598
G	13 (43.33%)	11 (36.67%)	1.32	0.468 to 3.721	

OR: Odd Ratio; CI: Confidence Interval-PF: Preventive Fraction; EF: Etiological Fraction.

4.4.5.3 c.361C>G and Hypothyroidism

The analysis for c.361C>G SNV of the (*TPO)* gene using the HWE test revealed statistically significant difference between the hypothyroidism group and the control group P= 0.020, P< 0.05, this finding indicates that the control group conformed to the HWE, the observed genotype counts for (GG), (GA), and (CC) were (6), (3), and (6) respectively, while the expected counts were (3.75), (7.50), and (3.75) respectively furthermore, the calculated HWE values for the genotypes were GG (25%), GC (50%), and CC (25%), as presented in Table (4-18).

Table (4-18): Observed and Expected Numbers and (HWE) of Genotypes *TPO* Gene in Hypothyroidism.

Case		Genotypes			*P*-value
	No.	GG	GC	CC	
Hypothyroidism	Observed	6	3	6	0.020*
	Expected	3.75	7.50	3.75	
	HWE %	25	50	25	

***: Difference of Statistical Significance, P≤ 0.05.**

4.4.5.4 Analysis of Genotypes and Alleles Frequencies of *TPO* Gene c.361C>G SNP in Hypothyroidism

The results in Table (4-19), detecting the (*TPO)* gene (c.361C>G) genotypes and allele frequencies in the hypothyroidism group and control group, confirmed that there were no statistically significant (P>0.05) differences in the hypothyroidism and control groups, the (GG) genotype were positively correlated with hypothyroidism (OR) of (2.66), these genotype were shown to be positively associated with the (EF) with a probability of (0.62), this indicates that individuals with these genotype is more likely to develop the disease than those with the (CC) and (CG) genotypes, as shown by ORs of (0.76) and (0.50) respectively the (CC) and (CG) genotypes appeared to be linked to a decreased incidence of hypothyroidism in this group.

The (C) allele lowers the risk of hypothyroidism, whereas the (G) allele may raise the likelihood of hypothyroidism, the (OR) for the (C) and (G) alleles were (0.57) and (1.72) respectively.

Table (4-19): Genotypes and Allele Frequencies of *TPO* Gene c.361C>G SNV in Hypothyroidism and Control Group.

Genotype	Study groups		OR	95% CI	*P*-value
	Hypor No. (%)	Control No. (%)			
CC	6 (40.00%)	7 (46.67%)	0.76	0.179 to 3.240	0.712
PF	0.24				
CG	3 (20.00%)	5 (33.33%)	0.50	0.095 to 2.627	0.412
PF	0.50				
GG	6 (40.00%)	3 (20.00%)	2.66	0.520 to 13.655	0.239
EF	0.62				
Alleles Frequency					
C	15 (50.00%)	19 (63.33%)	0.57	0.206 to 1.623	0.299
G	15 (50.00%)	11 (36.67%)	1.72	0.615 to 4.844	

OR: Odd Ratio; CI: Confidence Interval-PF: Preventive Fraction; EF: Etiological Fraction.

Thyroid disorders occur due to interaction between genetic and environmental causes, these causes, such as the environment, affect the health of the thyroid gland, including a deficiency or scarcity of I, exposure to chemicals and heavy metals, or lifestyle factors such as stress and smoking, which cause a malfunction in the thyroid gland (Cooper and Biondi, 2018).

This interaction and combination occur between environmental stimuli, genetic predisposition, genes, and multiple signaling pathways, resulting in a predisposition to thyroid diseases (Tyagi *et al*., 2024).

Since (TPO) is a membrane bound glycoprotein, it stimulates THs and thus regulates their function (Godlewska and Banga, 2019).

The (*TPO)* gene is one of the major autoantigens in thyroid diseases, the presence of (TPO) antibodies (TPOAb) in patients with autoimmune thyroiditis is one of the characteristics that distinguish them from this disease, it is one of the important diagnostic factors in the disease, these antibodies also play a pathological role in the development of this disease, several SNVs in the (*TPO)* gene have been investigated, which are associated with the infection with autoimmune thyroiditis, these include variants in the coding regions and also the promoter region of the gene, the presence of the SNV rs1126797 has been indicated and its association with thyroid diseases, including hypothyroidism and diseases related to autoimmunity (Lacka *et al*., 2025).

Many previous studies have been presented that focused on the importance of the region called the heme binding region, which encodes exons 7-11 which is considered the catalytic center of the (TPO) protein, as it was considered extremely important for the enzyme activity (Faam *et al*., 2012).

As for exons 7-14 which contained a center or point of accumulation of active mutations (Bikkere *et al*., 1995).

Studies have some that multiple mutations in this (*TPO*) gene may cause a defect and thus affect the function of this enzyme, resulting in many diverse human diseases (Yu *et al.*, 2021).

AITDs is associated with the (TPO) rs 2048722 polymorphism and has been reported in Polish Caucasian patients (Jendrzejewski *et al.*, 2016). The rs1126797 polymorphism has been associated with hypothyroidism and AITDs In West Bengal (Balmiki *et al*., 2014).

In the (*TPO*) gene rs1126797, specifically in exon (11), a relationship was found between the development of clinical autoimmune diseases of the thyroid gland in the Caucasian Polish population it indicates a difference in the

genetic patterns and size of the thyroid gland in people with thyroid diseases, which may play a role in the phenotype of the disease (Lacka *et al*., 2025).

The results of some research have found SNVs in the (*TPO)* gene that have been associated with AITDs these variants included several regions, including the coding region and the promoter region of the gene (Lacka *et al*., 2024).

Faam *et al*. (2012) reported that the (C) allele of this SNV was associated with higher levels of (TPOAb) in patients with the condition. They was found that the genotype (CC) showed a significantly higher level of (TPOAb) when compared with patients with AITDs which was studied in the japanese population (Tomari *et al*., 2017).

Since rs1126797, which is located in the coding region of the (*TPO)* gene in exon 11, does not lead to an aa change (Balmiki *et al*., 2014).

However, synonymous SNVs affect the gene and thus its function, as they affect mRNA stability, splicing efficiency, and translation dynamics, also, since rs1126797 is located near other polymorphisms within the gene, it increases the likelihood of developing the disease (Lacka *et al*., 2025).

Ghanooni *et al*. (2023) identified rs1126797 as a significantly associated genotype requirement for (TPOAb). Balmiki *et al*. (2014) also reported that the (G) allele rs1126797 was protective against hypothyroidism.The main cause of hormonal imbalance is mutations in the (*TPO)* gene, which is one of the causes of congenital hypothyroidism, often associated with an enlarged thyroid gland (Arteaga-Jacobo *et al*., 2024).

In a study on the (*TPO)* gene, specifically the SNVs rs1126797, in patients with AITDs, it was observed that the (CG) genotype was present in affected individuals, who had a smaller thyroid gland size when compared to

healthy individuals, which leads to this effect of heterozygosity being a genetic pattern that also needs more studies and research to understand it more and in larger groups, this result is consistent with several previous studies, for example, it was found that there is a relationship between rs and AITDs (Ahmed *et al*., 2021). There has also been a study that supported the role of the (*TPO*) gene in AITDs (Tomari *et al*., 2017).

While Su *et al*. (2015) contradicted this by mentioning that there was no association between rs1126797 and patients with congenital hypothyroidism due to the presence of a defect in the formation of THs in the population in China.

Thus, many SNVs have been studied in the (*TPO)* gene and when they are associated with AITDs, for example, there is a study of (SNVs) that appeared in the promoter region, such as rs2071399, or in exon (12), such as rs 732609, where multiple population locations were studied (Ahmed *et al*., 2021).

There is also a population study conducted in Iran that rs 732608 in exon (12) was associated with hypothyroidism (Khoshi *et al*., 2017). There is also a study conducted in Japan, where rs2048722 was shown to be associated with levels of (TPOAb) (12) in Korea, although there was no association with hypothyroidism, it was positively associated with (TPOAb) (Kwak *et al*., 2014).

For example, a study in Egypt was conducted on infected patients, where it was proven that (TC) in rs2071400 was associated with an increased risk of AITDs (Ahmed *et al*., 2021). There is an association between SNVs of the (*TPO*) gene and levels of (TPOAb) (Khoshi *et al*., 2017).

The heterozygous region Un Translated Regions UTR’3 in rs6605278 is the strongest contributor to (TPOAb) positivity and the multiple SNVs in the (*TPO)* gene (Ghanooni *et al*., 2023).

Also, the pathophysiology of AITDs and (GD) may overlap, for example, in japan, a link between rs2071400 and (GD) has been shown (12), in contrast, a link between rs11675434 and (GD) has been shown in the Polish population (Kuś *et al*., 2015; Kuś *et al*., 2017). A study was conducted in Asian populations, where a link between rs 1126797 and SNV levels was found, and hypothyroidism, which Justifies its ethnic context 15-17 (Balmiki *et al*., 2014). A study conducted showed that age and body mass index had a significant effect on SNV, especially in those with (TPOAb) (Ghanooni *et al*., 2021).

The BMI also increased, indicating that the (T) allele of this SNV increased the risk of infection, he explained that weight gain increases the incidence of AITDs, many studies have shown that there is a relationship between the increased prevalence of AITDs and obesity (Song *et al*., 2019; Habib *et al*., 2020). For the first time, a study has found that there is an important population based relationship between the (*TPO)* gene and (TPOAb), and it was positive, as it showed that age, body mass index, and gender were recorded as important factors that had a confusing effect when (TPOAb) was transformed (Ghanooni *et al*., 2021).

In a study conducted in Japan, they were shown that the rs2048722 (CT+TT) genotype of the (*TPO)* gene showed a significantly higher level of (TPOAb) when compared to patients with AITDs of the (CC) genotype (Tomari *et al*., 2017). Many studies have presented studies on the (*TPO)* gene, including that 7-11 of the exons encode the catalytic center of the (*TPO)* gene protein, the heme binding region, which is of great importance for the enzyme activity (Bikker *et al*., 1995). There is a study that showed that 7-14 of the exons contained hot spots for mutations (Bikker *et al*., 1995).

4.4.5.5 The Effect of Mutation in *TPO* Gene of Amino Acid changes in Thyroid Disorders

Several SNVs were found in the (*TPO*) gene, such as Glu 641 Lys, Asp 668 Asn, Thr725Pro,Asp620Asn,Ser398Thr,andAla373Ser mutations in several vital sites in the (*TPO*) gene, which led to its inactivation, these results helped patients with hypothyroidism in genetic screening when it was studied in the population of west Bengal (Guria *et al*., 2014).

In this current study, our results found was found that there is a change in the protein product due to a change in the aa, as a mutation was found in the (*TPO)* gene that led to a difference and change in one of the aa in the (TPO) protein, as the type of mutation, missense, showed the replacement of Cytosine with Guanine, and the aa leucine was also replaced with the aa Valine, as shown in Table (4-20).

Table (4-20): The Effect of Mutation in the *TPO* Gene on aa change and Translation.

Gene	Mutation	aa change	Effect on Translation
TPO	**c.361 C>G**	**121 Leu>Val**	**Missense**

G: Guanine, C: Cytosine, Leu: leucine, Val: Valine, aa: amino acid.

4.4.5.6 Protein Modeling and Structural Prediction:

We performed secondary and tertiary structure predictions of the (TPO) protein using phyre2.2, the secondary structure analysis revealed that the c.361C>G variant slightly altered the alpha helices 48% and the beta strands 0% and the random coils 38% in the Patient group mutant type, and the alpha helices 48% and the beta strands 0% and the random coils 32% in the control group wild type, this seemingly small change may affect the receptor membrane conformation and its functional interactions, 3D Model of structure further clarified these conformational changes between the wild type and variant forms

of c.361C>G indicating the potential effects on receptor binding to ligands and subsequent signaling, as shown in: Table (4-21), Figures (4-17), (4-18), (4-19).

Table (4-21): Prediction of Secondary Structure of TPO proteins by Phyre2.2.

Variant	Parameters		
	α-Helix	β-strand	Random coil
Mutant type	48%	0%	38%
Wild type	48%	0%	32%

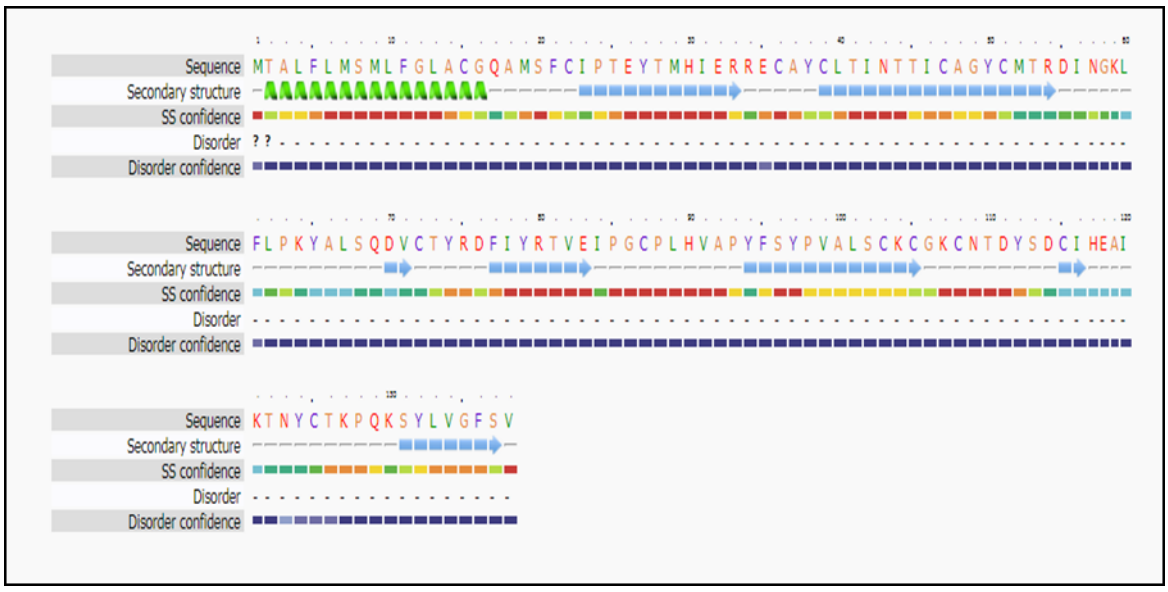


Figure (4-17): Analysis of TPO secondary structure in wild type.

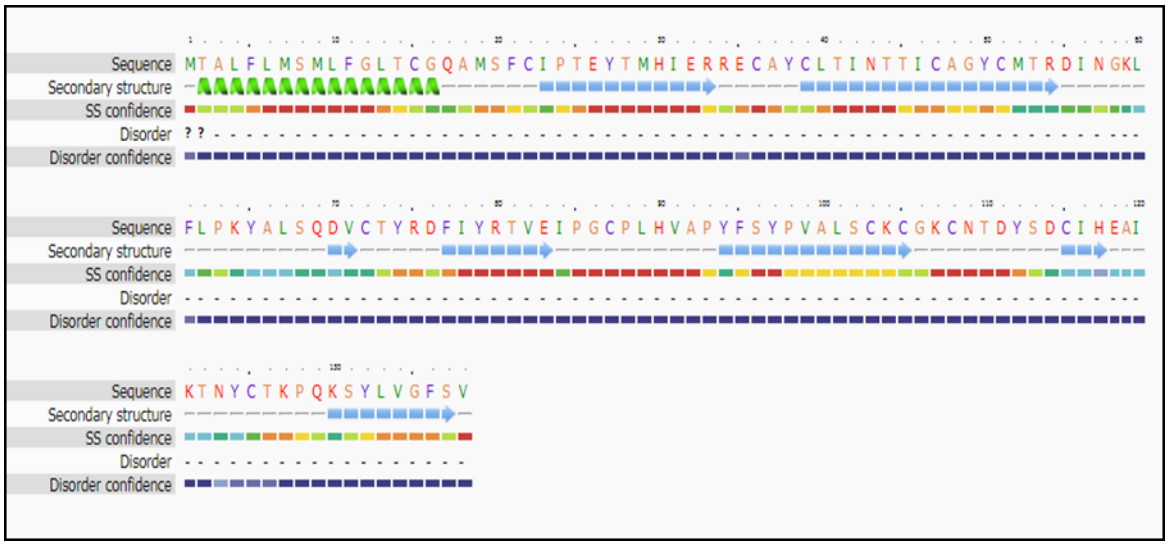


Figure (4-18): Analysis of TPO secondary structure in mutant type.

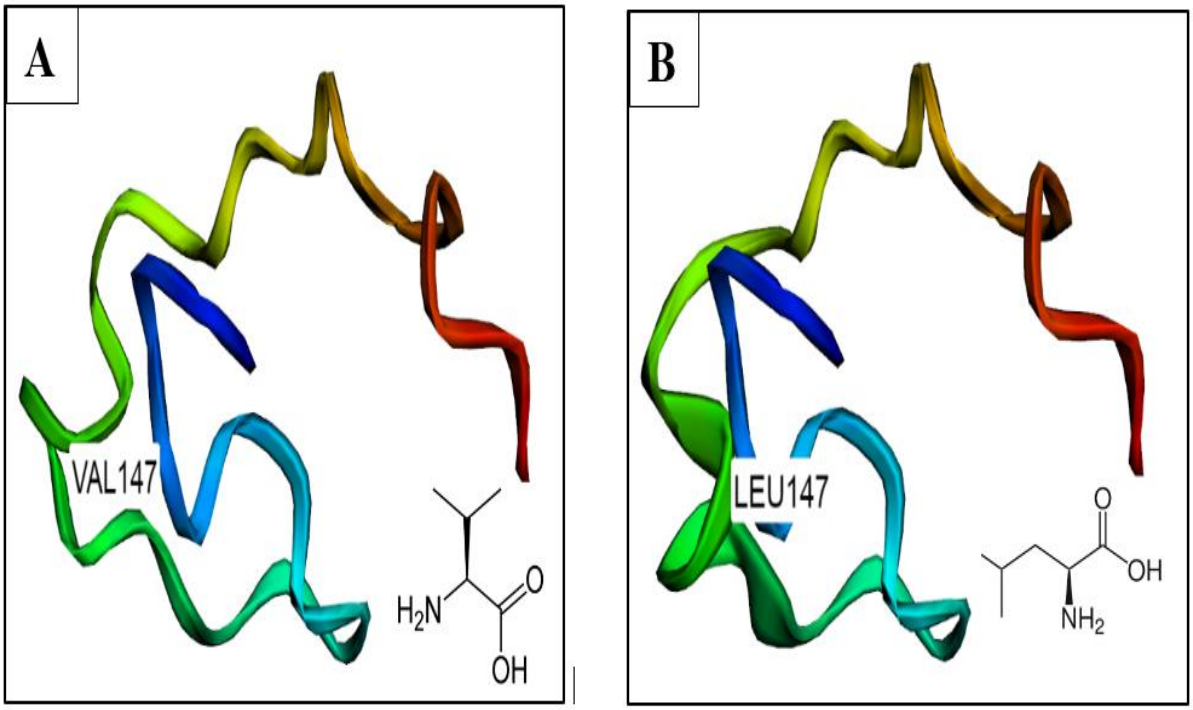


Figure (4-19): 3D Model of structure of TPO protein in patient. A: mutant type, B: wild type.

Some aa Glu 641 Lys - Thr 725Pro – Asp 620 Asn have been linked to hypothyroidism and hyperthyroidism (Guria *et al*., 2014).

As for the change in aa in the (TPO) protein, thus, it is expected that mutations in these regions will have significant effects on the activity of (TPO), and thus this leads to severe regulatory dysfunction and thus the occurrence of hypothyroidism, for example, in Glu 641 Lys, when a change occurs, i.e., a substitution between a negatively charged aa and another positively charged aa, and as we know, the aa threonine is considered a site for protein phosphorylation and thus is important for its activation (Krupa *et al*., 2004).

Thus, if any change occurs in the aa, such as a mutation that changes it in the protein product and thus a change in the aa, it will affect the activity of the (TPO) enzyme and thus may reduce the functional effectiveness, as is the case in some polymorphisms Glu641Lys - Thr725Pro -Asp620Asn, as it indicates an

effective and strong indicator of the presence of changes in active and vital sites in the (*TPO)* gene and thus leads to disruption to (TPO) (Guria *et al*., 2014).

A study found evidence of a link between rs732609 and rs121908087 in the (*TPO)* gene, as levels of (TPOAb) antibodies were found in patients with subclinical hypothyroidism, these regions showed that polymorphisms indicate a close association between (*TPO)* and subclinical hypothyroidism, as a significant increase in the level of (TPO) antibodies in the serum was found in the group of patients, especially in those infected who have the (C) allele rs732609 in the region, in addition, there was a substitution and change in amino acids such as Thr725Pro and Asn698Thr (Khoshi *et al*., 2017).

Therefore, that these regions are expected to have a significant impact on (TPO) activity, and thus the occurrence of mutations that led to severe regulatory dysfunction and thus the occurrence of hypothyroidism in Glu641Lys where the negatively charged aa, was replaced by the positively charged aa, since the aa, is considered a site for protein phosphorylation, Threonine is considered one of the important matters for its activation (Huse and Kuriyan, 2002).

These changes in the aa were considered mutations in (*TPO*) activity, which led to a reduction in its functional effectiveness, there is a study that showed that several polymorphisms of Glu641Lys, Thr725Pro and Asp620Asn, inactive enzymes and a decrease in (TPO) levels were considered a significant indicator of changes in the vital sites in the (*TPO*) gene, leading to its inactivation (Guria *et al.*, 2014).

Chapter Five

Conclusions and Recommendation

5.1 Conclusions

The present study demonstrated that single nucleotide variants (SNVs) in the *TSHR*, *TSHβ*, and *TPO* genes are associated with thyroid disorders among women in Misan Province. Sequence analysis revealed missense mutations that resulted in amino acid substitutions and structural alterations in the encoded proteins, as confirmed by 3D structural modeling.

1. *TSHR* gene: The C allele and the (TC/CC) genotypes were significantly associated with an increased risk of thyroid disorders, whereas the T allele and the TT genotype appeared to have a protective role.

2. *TSHβ* gene: The A allele and the (GA/AA) genotypes were associated with a higher risk of thyroid disorder, while the G allele and the GG genotype showed a protective effect.

3. *TPO* gene: The G allele and the (CG/GG) genotypes were associated with increased susceptibility to thyroid disorders, whereas the C allele and the CC genotype played a protective role.

4. The detected variants were found to correlate significantly with clinical and biological parameters including age, BMI, family history, and thyroid hormone levels, indicating that genetic factors contribute alongside physiological and environmental influences.

5. These findings support the role of genetic predisposition in the development of thyroid disorders and provide evidence that the studied (SNVs) may influence endocrine function through structural and regulatory alterations in key thyroid related proteins.

5.2 Recommendations

The current study presented the following Recommendations:

1. Incorporation of genetic screening for *TSHR*, *TSHβ*, and *TPO* variants in women with a family history of thyroid disorders.
2. Early hormonal evaluation (fT3, fT4, and TSH) in women presenting nonspecific symptoms such as fatigue, weight changes.
3. Furthermore, advanced molecular techniques including quantitative PCR can be applied to precisely measure the expression profiles of the *TSHR*, *TSHβ*, and *TPO* genes, thereby facilitating a clearer understanding of their contributory roles in the development of thyroid disorders among both males and females.
4. Further research with larger sample sizes and inclusion of additional genes involved in thyroid regulation (e.g., *TG*, *DIO2*, *PAX8*).

Chapter six
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Appendixes

Appendix

Republic of iraq
Ministry of Health
Misan Health Directorate
Training & Human Development Center
Research and Knowledge Management Unit

جمهورية العراق
وزارة الصحة
دائرة صحة ميسان
مركز التدريب والتنمية البشرية
وحدة ادارة البحوث والمعرفة

العدد: ٦٥٧ (الصادر)
التاريخ : ١٨ / ١١ / ٢٠٢٤

No :
Date :

الى/ مستشفى الصدر التعليمي
م.الحكيم التعليمي
م.الزهراوي الجراحي
المركز التخصصي لامراض السكري والغدد الصم
م/ تسهيل مهمة

تحية طيبة...

استنادا الى كتاب جامعة ميسان/ كلية العلوم المرقم ٣٦٠ في ٢٠٢٤/١١/١٤

يرجى تسهيل مهمة طالبة الماجستير الانسة (يمامة احمد عكلة)لغرض الحصول على العينات والمعلومات الخاصة ببحثها الموسوم:

(العلاقة بين التغيرات الوراثية في الجينات TSHR&TSHB&TPO لمرضى اضطرابات الغدة الدرقية في محافظة ميسان)

وفق الضوابط واصوليا.

مع التقدير...

رقم الوصل
٨٣٧٢٠٣

تاريخ الوصل
٢٠٢٤/١١/١٧

مدير المركز
علي اعنيد عبد الحسين
٢٠٢٤/١١/١٨

الدكتور محمد نعمة جيمان B.D.S

نسخة منه الى:
- وحدة البحوث مع الاوليات.

Appendix

Patient Questionnaire.

Patient No.( ).

Date of Collection (2024-2025).

Patient Name:	**Age: ( )**	**Gender: ( )**
Weight: ( ) K.gm	Length: ( )M	
Marital Status		
Family History		
Duration of Hypethyroidism		
Duration of Hypothyroidism		
Did you Take the Treatment ?		
Do you Suffer from Chronic Diseases? Diabetes, Blood pressure,…		
Comorbidities		

Other notes.

The Laboratory Tests.

Biochemical Analysis Type
T3
T4
TSH

الخلاصـــــة

الخلاصـــــة

اضطرابات الغدة الدرقية هي امراض الغدد الصم الشائعة ذات آثار سريرية واستقلابية كبيرة، تهدف هذه الدراسة إلى تسليط الضوء على بعض الأسس الجزيئية والجينية لخلل الغدة الدرقية لدى النساء من خلال معرفة العلاقة بين المتغيرات النوكليوتيدية المفردة SNVs في جينات مستقبلات هرمون تحفيز الغدة الدرقية (*TSHR*) وهرمون تحفيز الغدة الدرقية بيتا (*TSHB*) وبيروكسيديز الغدة الدرقية (*TPO*)، والتغيرات في مستوى هرمونات الغدة الدرقية وفرط نشاط الغدة الدرقية وقصور الغدة الدرقية. أجريت هذه الدراسة في محافظة ميسان بالعراق للفترة من 2024/10/17 إلى 2025/5/25 وشارك فيها (45) امرأة، تتراوح أعمارهن بين (18–46) سنة، تم تقسيمهن إلى ثلاث مجموعات: المجموعة الأولى (15) امرأة مصابة بفرط نشاط الغدة الدرقية، والمجموعة الثانية (15) امرأة مصابة بقصور الغدة الدرقية، والمجموعة الثالثة (15) امرأة ضابطة، تم جمع العينات من مركز السكري والغدد الصماء وبعض المختبرات الخاصة. في بعض المناطق والنواحي، مثل (الكميت، والمجر، والميمونة، والكحلاء)، تم تقييم بعض المعايير الديموغرافية والسريرية، بما في ذلك العمر، ومؤشر كتلة الجسم (BMI)، والتاريخ العائلي، والمعايير الهرمونية. أظهرت القياسات الأنثروبومترية ارتفاعًا في مؤشر كتلة الجسم ($P\leq0.05$) لدى مرضى قصور الغدة الدرقية، بينما لوحظ انخفاض في مؤشر كتلة الجسم ($P\leq0.05$) لدى مرضى فرط نشاط الغدة الدرقية مقارنةً بالمجموعة الضابطة. كما لوحظت زيادة ذات دلالة إحصائية في كل من متوسط العمر والتاريخ العائلي ($P\leq0.05$) لدى كل من مجموعتي فرط نشاط الغدة الدرقية وقصورها مقارنةً بالمجموعة الضابطة. أظهرت النتائج الهرمونية زيادة معنوية في مستويات ثلاثي يودوثيرونين الحر fT3 و الثيروكسين الحر fT4 في مصل مرضى فرط نشاط الغدة الدرقية $P\leq0.05$ مع انخفاض معنوي $P\leq0.05$ في هرمون تحفيز الغدة الدرقية TSH مقارنة بالمجموعة الضابطة، وفي مرضى قصور الغدة الدرقية، لوحظ انخفاض معنوي $P\leq0.05$ في مستويات fT3 و fT4، مع زيادة معنوية $P\leq0.05$ في هرمون تحفيز الغدة الدرقية TSH مقارنة بالمجموعة الضابطة. تضمن التحليل الجزيئي استخلاص الحمض النووي من مرضى يعانون من فرط نشاط الغدة الدرقية وقصورها، بالإضافة إلى مجموعة ضابطة. تم تضخيم منطقة محددة من الجين (*TSHR*)، والجين (*TSHB*)، والجين (*TPO*) باستخدام تفاعل البوليميراز المتسلسل (PCR)، ثم تم تحديد تسلسل النوكليوتيدات للمنتجات المضخمة باستخدام تقنية تسلسل الحمض النووي. تم تحليل التسلسلات الناتجة باستخدام أدوات المعلوماتية الحيوية

لتحديد المتغيرات النوكليوتيدية المفردة (SNVs)، ضمن المناطق المدروسة من الجينات. تم اكتشاف missense أحد الاختلافات الجينية في منطقة الترميز المدروسة لجين (*TSHR*)، في الموقع c.477T>C، حيث تم استبدال الثايمين (T) بالسيتوزين (C). كما ارتبط النمط الجيني المتغاير (TC) بزيادة خطر الإصابة باضطرابات الغدة الدرقية. من ناحية أخرى، تكشف الدراسة الحالية عن وجود طفرة missense ، وهي طفرة انتقالية في الموضع c.113G>A من جين (*TSHB*)، حيث يتم استبدال الكوانين (G) بالأدينين (A)، وارتبط النمط الجيني (GA) و(GG) بزيادة خطر الإصابة باضطرابات الغدة الدرقية.

بالإضافة إلى ذلك، تحدد هذه الدراسة وجود طفرة c.361C>G missense في جين (*TPO*)، حيث تم استبدال السيتوزين (C) بالكوانين (G)، وارتبط النمط الجيني (CG) و(GG) بزيادة خطر الإصابة باضطرابات الغدة الدرقية.

تشير هذه النتائج إلى أن الاختلافات الجينية في جينات *TSHR* و*TSHB* و*TPO* قد تساهم في تطور اضطرابات الغدة الدرقية لدى النساء، ويمكن استخدامها كعلامات جزيئية للتشخيص المبكر والتنبؤ بالمخاطر.

أدت جميع هذه المتغيرات إلى اختلافات في النموذج الهيكلي ثلاثي الأبعاد للبروتينات لدى النساء المصابات باضطرابات الغدة الدرقية مقارنة بالمجموعة الضابطة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعــة ميسان– كلية العلوم
قسم علوم الحياة

الارتباط الجيني لمتغيرات جينات *TSHR* و*TSHβ* و*TPO* مع اضطرابات الغدة الدرقية لدى النساء في محافظة ميسان

رسالة مقدمة إلى

مجلس كلية العلوم / جامعة ميسان

وهي جزء من متطلبات نيل شهادة الماجستير في علوم الحياة

من قبل
يمامة احمد عكلة

بإشراف

أ. م. د. صلاح حسن فرج

جماد الاول ١٤٤٧هـ

تشرين الثاني ٢٠٢٥ م