Ministry of Higher Education
and Scientific Research
University of Babylon
College of Medicine

Study of Association of Autoimmune Graves' Disease, Type 2 Diabetes Mellitus and Herpes Simplex Virus-1 (HSV-1)

A Thesis
Submitted to Council of the College of Medicine, University of the Requirements for the Degree of Babylon, in Partial Fulfillment of Doctorate of Philosophy in Science/Medical Microbiology

By

Mohammed Sahib Ismael Al-Basha

B.Sc. Laboratory Investigations/ University of al-Kufa (2013)

M.Sc. Medical Microbiology/ University of Babylon (2018)

Supervisor

Professor

Dr.Zaytoon
Abdulridha Al-Khafaji

Consultant doctor in immunology

Dr.Mohammed Salih Mahdi

July 2023 A.D

1444 A.H Thul-Hijjah

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل- كلية الطب

دراسة ارتباط مرض غريفز المناعي الذاتي ومرض السكري من النوع ٢ وفيروس الهربس البسيط-١ (HSV-1)

أطروحة

مقدمة الى مجلس كلية الطب/ جامعة بابل وهي جزء من متطلبات نيل
درجة دكتوراه فلسفة في العلوم / علم الأحياء المجهرية الطبية

من قبل
محمد صاحب اسماعيل ال باشا
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ماجستير احياء مجهرية طبية/ جامعة بابل (٢٠١٨)

الاشراف

الأستاذ الدكتور
د. زيتون عبدالرضا الخفاجي

الطبيب الاستشاري
د. محمد صالح مهدي

ذو الحجة ١٤٤٤ ه

تموز 2023 م

﴿ بِسْمِ اللَّـهِ الرَّحْمَٰنِ الرَّحِيمِ﴾

(فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ وَلَا تَعْجَلْ بِالْقُرْآنِ مِن قَبْلِ أَن يُقْضَى إِلَيْكَ وَحْيُهُ وَقُل رَّبِّ زِدْنِي عِلْمًا)

صدق الله العلي العظيم

سورة طه آية:١١٤

Certification

We certify that this thesis was prepared under our supervision at the Department of Medical Microbiology, College of Medicine, University of Babylon as partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Science of Medical Microbiology.

Supervisor
Professor
Dr.Zaytoon Abdulridha Ighewish
College of Medicine
University of Babylon
/ / 2023

Supervisor
Consultant doctor
Dr.Mohammed Salih Mahdi
Ministry of Health in Iraq
Hussein Hospital in Karbala
/ / 2023

In view of the available recommendation, I present this thesis for evaluation by the Examination Committee.

Professor
Dr. Hayam Khalis Al- Masoudi
Head of Department of Microbiology
College of Medicine
University of Babylon
/ / 2023

Dedication

I dedicate this work,

To the martyr hero, my father, who is present in his spirit with me.

To the one who supported me and believed in me, to the symbol of giving, my great mother

Mohammed al-Basha 2023

Acknowledgement

Praise to Allah and prophet mohammed who gave me power and motivation to finish this work. It is pleasure to express my deep appreciation to my supervisors **Prof Dr.Zaytoon Abdulridha Ighewish Al-Khafaji** and **Dr.Mohammed Salih Mahdi** for their invaluable advice, assistance, cooperation, and support throughout the course of preparing my thesis. Faithful thanks to the dean of the College of Medicine University of Babylon **Prof. Dr. Mohend Abbass Nori Al-shalah** and the Head of Department of Medical Microbiology **Prof. Dr. Hayam Khalis Al-Masoudi** for their help and great cooperation

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Summary

The study included 270 participants, with 180 females (66.7%) and 90 males (33.3%). Among them, 97 healthy individuals (33 males and 64 females) constituted the control group, and 173 patients (57 males and 116 females) were categorized into different groups: 38 in the HSV+ group, 99 in the Graves+ group, and 36 in the Graves+HSV+ group. The study focused on understanding the relationship between thyroid hormones, thyroid antibodies, and Herpes simplex virus (HSV-1) infection in Graves' disease, a common cause of hyperthyroidism.

Participants' ages ranged from 18 to 85 years, case information for each patient was taken from the report of the diagnosis, which was: name, sex, age, smoking habit, and other autoimmune disease (Diabetes mellitus type 2) attending Marjan Teaching Hospital in Babylon Province, Imam Al-Hussain Teaching Hospital in Karbala Province, from March 2022 to January 2023 with the majority falling within the 36–60 age group (57.8%), followed by 30.0% in the ≤35 age group and 12.2% in the ≥61 age group. There were no significant age-related differences observed among the study groups, as indicated by a p-value of 0.822.

Smoking status significantly differed, with 65 participants being smokers (24.1%) and 205 non-smokers (75.9%), resulting in a p-value of less than 0.001.

The primary objective of the study was to compare the average levels of thyroid hormones (TSH, T3, T4, anti-TG, anti-TPO, and anti-TSHR) among various groups (control, HSV+, Graves+, and Graves+HSV+). Significant variations were found between the groups for each of these hormones and antibodies.

For TSH, the highest mean values were in the control group (2.7±1.2), followed by the HSV+ group (2.8±1.3), Graves+ group (0.05±0.02), and Graves+ HSV+ group (0.04±0.011). Similarly, for T3 and T4, the Graves+ group had the highest mean values (6.56±1.8 and 617.3±217.8, respectively), followed by the Graves+ HSV+ group, the control group, and the HSV+ group.

The highest mean values for anti-TSHR, anti-TPO, and anti-TG were observed in the Graves+HSV+ group (5.52±2.5, 74.4±15.4, and 338±48.5, respectively), followed by the Graves+ group, the HSV+ group, and the control group.

Regarding HbA1c levels, the Graves+ group had the highest mean value (7.77±1.008), followed closely by the Graves+HSV+ group, the HSV+ group, and the control group.

Furthermore, mean CD25 and TSHR levels were highest in the Graves+HSV+ group (27.25±9.5 and 14.46±6.5, respectively), followed by the Graves+ group, the HSV+ group, and the control group, with statistically significant differences ($p<0.001$) among all the groups.

In conclusion, the study's findings indicated significant variations in thyroid hormone antibodies among the different study groups. This understanding may play a crucial role in diagnosing and managing autoimmune thyroid diseases like Graves' disease and may offer potential insights into therapeutic targets

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List of Abbreviations

AID	autoimmune diseases
ADA	American Diabetes Association
AITD	autoimmune thyroid disease
AIT	Amiodarone Induced Thyrotoxicosis
ANS	8-anilino-1-naphthalene sulfonic acid
APCs	antigen-presenting cells

BMR	basal metabolic rate
BCR	B cell receptor
cAMP	cyclic Adenosine Mono Phosphate
CD4	Cluster of differentiation 4
CD25	Cluster of differentiation 25
CFDS	Color Flow Doppler Sonography
EBV	Epstein-Barr virus
ECL	ElectroChemiLuminescence
ELISA	Enzyme Linked ImmunoSorbent Assay
FOXP3	forkhead box P3
gD	glycoprotein D
GLUT2	glucose transporter type 2 gene
GO	Graves orbitopathy
HHV-1	Human Herpes Virus-1
HbA1c	Hemoglobin A1c
HAART	Highly Active Anti-Retroviral Therapy
HT	Hashimoto's thyroiditis
HSK	herpes stromal keratitis
HSV-1	herpes simplex virus-1
HPT	hypothalamus-pituitary-thyroid
ICP0	Infected cell protein 0
IFNs	interferons
iTreg	induced regulatory T
KDa	Kilodalton
MCT	monocarboxylate transporter
MHC-I	major histocompatibility complex class I
MTOC	microtubule-organizing center
MVBs	multivesicular bodies

NHANES	National Health And Nutrition Examination Survey III Thyroid Stimulating Hormone
NK	natural killer
NPCs	nuclear pore complexes
nTregs	natural Tregs
OD value	optical density
PAMPs	pathogen-associated molecular patterns
PB	peripheral blood
PRRs	pattern recognition receptors
RAI	radioactive iodine
RAIU	radioactive iodine (RAI) uptake
RLRs	Retinoic acid-like receptors
SV40	simian virus 40
THs	thyroid hormones
TBII	Thyrotropin Binding Inhibitory Immunoglobulins
T2DM	Type 2 diabetes mellitus
TG	Thyroglobulin
TFCs	Thyroid Follicular Cells
TGN	trans-Golgi network
TMD	transmembrane domain
TRAb	thyrotropin receptor antibody
TRH	thyrotropin-releasing hormone
TRM cells	tissue-resident memory T cells
TPO	Thyroid Peroxidase
TPOAb	Thyroid PerOxidase Antibodies
TSAbs	thyroid stimulating antibodies
TSI	thyroid stimulating immunoglobulins
TSH	Thyroid Stimulating Hormone
TSHR	Thyroid Stimulating Hormone Receptor

UL	(unique long) gene
VP	virion protein
WHO	World Health Organization

الخلاصة

ملت الدراسة ٢٧٠ مشاركًا، منهم ١٨٠ إناث (٦٦,٧%) و٩٠ ذكور (٣٣,٣%). ومن بينهم، كان ٩٧ فردًا سليمًا (٣٣ ذكرًا و٦٤ أنثى) يشكلون المجموعة الضابطة، في حين تم تصنيف ١٧٣ مريضًا (٥٧ ذكرًا و١١٦ أنثى) في مجموعات مختلفة: ٣٨ في مجموعة+HSV ، و٩٩ في مجموعة+Graves ، و٣٦ في مجموعة .+Graves +HSV ركزت الدراسة على فهم العلاقة بين هرمونات الغدة الدرقية والأجسام المضادة للغدة الدرقية وعدوى فيروس الهربس البسيط (HSV-1) في مرض غريفز، وهو سبب شائع لفرط نشاط الغدة الدرقية.

تراوحت أعمار المشاركين بين ١٨ و ٨٥ عامًا، وتم الحصول على معلومات الحالة لكل مريض من تقرير التشخيص، والتي تضمنت: الاسم والجنس والعمر وعادة التدخين وأمراض الجهاز المناعي الذاتي الأخرى (مرض السكري من النوع ٢)، وتمت المشاركة من مستشفى مرجان التعليمي في محافظة بابل ومستشفى الإمام الحسين التعليمي في محافظة كربلاء من مارس ٢٠٢٢ إلى يناير ٢٠٢٣، حيث كان غالبية المشاركين في مجموعة العمر ٣٦-٦٠ عامًا (٥٧,٨%)، تليها مجموعة العمر ≤٣٥ بنسبة ٣٠,٠% ومجموعة العمر ≤٦١ بنسبة ١٢,٢%. لم تُلاحظ فروق عمرية معنوية بين مجموعات الدراسة، كما أشارت قيمة p إلى ٠,٨٢٢.

كما أظهرت حالات التدخين اختلافًا معنويًا، حيث كان هناك ٦٥ مشاركًا مدخنًا (٢٤,١%) و ٢٠٥ غير مدخنين (٧٥,٩%)، مما أدى إلى قيمة p أقل من ٠,٠٠١.

وكان الهدف الرئيسي للدراسة هو مقارنة المستويات المتوسطة لهرمونات الغدة الدرقية (TSH و T3 و T4 والأجسام المضادة لـ TG و TPO و TSHR) بين مجموعات مختلفة (الضابطة و +HSVو +Graves و +Graves+HSV) وتم العثور على اختلافات معنوية بين المجموعات لكل من هذه الهرمونات والأجسام المضادة.

بالنسبة لهرمونTSH ، كانت أعلى قيم متوسطة في المجموعة الضابطة (٢,٧±١,٢)، تليها مجموعة(2.8±1.3) +HSV ، ثم مجموعة(0.05±0.02) +Graves ، وأخيرًا مجموعة (0.04±0.011). +Graves+ HSVوبالمثل، كانت مجموعة +Graves هي التي كانت لها أعلى قيم متوسطة لهرمون T3 و T4(1.8±6.56 و٦١٧,٣±٢١٧,٨ على التوالي)، تليها مجموعة+Graves+ HSV ، والمجموعة الضابطة، ومجموعة.+HSV

وكانت أعلى قيم متوسطة للأجسام المضادة للـ TSHR والـ TPO والـ TG في مجموعة Graves+ HSV+ (5.52±2.5 و٧٤,٤±١٥,٤ و٣٣٨±٤٨,٥ على التوالي)، تليها مجموعة+Graves ، ثم مجموعة+HSV ، وأخيرًا المجموعة الضابطة.

بالنسبة لمستوياتHbA1c ، كانت مجموعة +Graves لها أعلى قيم متوسطة (٧,٧٧±١,٠٠٨)، تليها بالقرب مجموعة+Graves+ HSV+ ، ثم مجموعة+HSV ، وأخيرًا المجموعة الضابطة.

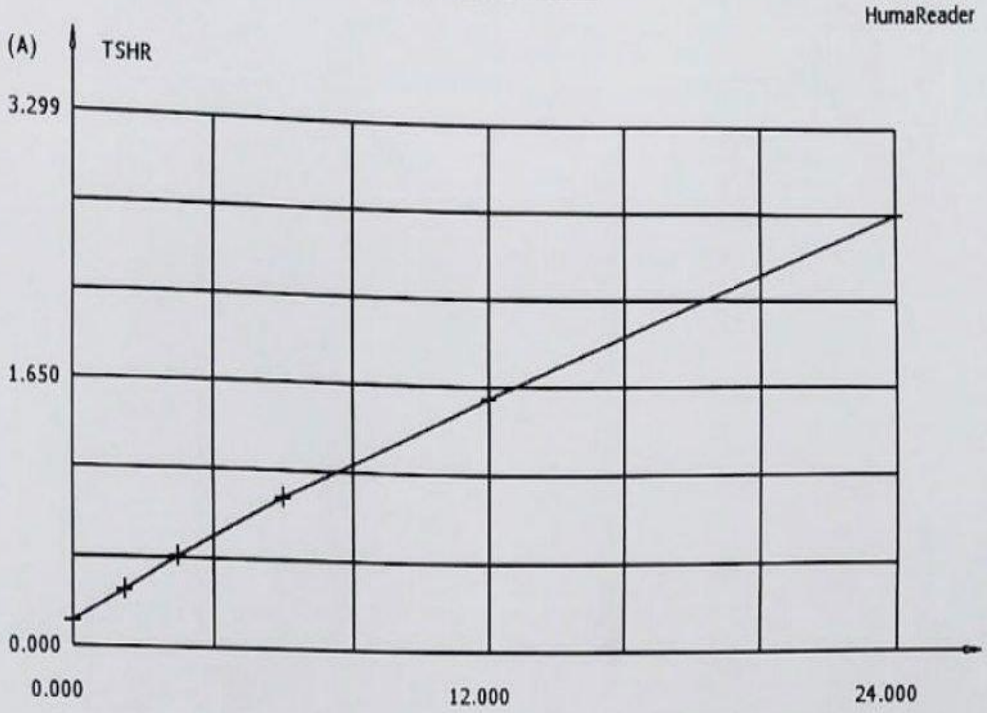
علاوة على ذلك، كانت مستويات المتوسط لـ CD25 و TSHR هي الأعلى في مجموعة (9.5±27.25 Graves+ HSV1+و١٤,٤٦±٦,٥ على التوالي)، تليها مجموعة +Graves، ثم مجموعة+HSV ، وأخيرًا المجموعة السيطرة، مع وجود اختلافات ذات دلالة إحصائية(p <٠,٠٠١)بين جميع المجموعات.

في الختام، أشارت نتائج الدراسة إلى وجود اختلافات معنوية في أجسام المضادات لهرمونات الغدة الدرقية بين مجموعات الدراسة المختلفة. قد تلعب هذه الفهم دورًا حاسمًا في تشخيص وإدارة أمراض الغدة الدرقية المناعية الذاتية مثل مرض غريفز وقد تقدم رؤى محتملة فيما يتعلق بالأهداف العلاجية.


500µL
500µL
500µL
500µL
500µL
500µL
Reference
Standard
2000
1000
500
250
125
62.5
31.25
0

Standard curve

HumaReader



Program: TSHR TSHR Caculation: Curve Wavelength(nm): 450

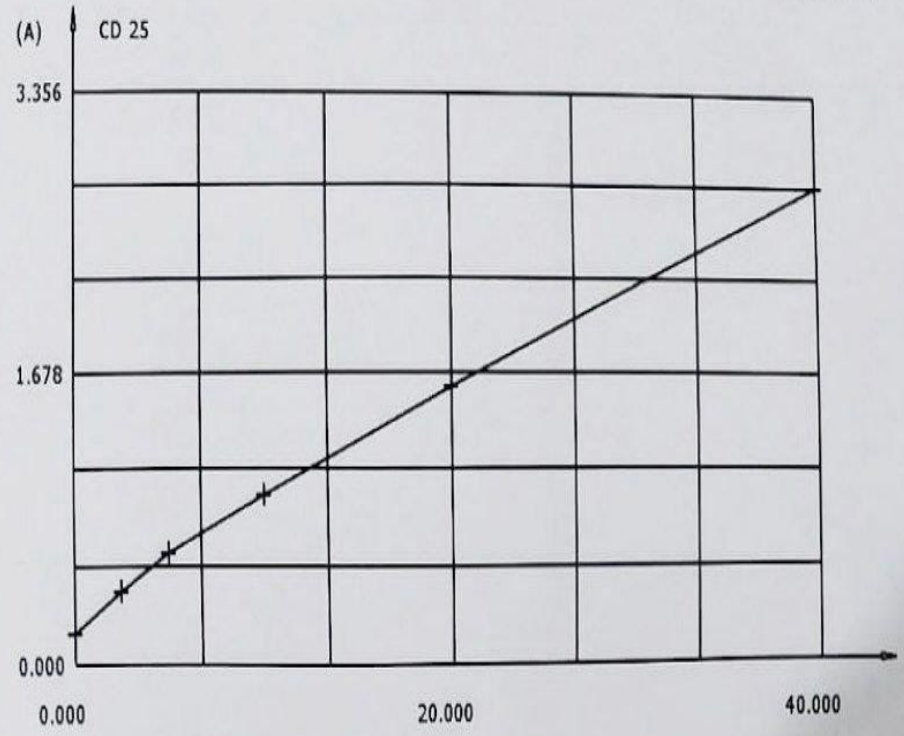
Standard sample	#1	#2	#3	#4	#5	#6
Concentration(ng/ml)	0.000	1.500	3.000	6.000	12.000	24.000
Abs(A)	0.156	0.342	0.552	0.926	1.572	2.749

Operator: Checker:

Print date: 04/22/2009

Standard curve

HumaReader



Program: CD 25 CD 25 Caculation: Curve Wavelength(nm): 450

Standard sample	#1	#2	#3	#4	#5	#6
Concentration(ng/ml)	0.000	2.500	5.000	10.000	20.000	40.000
Abs(A)	0.176	0.414	0.630	0.964	1.585	2.797

Operator: Checker:
Print date: 04/22/2009

1.1 Introduction

Graves' disease (GD), or hyperthyroidism, is a type of autoimmune illness marked by increased thyroid hormone production. One of the typical features of GD is the existence of hyperthyroidism. Several risk factors, including genetics, smoking, stress, and viral infections, have been proposed to contribute to the disease's onset. An immune-related condition is GD. (Cao *et al*., 2023). One of the most prevalent autoimmune diseases is Graves' disease, which affects 13 million people. Females are seven times more likely than males to be affected. Although it can occur at any age, it is most commonly observed in young to middle-aged individuals and accounts for 60–80% of cases of hyperthyroidism. (Kahaly *et al.*, 2018)

According to estimations from the World Health Organization (WHO), 67% of people under 50 worldwide have an HSV-1 infection (WHO, 2015). By geography and age group, prevalence rates might, however, differ significantly. According to certain research, HSV-1 infection may contribute to the development or aggravation of autoimmune diseases like Graves' disease(Weetman, 2003; Harris, *et al*., 2015). It is hypothesized that viral antigens may trigger an immune response that leads self-tissues to be attacked, even if the precise mechanisms driving this link are not entirely understood (von Herrath *et al*., 2009).

Moreover, chronic herpes simplex virus (HSV) infections, particularly HSV-1 and varicella-zoster virus infections, are more common in patients with diabetes , the immune response to viruses appears to be compromised in patients with diabetes as a result, and these patients require greater attention when they are infected with viruses(Ke *et al.*, 2016 ; Woelfle *et al*., 2022).

Type 2 diabetes mellitus (T2DM) has been associated with a number of viral diseases, including Epstein-Barr, CMV, and the Herpes virus (Casqueiro *et al*., 2012). Several studies have suggested that the duration of a CMV infection brought on by the herpes virus may be a risk factor for type 2 diabetes. By encouraging the expansion of late-differentiated CD4+ and CD8+ T-cells that generate pro-inflammatory cytokines and hence provide an inflammatory milieu, the virus may hasten the immunosenescence process. (Tu *et al*., 2016). Type 2 diabetes is marked by high blood sugar levels, which often arise from the coexistence of two factors: insulin resistance and reduced beta cell function (Esser *et al.*, 2020).

The chance of having T2D is increased by a number of factors, including aging, genetics, environmental factors, lifestyle choices, and infections, among others. It appears that viral infections are strongly linked to non-autoimmune diabetes, given that viruses can either directly or indirectly, through viral-mediated pathways, contribute to the development of T2D. (Karim *et al.*, 2014 ; Lontchi-Yimagou *et al*., 2021). Type 1 and type 2 diabetics have higher rates of thyroid dysfunction compared to non-diabetics, demonstrating their close association (Nishi, 2018).

Thyroid problems and type 2 diabetes are connected. Studies have indicated that those with diabetes mellitus are more likely than those without to experience thyroid problems. A diabetic patient's capacity to regulate their metabolism can be impacted by untreated thyroid dysfunction, and the interaction between these two disorders may have a considerable effect on how both of these conditions develop(Biondi *et al*.,2019).

Aim of the study: The aim of the present study was to a prove the role of herpes virus 1 infection as triggering factors of Graves' disease and Type 2 diabetes mellitus

Estimations of the study:

1. Immunological detection of IgG for herpes viruses

2: Study Thyroid Peroxidase (TPO) Antibodies.

3 Thyroglobulin (TG) Antibodies

4 Thyroid Stimulating Hormone (TSH) Antibodies

5: Study T3 and T4.

6-Thyroid Stimulating Hormone Receptor (TSHR) Antibodies

7: Study the correlation between HSV 1 and Graves' disease.

8: Study for CD25 marker in Graves' disease

9: Study HbA1c levels.

1.2 Literature review

1.2.1 Graves' disease

1.2.1.1 Definition

Graves' disease is an autoimmune thyroid disorder clinically characterized by the presence of hyperthyroidism, diffuse goiter, and, in some patients, ophthalmopathy (Smith, *et al*.,2016).GD is finally caused by auto-antibodies directed to TSH-R, which mimic the effect of TSH peptide (physiologically released by the pituitary gland) and induce the uncontrolled synthesis of thyroid hormones (THs), as well as the hypertrophy and hyperplasia of thyroid cells, resulting in diffuse goiter (Kahaly, *et al., 2020*).

1.2.1.2 History of Autoimmune Thyroid Disease.

The Persian physician Avicenna (Ibn Sina) first described in 1000 AD the coexistence of goiter and exophthalmos in patients with increased appetite. A century later, another Persian physician, Sayyid Ismail al-Jurjani, reported this association in the medical dictionary of its time (Thesaurus of the Shah of Khwarazm). Centuries would pass before this clinical picture again attracted scientific attention. Graves' disease (GD), also called Basedow's disease, owes its names, respectively, to the Irish physician Robert James Graves, who described the disease in 1835, and to Karl Adolph von Basedow, who reported the same clinical picture in Germany in 1840. Indeed, it was the Englishman Caleb Hillier Parry who first reported a case of hyperthyroidism and goiter in 1786, but his report was not published until 1825. Earlier, in 1802, the Italian physician Giuseppe Flajani described a disease characterized by the coexistence of palpitations and exophthalmos (Lindholm and Laurberg 2010).

1.2.1.3 Anatomy And Histology of Thyroid Gland .

The thyroid gland is a crucial endocrine gland with a distinctive butterfly shape, Located in the lower portion of the neck. Positioned in the front and on the sides of the trachea, it is situated below the larynx. One of its primary functions is to regulate the basal metabolic rate (BMR) and promote both physical and mental growth. Additionally, Calcitonin it plays a crucial role in regulating calcium homeostasis. When serum calcium levels are elevated, calcitonin is released to inhibit osteoclast activity, thereby reducing bone resorption and promoting the deposition of calcium in the bones the thyroid gland is responsible for calcium metabolism . Comprising two lobes, namely the left and right lobes, the gland is connected by an intermediate structure called the isthmus. Occasionally, a third lobe known as the pyramidal lobe may protrude from the isthmus. A fibrous or fibromuscular band, called the levator glandulae thyroideae, runs from the body of the hyoid to the isthmus. (Khan *et al*., 2022) Figure (1-1).

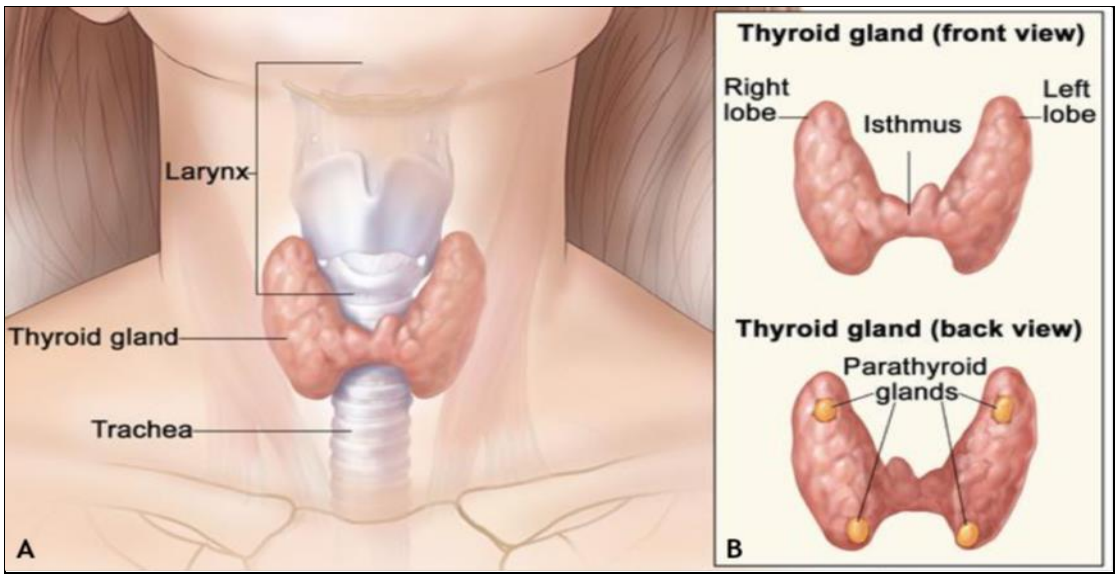


Figure 1-1. Thyroid anatomy .Adapted from © 2012 Terese Winslow LLC. Panel A: localization of the thyroid in the neck. Panel B: details of thyroid gland ; American Thyroid Association. 2018) .

1.2.1.4 Prevalence

Thirteen million people throughout the world are afflicted by Graves' disease, an autoimmune thyroid illness that is one of the most common autoimmune diseases (AID). Seven to eight times more females than males are likely to be impacted. Even though it typically affects young to middle-aged adults, it can happen to anyone (Utiger , 2015). Epidemiologic studies are carried out in school-age children (6–12 years of age) owing to their high physiologic vulnerability and their accessibility through schools for studies on baseline health parameters as well as the lasting impact of public health programs and interventions (Clark *et al*.,2016). The prevalence of hyperthyroidism in women is between 0.5 and 2%, and is 10 times more common in women than in men in iodine-replete communities (Vanderpump, 2011).

In Iraq, some studies have found that the incidence of Graves' hyperthyroidism disease increased in females compared with males (AL-Fatlawi, 2014). In a study conducted in Baghdad, 64.6% had multinodular goiter, 22% had diffuse goiter, and 12.6% had solitary nodules. Thyrotoxicosis was noticed in 21.5%. Thyroid malignancy was diagnosed in 6.3% of patients, with a female predominance (80%). Papillary carcinoma was found in 80% of patients with malignancies. (Ghadhban *et al*., 2018)

The prevalence of hyperthyroidism in women is between 0.5 and 2% and is 10 times more common in women than in men in iodine-replete communities (Ighewish *et al*., 2020).

1.2.1.5 Etiology

The etiology of AITD is multifactorial; interactions between genetic and environmental predisposing triggers lead to deregulation of immune tolerance. The incidence of the two main clinical presentations of AITD, Graves' disease (GD) and Hashimoto's thyroiditis (HT), is estimated at 5% of the population (Antonelli *et al*., 2015; Yoo and Chung, 2016). The thyroid's functions are regulated by a hormone released by a very small gland at the base of the brain (the pituitary gland). The antibody associated with Graves' disease, thyrotropin receptor antibody (TRAb) acts like the regulatory pituitary hormone and overrides the normal regulation of the thyroid, causing an overproduction of thyroid hormones (hyperthyroidism). (Kadhum, *et al*., 2014)

Like all autoimmune diseases, it occurs more commonly in patients with a positive family history. It is more common in monozygotic twins than in dizygotic twins. It is precipitated by environmental factors like stress, smoking, infection, iodine exposure, and postpartum, as well as after highly active antiretroviral therapy (HAART) due to immune reconstitution(Hussain *et al*., 2017).

1.2.1.6 Environmental risk factors for GD

The genetic predisposition accounts for 79% of the risk for GD, while environmental factors account for 21%. Among GD environmental risk factors, smoking, iodine excess, selenium and vitamin D deficiency, smoking, infections, and occupational exposure to Agent Orange have been associated with GD (Antonelli *et al*., 2020).

1.2.1.6.1 Stress

The role of stress in the pathophysiology of Graves' disease is suggested by several clinical observations in immunology, and a better understanding of autoimmune diseases, which provides new insights into the potential effects of stress hormones on the T helper cell imbalance involved in the pathogenesis of autoimmune diseases. Stress management should therefore be an important part of the treatment of Graves' disease, as stress reduction may improve the effect of therapy. (Geraldine *et al.*, 2013)

Mental disorders merge strongly with thyroid diseases. Because of its regulatory effects on serotonin and noradrenaline, T3 has been closely linked to depression and anxiety. It is known that in many cases, the mental symptoms persist even after normalization of thyroid function through treatment. Psychosocial factors, including stress, have been associated with mental symptoms even after thyroid function normalization in Graves' disease, and a combination of mental disorders has been related to the exacerbation of hyperthyroidism. These findings suggest that psychosomatic approaches based on the bio-psycho-social medical model are important for the treatment of mental disorders associated with Graves' disease (Fukao *et al*., 2020).

1.2.1.6.2 Nutrition

The interplay between nutritional elements and immunological issues plays a significant role in the development of AITD. Researchers are actively investigating how iodine, iron, and selenium affect the risk, causes, and treatment of thyroid disorders.

A-Iodine:

Iodine is a crucial component of the thyroid hormones tri-iodothyronine (T_3, active hormone) and thyroxine (T_4, pro-hormone), as seen in Figure 1–2. This figure also shows the key players who contribute to the production of thyroid hormones within thyroid follicular cells.

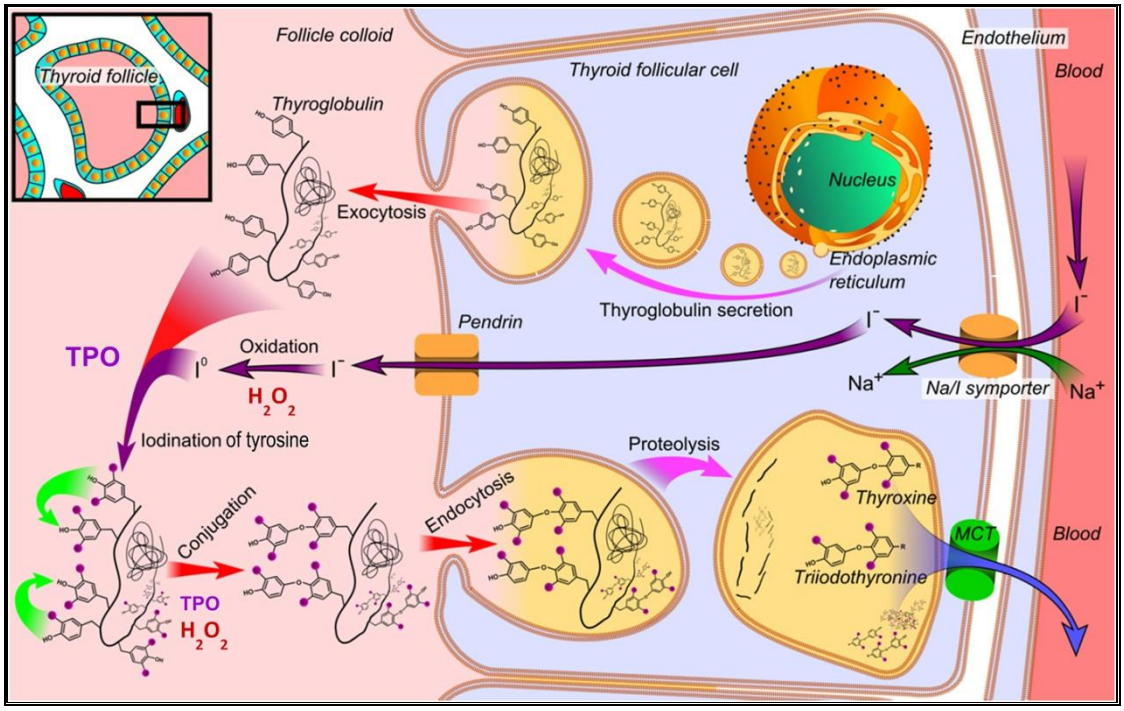


Figure 1-2. Synthesis of the thyroid hormones in the thyroid follicle (modified from Häggström 2014) Thyroglobulin is synthesized in the rough endoplasmic reticulum and follows the secretory pathway to enter the colloid in the lumen of the thyroid follicle by exocytosis. Meanwhile, a sodium-iodide (Na/I) symporter pumps iodide (I^-) actively into the cell, which previously crossed the endothelium by largely unknown mechanisms. This iodide enters the follicular lumen from the cytoplasm by the transporter pendrin in a purportedly passive manner. In the colloid, iodide (I^-) is oxidized to iodine (I^0) by hydrogen peroxide (H_2O_2) with the help of an enzyme called thyroid peroxidase (TPO). Iodine (I^0) is very reactive and iodinates the thyroglobulin at tyrosyl residues in its protein chain (in total, containing approximately 120 tyrosyl residues). In conjugation, adjacent tyrosyl residues are paired together, again under the influence of TPO and H_2O_2. The entire complex re-enters the follicular cell by endocytosis. Proteolysis by various proteases liberates thyroxine and triiodothyronine molecules, which enter the blood *via* a monocarboxylate transporter (MCT).

Chronic intake of excess iodine can cause autoimmune thyroiditis by increasing the immunogenicity of highly iodinated thyroglobulin (Tg).

The implementation of universal salt iodization can also have a similar effect, albeit temporarily. Environmental factors, including iodine intake, may contribute to the prevalence of Graves' disease. A study conducted in China discovered a higher incidence of Graves' disease in regions with elevated iodine consumption. (Teng *et al.*, 2006).

A cross-sectional study in China reported a higher prevalence of overt and subclinical hyperthyroidism in an iodine-sufficient area than in an iodine-deficient area (1.2% versus 1.0%; $P < 0.001$)(Du *et al*., 2014).

These differences were, however, not observed either in China or Japan when iodine-sufficient areas were compared with areas where the populace has an excessive iodine intake(Tan *et al.*, 2015). In Africa, the epidemiology of thyroid dysfunction has proved more challenging to monitor owing to a lack of comprehensive population-based studies (Taylor *et al*., 2018).

B-Iron:

The haem-dependent enzyme thyroperoxidase (TPO), whose active center contains iron and is necessary for the manufacture of thyroid hormone, depends on iron for proper function. Iron is a crucial component of this enzyme. The necessity of a sufficient iron status in the manufacture of thyroid hormones is highlighted by the fact that TPO only becomes active on the apical surface of thyrocytes after binding a prosthetic haem group. In cases of iron insufficiency, thyroid metabolism is seen to be impaired. AITD patients frequently have iron deficiency due to frequent co-morbidities such as autoimmune gastritis, which reduces iron absorption, and celiac disease, which causes iron loss. In fact, In two-thirds of women with persistent symptoms of hypothyroidism despite

appropriate levothyroxine therapy, restoration of serum ferritin above 100 µg/l ameliorated symptoms. (Dunn *et al*., 2001; Hess *et al*., 2002; Fayadat *et al.,* 1999)

C-Selenium:

Even in cases of severe insufficiency, the thyroid gland, which has the highest concentration of selenium in the human body, is able to maintain selenium levels (Kohrle, 2013). Thyrocytes express a number of selenoproteins, some of which are crucial for thyroid function (Schmutzler *et al*., 2007). Selenoproteins, in particular the glutathione peroxidases, which eliminate excess hydrogen peroxide created during the iodination of thyroglobulin to form thyroid hormones, play a crucial role in thyroid function. Selenium, probably in the form of selenoproteins, can lower TPO-antibody concentrations, hypothyroidism, and postpartum thyroiditis, according to observational studies and randomized controlled trials. For the thyroid to remain healthy, iodine, iron, and selenium levels must be appropriate (Rayman *et al*., 2019).

Probiotics have shown beneficial effects in thyroid diseases and are able to have a positive effect on trace elements such as selenium, zinc, and copper. Additionally, microbes function as a reservoir for T_3 and are able to prevent thyroid hormone from fluctuating, thus reducing the need for T4 supplementation. Probiotics could constitute an adjuvant therapy for thyroid diseases (Knezevic *et al*., 2020).

1.2.1.6.3 Sex steroid

There is some evidence to suggest that sex steroids (such as estrogen and testosterone) may play a role in the development and/or severity of hyperthyroidism (an excess of thyroid hormone in the body)(Kjaergaard *et al*., 2021). Estrogen has been shown to stimulate the thyroid gland and increase thyroid hormone synthesis and secretion. This may be one reason why women are more likely to develop hyperthyroidism than men. (Santin *et al.*, 2011)

Testosterone may have the opposite effect and inhibit thyroid hormone synthesis and secretion. This may be why men are less likely to develop hyperthyroidism than women. (Krassas *et al*., 2003)

Hyperthyroidism has also been associated with abnormalities in sex hormone metabolism and levels, such as an imbalance of estrogen and testosterone. In patients with hyperthyroidism, treatment with thyroid hormone replacement therapy has been shown to improve sex hormone levels and sexual function (Ren *et al*., 2022).

1.2.1.6.4 Smoking

suggest that cigarette smoking is related to a significant decline in the concentrations of TSH, TPOAb, and TgAb. In addition, daily smoking and long-term smoking decrease serum TSH and TPOAb levels. Cigarette smoking plays a significant role in the development of thyroid dysfunction (Zhang *et al*., 2019). Smoking cigarettes has been linked to an overactive thyroid, and studies have shown that women who smoke regularly have a roughly 2-fold higher risk of developing Graves' disease than non-smokers do, while heavy smokers (those who smoke more than 25 cigarettes per day) are nearly 3-times more likely to have the

disease.(Sawicka-Gutaj *et al*., 2014). Smoking is known to be a risk factor for the autoimmune condition Graves' disease, which affects the thyroid gland. Numerous studies have shown that smokers have a higher risk of Graves' disease than non-smokers. (Bartalena *et al*., 2019; Effraimidis *et al*., 2014).

1.2.1.6.5 Infections

A-Viral Causes :

1- COVID-19 infection can cause Graves' disease and thyrotoxicosis. The onset of this disease after SARS-CoV-2 does not depend on the presence of pre-existing thyroid pathology and requires the appointment of glucocortisteroids (Urbanovych, *et al.,* 2021)

2- HCV infection has been identified as a risk element in the development of G.D. (Al-ziadi *et al.,* 2020). A connection of noteworthy importance has been demonstrated between HCV-related mixed cryoglobulinemia and the risk of GD in patients with CHC hepatitis (Antonelli *et al*., 2020).

B- Bacterial Causes :

The considerable correlation observed between Helicobacter pylori and Graves' disease hints at the potential involvement of this bacterium in either the commencement or sustenance of the autoimmune disorder (Bassi *et al*., 2012).

1.2.1.7 Hypothalamus-Pituitary-Thyroid Axis

The hypothalamus-pituitary-thyroid (HPT) axis establishes the level of thyroid hormone (TH) production, with hypothalamic thyrotropin-releasing hormone (TRH) stimulating the synthesis and secretion of the

pituitary gland, leading to the formation of the protein precursor thyroglobulin (TG) in the thyroid. The thyroid accumulates iodide (I-) in both the cytoplasm and the lumen of thyroid follicular cells (colloid), into which TG is secreted in significant amounts. (Ortiga-Carvalho *et al*., 2016) Polyclonal thyroglobulin autoantibodies of the IgG class, encompassing all four subclasses, are detected in patients with lymphocytic thyroiditis and those with Graves' disease. (Czarnocka *et al*., 2014)

The regulation of thyroid peroxidase (TPO) and thyroglobulin (TG) cell surface expression, as well as the mRNA transcription of these two proteins, is influenced by thyroid stimulating hormone (TSH). However, autoantibodies present in the serum of patients with Graves' disease (GD) can replicate these effects, including both inhibitory and stimulatory actions. (Iddah *et al*., 2013)

1.2.1.8 Thyroid autoantibodies

Thyroid gland function is altered, and cellular damage occurs due to autoantibodies. This happens when auto-antibodies and/or sensitized T-lymphocytes attach to thyroid cell membranes, leading to cell lysis and inflammatory responses (Bogusławska *et al*., 2022). The function of the thyroid gland can also be affected by stimulating or blocking autoantibodies that target cell membrane receptors. The three main thyroid auto-antigens implicated in autoimmune thyroid disease (ATD) are TPO, Tg, and the TSH receptor. (Weetman *et al*., 2016)

1.2.1.8.1 Thyroid Stimulation Hormone Receptor Antibody (TSHR-Abs)

The thyroid stimulating hormone receptor (TSHR) is prominently expressed on the plasma membrane of thyroid epithelial cells and is a pivotal component in the regulation of both the growth and physiological processes of the thyroid gland (Kahaly *et al*., 2020) .The TSH-R serves as the primary and fundamental autoantigen in the autoimmune hyperthyroidism characterized by Graves' disease, where in T cells and autoantibodies are targeted against the TSHR antigen. The activation of the receptor necessitates the binding of the cognate hormone to the extensive ectodomain of the TSHR, followed by the interaction between the receptor and the transmembrane domain (TMD), which ultimately triggers multiple signaling pathways, leading to the synthesis and secretion of thyroid hormones. (Davies, *et al*., 2019)

Graves' disease is characterized by thyroid-stimulating antibodies (TSAbs) that imitate the actions of TSH by binding to the receptor and prompting the thyroid cell to overproduce thyroid hormones, leading to hyperthyroidism. While the mechanism behind the production of TSHR-autoantibodies is relatively well understood, the gene that predisposes individuals to their development remains unknown. Genetic investigations have failed to establish any link between Graves' disease and the TSHR gene. (Chistiakov *et al.*, 2003)

1.2.1.8.2 Thyroglobulin (TG) antibodies

Thyroglobulin is the protein precursor of thyroid hormones, which are essential for growth, development, and the control of metabolism in vertebrates (Coscia *et al*.,2020).Anti-thyroglobulin (TG) antibodies are

against thyroglobulin, a thyroid hormone precursor , anti-TG antibodies are considered diagnostic of AITDs because they are present in over 90% of cases of Hashimoto's thyroiditis and over 80% of cases of Graves' disease (Stathatos *et al*., 2012). Hormone synthesis from thyroglobulin (TG) occurs in the thyroid gland via the iodination and coupling of pairs of tyrosines and is completed by TG proteolysis (Zhang *et al.*, 2021).

1.2.1.8.3 Thyroid Peroxidase (TPO) Antibody

Thyroid peroxidase (TPO) antibodies are a type of thyroid antibody, Thyroid peroxidase is an enzyme that helps make thyroid hormones (T_3, T_4, and TSH). (Dong, *et al.*,2020)TPO is also the key enzyme involved in the generation of thyroid hormones on the apical surface of thyroid epithelial cells (Salvatore Benvenga *et al*. 2018). TPOAbs in patients' sera are polyclonal and belong predominantly to the IgG class. They usually have a high affinity for TPO and preferentially bind to conformationally intact proteins (Godlewska *et al.*, 2018).

1.2.1.9 Graves' Disease Immunity

1.2.1.9.1 Role of B Cells in Graves' Disease

The role of B cells in autoimmune diseases involves different cellular functions, including the well-established secretion of autoantibodies, autoantigen presentation and ensuing reciprocal interactions with T cells, the secretion of inflammatory cytokines, and the generation of ectopic germinal centers. Through these mechanisms, B cells are involved both in autoimmune diseases that are traditionally viewed as antibody-mediated and in autoimmune diseases that are commonly classified as T-cell-mediated. (Hampe, 2012).

The thyroid-stimulating hormone receptor (TSHR) is the target of autoantibodies in Graves' disease (GD), which are produced by B lymphocytes. Thyrotropin Receptor Antibody (TRAb) causes long-term stimulation of the receptor by attaching to it. Thyroid hormones T_4 and T3 are produced and secreted in greater amounts as a result of this continuous stimulation because thyroid follicular cells express TSHR. GD pathogenesis may be better understood through the characterization of autoimmune B-cell expression profiles. (Davies *et al*., 2005; Diana *et al.*, 2017; Spencer 2017; Jiang *et al*., 2020)

B cells can act as APCs as well. Their surface immunoglobulin, or B cell receptor (BCR), is a transmembrane receptor that allows them to recognize particular antigens, which they then use to trigger an immune response and produce antibodies against. They then use MHC class II molecules to transmit pieces of these antigens to CD4+ T cells (Kambayashi *et al*., 2014; Ramos-Leví *et al*., 2016; Rastogi *et al.*, 2022*).*

Support for B cell activation is reciprocated by T helper (Th) cells. Thyroid antibody sequencing and the identification of B cell epitopes on the TSH receptor received special attention. The pathogenesis of GD, which is a factor in the triggering of TSHR and the subsequent development of this disease, may then be better understood as a result. (Rydzewska *et al.,* 2018). However, the autoimmune reaction in AITD typically proceeds slowly, which encourages the proliferation and differentiation of many polyclonal B and T cells. (Zouali, 2022). AITD (autoimmune thyroid disease) is commonly considered an archetypal B-cell-mediated autoimmune disorder, occurring via a breach in tolerance that allows autoreactive B cells to be activated and expand at disease

onset. Patients with AITD, including those with GD, have autoreactive B cell infiltration of the thyroid tissue. (Smith *et al.*, 2018)

1.2.1.9.2 Role of T-cell Response in Graves' Disease

T regulatory (Treg) and T helper (Th) cells play a significant role in the development of autoimmune thyroid disease (AITD), particularly Graves' disease. T-cells in the immune system of people with Graves' illness respond to all thyroid auto-antigens that have been properly processed peptides. These T cells that have been activated encourage B cells to produce antibodies that are specific to the thyroid. A heterogeneous pattern of cytokine production found in T cell identification investigations suggests that both Th1 and Th2 subtypes of T helper responses are involved in all manifestations of AITD. The etiology of AITD is further complicated by the critical roles played by the T cell subtypes Th17 and Treg. Thyroid-specific T lymphocytes are mostly helpful in Graves' disease. (Rydzewska *et al.*, 2018).

CD8+ cytotoxic cells function mainly as damaging T cells, which are regulated by Th2 and Treg cells. Although all types of T cells are present in the thyroid glands of Graves' hyperthyroidism patients, it is beneficial to view Graves' disease mainly as a Th2-type of autoimmune disease due to the fact that the main pathophysiology is associated with the production of TSHR-Ab. (Luty *et al*., 2019)

1.2.1.9.3 The role of CD4+CD25+ regulatory T cells in Graves' disease

For the host to survive and for an immune system to function effectively against infections, CD4+ T cells are crucial. A naive CD4+ T cell's ability to differentiate into Th1, Th2, Th17, and induced regulatory

T (iTreg) cells is dependent on the interaction of the antigen with the MHC complex. (Zheng , 2013). The diverse functions of CD4+ T cells in controlling viral infections and shaping immune responses are mediated by distinct cytokine profiles and regulatory mechanisms that depend on specific antigenic stimuli and microenvironmental cues (Chen, *et al.*, 2013). Regulatory T cells (Tregs) are a subtype of CD4 T cells that are distinguished by their expression of CD25, while the forkhead box P3 (FOXP3) molecule is a typical marker of Tregs (Jiang *et al*., 2018). Tregs constitute approximately 5–10% of CD4 T cells in peripheral blood (PB) and are further classified into two subtypes: natural Tregs (nTregs), which primarily originate from the embryonic or newborn thymus, and inducible Tregs (iTregs), which are predominantly generated in vitro (Goswami *et al.*,2022). The breakdown of central and peripheral immune tolerance to autoantigens in autoimmune diseases, such as Graves' disease (GD), is largely due to deficient immune regulation. Treg cells play a critical role in preventing autoimmune responses and maintaining homeostasis by suppressing autoreactive T cell proliferation, promoting autoreactive T cell anergy, inhibiting antibody production from autoreactive B cells, and maturing dendritic cells (McLachlan *et al*., 2014).

In addition, a defect in CD4(+)CD25(+) T regulatory cells would break the immunologic tolerance of the host and induce an abnormal production of cytokines, but this would also facilitate the initiation of Treg apoptosis itself and thus lead to immune activation by decreasing the number of Treg cells, as previously described in GD patients (Mao *et al*., 2011).

1.2.1.10 Diagnosis of Graves' disease:

A- Clinical signs and symptoms :

The overproduction of thyroid hormones by the thyroid gland is a defining features of hyperthyroidism. Clinical symptoms, physical exam findings, laboratory results, and other considerations can all be used to make the diagnosis of hyperthyroidism. Palpitations, exhaustion, trembling, anxiety, restless sleep, weight loss, heat intolerance, sweating, and polydipsia are symptoms that are frequently described. Tachycardia, trembling in the extremities, and weight loss are common physical symptoms (Boelaert *et al*., 2010; Devereaux *et al*., 2014 ; Goichot *et al.,* 2015) Ophthalmopathy, thyroid dermopathy, and thyroid acropachy are some of the signs and symptoms of Graves' illness (De Leo *et al.*, 2016).

B- Laboratory investigations :

Low blood TSH and high levels of free thyroxin (T_4) or triiodothyronine (T_3), together with thyrotoxicosis, should raise the possibility of Graves' disease. The diagnosis can be made only based on the presence of pathognomonic signs, such as diffuse goiter associated with orbitopathy, dermopathy, or acropachy. In the absence of these symptoms, TSH receptor antibodies (TRAbs) can be measured with 97% sensitivity and 98%–99% specificity for GD (Cooper *et al.,* 2012; Tozzoli *et al.,* 2012; Ross *et al*., 2015). Can be beneficial, especially when a nodular goiter is present (Pedersen *et al.,* 2001).

C- Radiological examinations :

The diagnosis can also be confirmed by normal or increased radioactive iodine uptake (RAIU) with diffuse distribution on the scan, which

separates GD from other thyrotoxicosis causes, thyroid ultrasonography and color flow Doppler sonography (CFDS) have been used to diagnose GD with good accuracy (sensitivity of 87% and specificity of 100%) (Surks *et al.,* 2004; Hiraiwa *et al.,* 2013).

1.2.1.11 Treatment of Graves' disease:

Treatment options for hyperthyroidism, which is characterized by excessive thyroid hormone production and secretion, focus on lowering thyroid hormone levels. Surgery, radioiodine therapy, and medication are available as treatments. Antithyroid medications, such as methimazole and propylthiouracil, which function by preventing the generation of thyroid hormone, are often the first-line treatment for Graves' disease. After 6–24 months of treatment, these medications have been shown to be effective in bringing thyroid hormone levels back to normal in 30–50% of patients. (Cooper *et al*., 2012 ; RossDouglas *et al*., 2016). The overactive thyroid cells are eliminated with the use of radioactive iodine in radioiodine therapy, a non-invasive treatment approach. In 80–90% of patients, this medication is successful in reaching euthyroidism, but it may take several months to observe the entire impact. (Sharma *et al*., 2019)

Another Graves' disease treatment option is surgery, which involves partial or complete thyroid gland removal. Surgery is normally only performed on individuals who are unable to tolerate or do not respond to radioiodine therapy or antithyroid medications or who have big goiters or suspicious nodules. Overall, a number of variables, such as the severity of the disease, the presence of concomitant conditions, patient preferences, and the accessibility of resources, influence the treatment option. (Kravets, 2016)

1.2.2 Herpes simplex virus-1 (HSV-1)

1.2.2.1 Historical Background of HSV-1

The origin of the word is derived from the ancient Greek herpein, meaning "to creep" or "move slowly," which characterizes the latent onset of the infection and the inconspicuous, recurrent spread of lesions on the skin (Ighani, 2017).

1.2.2.2 Taxonomy and Classification of Herpesviridae

Members of the family Herpesviridae have enveloped, spherical virions with characteristic complex structures consisting of symmetrical and non-symmetrical components. The linear, double-stranded DNA genomes of 125–241 kbp contain 70–170 genes, of which 43 have been inherited from an ancestral herpes virus (Gatherer, 2021).

There are eight types of human herpes viruses. These viruses belong to three subgroups: alphaherpesviruses, betaherpesviruses, and gammaherpesviruses. As illustrated in Table 1-1,

Table 1-1: Classification of Human Herpesviridae (Jawetz, *et al.*, 2019)

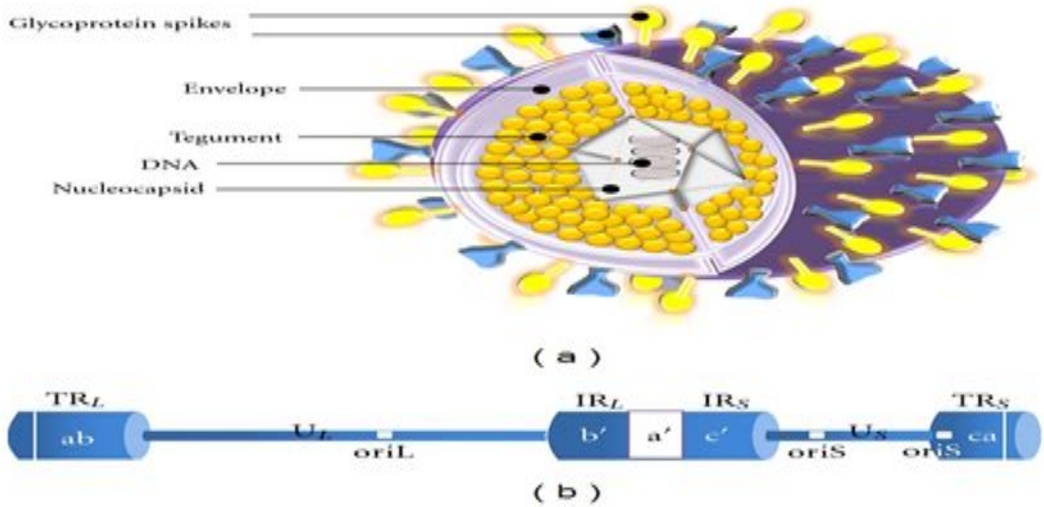
Sub family (" herpesvirinae")	Biological properties		Genus ("virus")	Examples	
	Growth cycle and cytopathology	Latent infection		Official name ("human herpesviridae")	Common name
Alpha	Short, cytolytic	Neurons	Simplex Varicello	1 2 3	Herpes simplex virus type 1 Herpes simplex virus type 2 Varicella-zoster virus

Beta	Long, cytomegalic Long, lymphoproliferative	Gland, Kidneys Lymphoid tissue	Cytomegalo Reseolo	5 6 7	Cytomegalovirus Human herpesvirus 6 Human herpesvirus 7
Gamma	Variable, lymphoproliferative	Lymphoid tissue	Lymphocrypto Rhadino	4 8	Epstein-Barr virus Kaposi sarcoma-associated herpesvirus

1.2.2.3 Structural Characteristics of HSV-1

1.2.2.3.1 Viral Structure

Herpes viruses have a comparable structure, consisting of a linear double-stranded DNA center encased in an icosahedral capsid at the core (as in Figures 1–3). Surrounding this core is a region known as the tegument, which contains proteins and enzymes that facilitate the initiation of replication. The tegument is then enveloped by a membrane containing various glycoproteins. (Hadi *et al*., 2022).



Figures 1–3: HSV-1 model structure and genome arrangement (a) The icosahedral, DNA-containing capsid is asymmetrically located within the virion and surrounded by an amorphous protein layer called

the tegument and a membrane envelope heterogeneously studded with morphologically distinct spikes formed by 12 different glycoprotein species. (b) The HSV-1 genome arrangement shows repeats surrounding UL designated ab and b′a′ and those surrounding US designated a′c′ and ca. There are two different origins of replication: oriL in the long segment and oriS in the short segment. Abbreviations: UL: long unique sequence; US: short unique sequence; TRL: terminal repeats of the long segment; TRS: terminal repeats of the short segment; IRL: internal repeat of the long segment; IRS: internal repeat of the short segment. (Elbadawy *et al*., 2012)

The envelope is derived from the host cell membrane and contains viral glycoproteins, while the capsid contains the viral DNA. The capsid is composed of capsomeres arranged in an icosahedral pattern (Heming *et al*., 2017).

1.2.2.3.2 Viral genome

The HSV-1 genome consists of a linear, double-stranded DNA molecule that is approximately 152,000 base pairs in length. The genome is organized into a unique region and a repeat region, which contain the genes that encode for the virus' structural proteins. The unique region of the genome contains the genes that encode for the virus' non-structural proteins, which are involved in the virus' replication and pathogenicity (Roizman, 2013). Both replicating DNA and encapsulated viral genomes contain nicks and gaps. Gaps in virion-isolated DNA were estimated to be between 3 and 13 per genome (Smith *et al*., 2014).

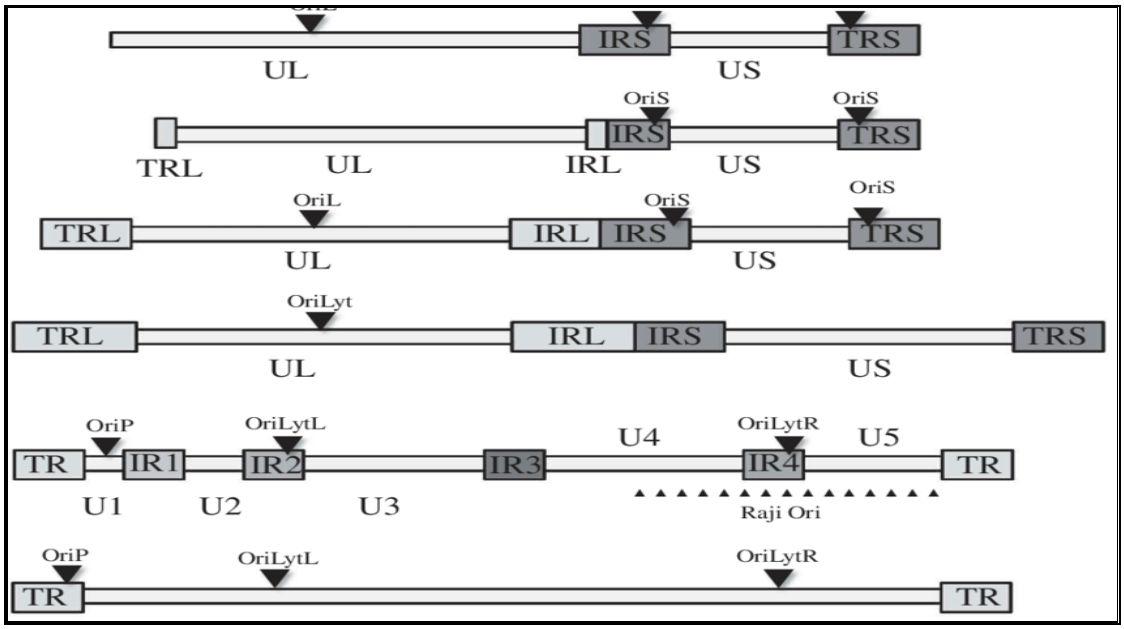


Figure 1–4: The genomic structure of various herpesviruses The genomes of herpesviruses are composed of varying numbers of unique and repeat regions. The replication origins can be located either in the unique regions, in the repeat regions, or in both. Abbreviations: PRV: pseudorabies virus; VZV: varicella-zoster virus; HSV-1: herpes simplex virus type 1; HCMV: human cytomegalovirus; EBV: Epstein-Barr virus; KSHV: Kaposi's sarcoma herpesvirus; UL: unique long region; US: unique short region; TRL: terminal repeat of UL region; TRS: terminal repeat of US region; TR: terminal repeat; IR: internal repeat; LUR: long unique coding region; Ori: replication origin. (Boldogkői Z. *et al*., 2019)

1.2.2.3.3 Viral envelope

The host cell's plasma membrane provides the lipid bilayer that the herpes simplex virus (HSV) uses as its envelope. Embedded inside the lipid bilayer of this envelope are glycoproteins that are essential to the virus' infectivity and morphogenesis. The capacity of the virus to avoid the host immune system is also facilitated by these glycoproteins, which also aid in the virus' attachment to and penetration into host cells,Herpes simplex virus 1 comprises 15 viral proteins, 12 glycosylated proteins, and 3 unglycosylated proteins in its lipid envelope, Four of these glycosylated proteins—gD, gH, gL, and gB—are important for entry into target cells in tissue culture and animal models, while the remaining 11 proteins are

usually referred to as "non-necessary" for entry because they have mild phenotypes when removed (Stiles *et al*.,2010).

When gD binds to certain receptors on the surface of host cells, the protein undergoes a conformational change that allows it to connect with other viral glycoproteins (the gH/gL complex). By causing the viral envelope and host cell membrane to fuse, this interaction makes it easier for the viral genome to enter the host cell, where it can start the replication process. (Di Giovine *et al., 2011*)

Herpes simplex virus type 1 glycoprotein D binds to the N-terminal regions of nectin-1 and nectin-2 and to human dendritic cells. There is interaction between herpes simplex virus type 1 (HSV-1) glycoprotein D and nectins, which are cell surface proteins that are expressed in various tissues. These findings suggest that glycoprotein D plays a role in the attachment of HSV-1 to host cells and in the entry of the virus into host cells (Kirkland *et al*., 2020).

1.2.2.3.4 Viral Tegument

The tegument, which is mainly located between the capsid and the envelope, is a significant component of viruses. Tegument proteins have critical functions in various viral processes, such as regulating viral gene transcription, promoting viral replication and virulence, facilitating viral assembly, and even modulating the interaction between the virus and the host immune system. (Xu *et al*.,2016). According to Metrick *et al.* (2020), it is believed that the UL37 protein located in the inner tegument acts as a bridge between the viral capsid and envelope by binding to UL36, gK, and UL20. Additionally, research conducted by Oda *et al.* (2016) showed that there is a necessary interaction between UL51 and

UL14 for the proper localization of these viral proteins in infected cells. The UL51-UL14 complex also plays a role in regulating final viral envelopment, which is essential for efficient viral replication.

In particular, the VP1/2 protein, also known as pUL36, is a significant component of the tegument, weighing 4330 kDa, and is crucial for both the virus's entry and assembly. This protein has binding regions located at its N-terminus that bind to the inner tegument protein pUL37 and the main tegument protein VP16, while the C-terminal section has two binding regions for the outer capsid protein pUL25. As a result, HSV1-pUL36 acts as a connector between the capsid and other tegument proteins (Fan *et al*. 2015). The function of PUL36 is essential for directing incoming capsids to nuclear pores and facilitating the release of genomes into the nucleoplasm, where viral replication and transcription occur, as demonstrated by experiments carried out by Abaitua *et al*. (2012) and Schipke *et al*. (2012).

Another crucial inner tegument protein, weighing 120 kDa and consisting of 1123 amino acids, is encoded by the open reading frame UL37 in HSV1. pUL36 and pUL37, the inner tegument proteins of HSV1, can interact with each other directly, and both rely on each other for their incorporation into L-particles. Kelly, *et al*. (2014) HSV1-pUL37 requires the assistance of HSV1-pUL36, but not capsids, to target the secondary envelope membranes. The interaction between pUL37 and VP11/12 and pUL36 and VP16 at the secondary budding site likely explains this phenomenon. Unlike VP1/2 and pUL37, VP16, one of the main tegument proteins, is believed to be closer to the viral envelope and is a component of the outer tegument. VP16 interacts with VP1/2 and triggers immediate-early transcription of viral genes, which is crucial in

the viral life cycle. Due to its numerous interactions with other viral proteins, VP16 has been proposed as the central tegument organizer. (Fan *et al*., 2020)

Following that, a number of tegument proteins associated with viral DNA were discovered and found to be involved in the assembly and egress of the virus from the nucleus. One such protein is VP22, which plays a critical role in both viral gene transcriptional regulation and the formation of the virion capsid and envelope. Additionally, VP22 is able to bind to other viral proteins, including ICP0, pUL16, gD, gE, and gM. (Wu *et al*., 2020) UL31, Us3, ICP34.5, and UL51 are essential proteins for controlling nuclear viral egress, while UL11, UL20, and UL46–49 are associated with the primary envelope of HSV-1. The formation of functional complexes by HSV1-pUL20 and gK enables them to bind with capsids through pUL37 and the small capsid protein VP26. (Liu *et al*., 2014)

1.2.2.3.5 Viral capsid

The herpes simplex virus type 1 (HSV-1) is a virus that causes infections in humans. One of the key components of the virus is its capsid, which is a protein shell that encloses the viral genome. The capsid is composed of proteins called capsomeres, which are arranged in a specific pattern to form the spherical shape of the capsid (Heming *et al*.,2017).The virus's capsid has a number of crucial functions during its life cycle. It aids the virus in evading the host's immune system and guards against damage to the viral DNA. Because it contains proteins that interact with receptors along the surface of the cells that host it, the capsid also aids the virus in entering host cells. (Banerjee *et al*.,2020).

1.2.2.4 Herpes Simplex Virus Replication

The replication of HSV-1 can be divided into seven steps (1.2.2.4.1–1.2.2.4.7), all of which are shown in (Figure 1–5.)

1.2.2.4.1 Attachment of the Herpes Simplex Virus to the Host Cell

The capsid of HSV-1 can be modified in order to improve its ability to infect host cells or to evade the host's immune system. For example, researchers have engineered HSV-1 capsids to target specific types of cells or to express specific proteins that enhance the virus's ability to infect host cells (Waehler *et al*., 2007).

First, the virus attaches to the host's cell surface receptor, heparan sulfate proteoglycans (HSPGs), via its viral glycoproteins gB and/or gC (Madavaraju *et al*., 2021). The virus then slides on the cell surface and reaches the cell body, a movement termed "viral surfing (Thakkar *et al*., 2017).

Like all enveloped viruses, herpesviruses enter cells by joining their lipid envelopes to the host cell membrane. Although specific to herpesviruses, the procedure of receptor activation and membrane fusion is carried out by a wide variety of glycoproteins. Among these glycoproteins, two complexes are shared by the three herpesvirus subfamilies:

The heterodimeric gH/gL complex and the trimeric gB, which functions as a membrane fusogen. This work investigates the interactions of gH/gL with different accessory viral proteins, host cell receptors, and neutralizing or inhibitory antibodies to determine the conserved and

distinctive roles of gH/gL in the three subfamilies of human herpesviruses. According to the authors, the structural adaptability of gH/gL enables it to serve as a signal integration device that can take in various regulatory inputs and convert them into a "trigger" signal, thereby triggering gB's fusogenic capability. (Gonzalez-Del Pino *et al*.,2022).It is noteworthy that the gH/gL heterodimer has the ability to interact with certain integrins present on the cell surface, including αvβ6, αvβ8, and αvβ3. Of particular interest is the interaction between gH/gL and αvβ6 and αvβ8, which has been shown to induce dissociation of the heterodimer and activate gH independently. This activation of gH facilitates the entry of the herpes simplex virus into host cells through acid endosomes. (Gianni *et al*., 2015) HSV-1 gB, gD, and gH/gL are the "core roles" in virus-induced fusion (Sathiyamoorthy *et al*., 2017). gM is a modulator in this process, which implies that gM is involved in virus entry into the cell (El Kasmi and Lippé, 2015). The envelope glycoprotein M (gM), a surface virion component conserved among alpha herpes viruses, is a multiple-transmembrane domain-containing glycoprotein with a complex N-linked oligosaccharide. The gM mediates a diverse range of functions during the viral life cycle (Li *et al*., 2021).

1.2.2.4.2 Penetrations of Herpes Simplex Virus into Host Cell

Herpes Simplex Virus-1 (HSV-1) uses its glycoprotein D (gD) to interact with particular cell surface receptors when it comes into contact with a host cell. According to the type of cell the virus is attacking, a different receptor may be used. Nectin-1 (also known as HveC) or nectin-2 (also known as HveB) receptors are strongly bound by gD in non-immune cells. (Akhtar *et al.*, 2009). The complex process of herpes

simplex virus (HSV) entry into host cells involves multiple steps, and several viral glycoproteins have been identified as crucial players. Among these, viral glycoprotein D (gD) has been extensively studied and confirmed to play a critical role in the initial stages of HSV entry.

Interaction between gD and the host cell surface receptor nectin-1 (HveC) is required for promoting HSV-1 entry into neuronal cells (Bhargava *et al*., 2016). Additionally, other viral glycoproteins such as gB, gH, and gL are also essential for efficient HSV entry into host cells.

Previous research has revealed that gB and gH/gL form a stable pre-fusion complex, which is necessary for efficient HSV-1 entry into target cells (Rice SA *et al.*, 2021). Moreover, gH/gL can bind to the host protein BILF1, which enhances HSV-1 entry into immune cells (Pataki *et al*., 2022).

Additionally, the transmembrane protein neuropilin-1 has been identified as a co-receptor for HSV-1, promoting virus entry into cells that express low levels of nectin-1 (Simpson *et al.*, 2005). The viral protein fusion gB is activated as a result of gD's binding to the particular receptors on the surface of the host cell. The internalization of the viral components inside the cytoplasm of the host cell is thus made possible by the active gB, which also aids in the fusing of the viral envelope with the cell membrane. (Hogue *et al*., 2014).

The activation of gB is facilitated by the gH/gL glycoprotein complex, and recent studies have shown that gB undergoes pH-dependent conformational changes during the fusion process (Stampfer *et al*., 2010). These changes expose hydrophobic residues that allow gB to interact with the host cell membrane and initiate the fusion process. Specific mutations in gB have been found to impair the viral entry process, indicating its

critical role in HSV entry (Cairns *et al*., 2020). The complex mechanism of HSV entry highlights the importance of understanding the structural changes and activation of viral fusion proteins for the development of effective treatments and vaccines.

1.2.2.4.3 Capsid Delivery To The Nucleus And Genome Delivery

As soon as HSV particles enter the cytoplasm, certain tegument proteins connected to the capsid work there, while others, like the VP16 transactivator, travel to the nucleus to facilitate viral gene transcription, which is essential for the virus's reproduction cycle. (Fan *et al*., 2020). Fusion is followed by the release of the viral capsid into the cytoplasm. Once discharged into the cytoplasm, viral capsids are imported along with the microtubules towards the microtubule-organizing center (MTOC) and hence to the nuclear envelope. (Alandijany ,2018)

Herpes simplex viruses (HSVs) employ dynein and kinesin-1 to travel along polarized microtubules, which suggests that different cell types have different distributions of microtubule-organizing centers. As a result, the virus is able to select the ideal motor proteins for capsid transportation to the nucleus based on the cellular environment (Musarrat *et al*., 2021).

VP26 is a viral tegument protein, promote process of interaction among capsids and dynein. In experiments with a library of viral proteins tested for interactions with dynein subunits, it was demonstrated that VP26 interacts with the dynein light chains RP3 and Tctex1, enabling the virus to use the host's dynein motor protein for moving the viral capsids along microtubules towards the cell nucleus. (Kobayashi *et al.*, 2017).

Despite the fact that VP26 is involved in viral capsid transport to the nucleus, an HSV-1 mutant lacking the VP26 protein was still able to enter the nucleus at levels comparable to the wild-type virus, indicating the involvement of other viral proteins and host receptors (Nagel *et al*., 2012).

Eventually, the viral capsid and remaining tegument proteins make their way to the nuclear pore complex (NPC), where their binding triggers the delivery of viral DNA to the nucleus, aided by the high pressure inside the viral core. (Brandariz-Nuñez *et al.*, 2019)

1.2.2.4.4 Gene Expression And Virus Replication

According to Pesola *et al*. (2006), the inherent infectivity of the HSV DNA genome enables the generation of live particles from cells transfected with pure viral DNA without the requirement for accompanying viral proteins. Host-encoded factors can transcribe immediate early viral genes, also known as alpha genes, some of which are responsible for promoting the expression of further viral genes regulated by these viral factors. According to studies undertaken by Sun *et al*. (2021), these results in the sequential development of two waves of viral proteins: one that triggers the expression of early viral genes, also known as beta genes, and a second wave of late viral genes, also known as gamma genes.

1.2.2.4.5 Viral Capsid Assembly

The formation of the viral capsid is a vital phase in many viruses' reproduction cycles. According to research, this process takes place within the nucleus and is then transported to the cytoplasm. For example, research on the herpes simplex virus (HSV) has revealed that capsid

construction occurs in infected cells' nuclei before being transported to the cytoplasm. (Mettenleiter, 2002). Similarly, a study on the human cytomegalovirus (HCMV) has demonstrated that the assembly of the viral capsid takes place in the nucleus, and this process requires the involvement of viral and host-encoded factors (Tandon *et al*., 2015).

The mechanism by which the viral capsid is transported from the nucleus to the cytosol is not fully understood, but studies have suggested that it may involve the involvement of nuclear pore complexes (NPCs). A study on the simian virus 40 (SV40) has shown that the transport of viral capsids through NPCs requires the presence of specific viral proteins (Eibauer *et al*., 2015).

1.2.2.4.6 maturation of the virion

Herpes virus capsids acquire additional tegument proteins, namely UL7, UL11, UL16, and UL51, after leaving the nucleus (Roller *et al.*, 2015). The complex structure of the tegument layer in herpesviruses implies that some internal tegument proteins, like VP1/2, may act as scaffold proteins that hold the capsid in place and assist in the assembly of other tegument proteins. Research has supported the notion that VP1/2 interacts with capsid proteins, including UL19 (VP5), as well as other tegument proteins like UL17 and UL25, which are essential for herpesvirus assembly and egress. (Draganova *et al*., 2021).

Research on Herpes Simplex Virus 1 (HSV-1) revealed that VP1/2 interacts with UL37, a tegument protein of the virus. This interaction indicates that VP1/2 has a significant role in connecting the tegument layer and viral capsids, thereby facilitating virus assembly and egress. (Hogue *et al*., 2016).

1.2.2.4.7 Virus Maturation And Release

The viral capsids of the herpes simplex virus (HSV) are produced in the nucleus and subsequently released into the cytoplasm. (Campadelli-Fiume *et al.*, 1999) The endoplasmic reticulum simultaneously produces glycoproteins necessary for this process, transports them via the trans-Golgi network (TGN), and directs them toward multivesicular bodies (MVBs). Later, the plasma membrane membrane receives these glycoproteins. (Wild *et al.*, 2017) and are subjected to endocytosis if they are present on the cell surface, resulting in their return to early endosomes (Tebaldi *et al.*, 2020). The cytoplasmic viral capsids fusion with the HSV glycoprotein-containing endosomes results in the formation of infectious virions inside vesicles. (Butt *et al.*, 2020). The virions present in these vesicles are released into the extracellular medium eventually, following their transit through the actin mesh present in the plasma membrane. (Bearer *et al*., 2019).

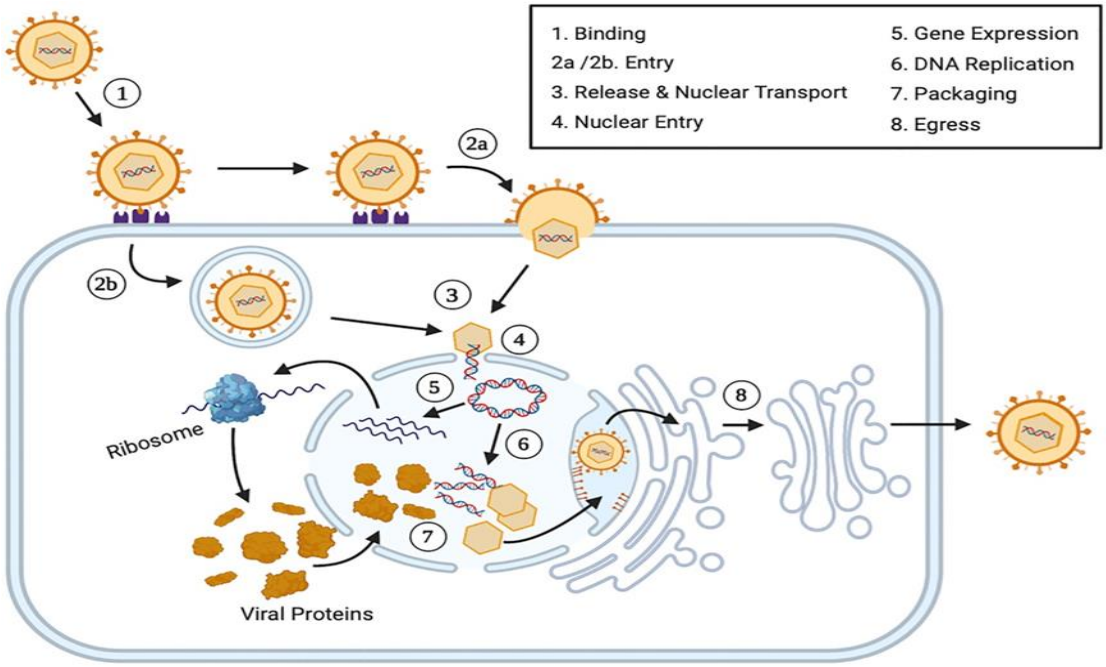


Figure 1-5: Herpes simplex replication cycle (Verzosa, *et al*., 2021).

1.2.2.5 Pathogenesis of HSV-1 Infections

Like all herpes viruses, herpes simplex virus 1 (HSV-1) establishes a persistent infection in an individual that persists throughout their life. In this state, the virus is latent and resides within the nuclei of sensory neurons as silenced DNA, with only the latency-associated transcript RNA being abundantly transcribed. Although this silencing of the virus can persist, reactivation of HSV-1 can occur through the reversal of this silencing in individual neurons, leading to full virus replication (Cohen *et al*., 2020). HSV can infect and replicate in epidermal and dermal cells after exposure to mucosal membranes or abraded skin sites. The initial HSV infection is usually subclinical and does not produce any visible lesions. Studies in animals and humans have shown that the presence of sufficient levels of the virus is associated with both clinical and subclinical acquisition, allowing for infection of sensory or autonomic nerve endings. (De La Cruz *et al.*, 2021). During the acute infection of the oral, nasal, or ocular mucosal epithelium, the herpes simplex virus (HSV) gains entry into sensory neurons and travels retrograde inside the axon to reach the cell body in the sensory ganglia, where it establishes latency. (Bello-Morales *et al*.,2020). During latency, viral gene expression is mostly suppressed, except for latency-associated transcripts (LATs), which aid in the establishment of latency and prevent the apoptosis of host cells. (Vanni *et al*., 2020).

1.2.2.5.1 Herpes Simplex Virus Type (1) Lytic Cycle

Lytic infections are typically initiated by cold sores and other lesions that affect the oral mucosal epithelial cells. The progeny virus produced during this primary outbreak can then travel to the sensory neurons

present in the trigeminal ganglion, leading to a latent infection (Mertens *et al*., 2021).

1.2.2.5.2 Latency and reactivation of Herpes Simplex Virus Type 1

HSV infection can replicate lytically or latently. While minimal gene expression and the absence of viral particle generation take place during latency, coordinated viral gene expression during lytic replication produces infectious viruses. Despite this, the viral genome is still capable of reactivation, which can be prompted by a suitable stimulus and result in the creation of infectious virions ,there are a number of models that have been used in research on the latency and reactivation of HSV, each with advantages and disadvantages. Alphaherpesvirus active transcription and reactivation during latency were covered in a review by Bloom (Bloom, 2016). However, susceptible non-neuronal cells typically experience lytic replication, whereas HSV develops latency in neurons. A recent study, however, suggested that some non-neuronal cells may exhibit latency when cultured in vitro (Cohen *et al*., 2020). If this phenomenon also occurs in vivo, additional study is required to validate it.

1.2.2.6 HSV-1 Transmission

According to AlMukdad *et al.* (2023), herpes simplex virus type 1 (HSV-1) primarily infects the mouth and causes oral herpes or cold sores, but it can also cause genital herpes. The virus can be transmitted even when there are no visible symptoms or sores, making it difficult to prevent the spread of the virus.

Looker *et al*. (2015) state that HSV-1 is a highly contagious virus that can be transmitted through close personal contact, including kissing and sexual activity.

James *et al*. (2010) further explain that certain factors increase the risk of transmission of HSV-1, such as having multiple sexual partners, engaging in unprotected sex, and having sexual activity at a young age. People with weakened immune systems are also at increased risk of transmission. Preventing the transmission of HSV-1 can be challenging, but there are several strategies that can help. These strategies include avoiding close personal contact with people who have visible cold sores or genital herpes, using condoms or dental dams during sexual activity, and practicing good hygiene, such as washing hands frequently and avoiding sharing utensils or personal items with others, as discussed by (Bello *et al*., 2020; Barrow *et al*., 2020).

1.2.2.7 HSV-1 immunity

The host's innate and adaptive immune systems, which cooperate to stop the virus from replicating and spreading, are crucial to the effectiveness of a viral infection. The innate immune response acts as the initial line of defense in the continuing and dynamic host-virus conflict. It is essential for preventing viral propagation and controlling how the adaptive immune system is activated. (Zhu *et al*., 2020). Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I) receptors, are crucial in the context of viral infection for identifying viral pathogen-associated molecular patterns (PAMPs) in infected cells. The innate immune response is triggered by the activation of PRRs, which causes the induction of type I interferons (IFNs) and other pro-inflammatory cytokines (Zhao *et al*.,

2021). Toll-like receptors (TLRs) recognize viral pathogen-associated molecular patterns (PAMPs) present on the surface of infected cells, whereas retinoic acid-inducible gene-I (RIG-I) receptors recognize viral RNA in the cytoplasm. (Ma *et al*., 2018). Activation of pattern recognition receptors (PRRs) initiates downstream signaling pathways that lead to the upregulation of genes involved in antiviral immunity. (Garcia-Sastre *et al*., 2006). Virus-specific CD8+ T cells, which are part of the adaptive immune response, play a crucial role in eliminating infected cells and preventing the spread of the virus (Gebhardt *et al*., 2009; Zhang, *et al*., 2017). Type I interferon (IFN) signaling induces the upregulation of interferon-stimulated genes (ISGs), which can enhance the adaptive immune response and provide long-lasting protection against viral infections. (Durbin *et al.,* 2013).

Furthermore, research has suggested that dysregulation of the type I interferon (IFN) signaling pathway may contribute to the severity of viral infections in certain individuals, particularly those with autoimmune disorders. (Cao, 2020).

1.2.2.7.1 CD8+ T cell immune response to HSV1 infection.

The adaptive immune response to intracellular infections like viruses depends heavily on CD8+ T cells. Naive CD8+ T cells differentiate into Tc1, Tc2, or Tc17 cells after identifying viral peptides given by antigen-presenting cells (APCs) that carry the MHC-I class I antigen. (Zhang *et al*., 2017). Upon viral infection, CD8+ T cells secrete interferon-gamma (IFN-γ), which promotes antigen presentation to CD8+ T cells and enhances the immune response while inhibiting viral infection. (Nakiboneka *et al.,* 2018).

CD8+ T cells respond to various antigen-presenting cells (APCs), such as dendritic and ganglionic cells, during herpes simplex virus type 1 (HSV-1) infection. The choice of APC type and experimental conditions are essential to limiting HSV-1 infection when studying CD8+ T cell responses. Furthermore, the generation of virus-specific CD8+ tissue-resident memory T cells (TRM cells) occurs in both ganglia and mucosal tissues following HSV-1 infection. TRM cells represent a subgroup of memory lymphocytes that are located in non-lymphoid tissues and provide long-term protection against recurrent infections. (Pollara *et al*., 2003).

1.2.2.7.2 CD4+ T cells respond to HSV1 infection.

Following interaction with the antigen-MHC complex, naive CD4+ T cells differentiate into a number of subsets, including Th1, Th2, Th17, and induced regulatory T (iTreg) cells, depending on the milieu. In controlling effective immune responses to pathogens and host survival, CD4+ T cells are essential. (Ike *et al*., 2013). The various roles of CD4+ T cells in preventing viral infections and regulating immune responses are mediated by varied cytokine profiles and regulatory mechanisms that depend on particular antigenic stimuli and microenvironmental cues. (Chen *et al.,* 2013).

Th1 cells are responsible for providing immunity against intracellular pathogens, while Th2 cells are crucial for immunity against various extracellular pathogens. Treg cells play a vital role in minimizing the intensity of inflammatory lesions caused by viruses, suggesting that Tregs could be therapeutically useful in terms of expansion and activation. In the case of HSV-1 infection, Th17 cells are primarily responsible for upregulating IL-17 expression in the host cornea, while Th1 cells

orchestrate herpes stromal keratitis (HSK) during viral infection. (Abbas *et al*., 2021).

1.2.2.8 Herpes simplex virus-1 in Graves' disease

TH, a hormone that acts on almost every cell in the body, including neurons, is likely to participate in the regulation and maintenance of viral latency and reactivation, including HSV-1 (Hsia *et al*., 2011).

This idea is supported by studies that suggest a potential link between other herpesviruses, such as Epstein-Barr virus (EBV) and human herpesvirus-6 (HHV-6), and neurological diseases like multiple sclerosis (MS) and Alzheimer's disease (AD) (Figliozzi *et al*., 2017). Despite the fact that almost 60% of the world's population has antibodies against HSV-1, only 20%–40% of those infected with the virus develop symptoms (Looker *et al*., 2015).

1.2.3 Diabetes mellitus type 2 (T2DM)

Type 2 diabetes mellitus (T2DM) is one of the most common metabolic conditions in the world and results from two essential indicators: impaired insulin sensitivity of tissues and hypoinsulinemia generation by pancreatic islets (Roden *et al*., 2019). When insulin resistance and impaired beta cell activity coexist, hyperglycemia, a defining feature of type 2 diabetes, is frequently triggered (Esser *et al.*, 2020).

Saeedi *et al.* (2019) predict that between 2017 and 2045, the number of people with diabetes globally is likely to rise by 110 percent, to 629 million. According to DeFronzo *et al.* (2015) and Kyrou *et al.* (2020), T2DM risk factors include genetic susceptibility, obesity, family history, sedentary lifestyle, ethnicity, and other variables. T2DM may result

from a variety of variables, including aging, genetics, environmental factors, lifestyle decisions, and infections. According to research by Lontchi-Yimagou *et al.* (2021) and Karim *et al.* (2014), viral infections have been demonstrated to have a substantial connection with non-autoimmune diabetes, possibly through direct or indirect viral-mediated pathways.

Given that red blood cells have a predicted half-life of two to three months, glycated hemoglobin (HbA1c), a key indicator of long-term glycemic control, is used to diagnose diabetes (Sherwani *et al.*, 2016). Diabetes is also associated with an elevated HbA1c level (Dave *et al.*, 2019). Lifestyle modifications such as regular physical activity, a healthy diet, weight management, and smoking cessation have been shown to improve glycemic control, reduce insulin resistance, and lower the risk of cardiovascular disease and mortality. Pharmacotherapy includes several classes of antidiabetic agents, such as metformin, sulfonylureas, thiazolidinediones, dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-1 receptor agonists, and sodium-glucose cotransporter-2 inhibitors, which target different aspects of the pathophysiology of T2DM. Monitoring of blood glucose levels, either by self-monitoring or laboratory testing, is essential to assess the effectiveness of the treatment and adjust it accordingly (Abusaib *et al*., 2020).

In conclusion, T2DM is a complex metabolic disorder that requires a comprehensive understanding of its risk factors, pathogenesis, and management. Addressing lifestyle modifications, pharmacotherapy, and monitoring of blood glucose levels are crucial components of its management. Research into effective and personalized strategies to

prevent, diagnose, and treat DM2 and its complications is ongoing (Goyal *et al*., 2018).

1.2.3.1 Correlation between herpes simplex virus-1 and diabetes mellitus type 2 .

Although the primary cause of type 2 diabetes is unknown, two breakthroughs have been made regarding its development (Taylor, 2004). First, insulin resistance in muscle is the earliest detectable defect in people in whom type 2 diabetes will later develop. Second, β-cell function has to be abnormal before hyperglycemia develops. One of the risk factors for diabetes development might be virus infection (Jun *et al*., 2003). Preexisting hepatitis C virus infection may increase the risk of type 2 diabetes (Mehta *et al*., 2003; Lecube *et al*., 2004).

Herpes simplex virus 1 (HSV-1) is a prevalent virus that infects up to 90% of the adult population worldwide. Diabetes mellitus type 2 is a metabolic disorder characterized by insulin resistance and hyperglycemia. Recent studies have shown a potential correlation between the two diseases.

A study by Kute, (2019) found that patients with T2DM had a higher prevalence of HSV-1 infection than those without T2DM. The study involved 2,361 participants, and the results showed that 53.6% of T2DM patients had a positive HSV-1 IgG antibody, compared to only 35.6% of those without T2DM. The researchers suggested that the correlation may be due to the role of inflammation in both diseases, as both T2DM and HSV-1 infections are associated with increased levels of inflammatory cytokines. However, further studies are needed to determine the exact

mechanisms underlying this relationship and explore potential therapeutic strategies.

According to Casqueiro *et al*. (2012), T2DM has been associated with a number of viral diseases, including Epstein-Barr, CMV, mumps and rubella . The double-stranded DNA virus known as human herpes virus 5 (HHV 5), also referred to as cytomegalovirus (CMV), is a member of the Herpesviridae family and can cause a wide range of human illnesses. In the words of Lazim *et al.* (2018), the CMV replication cycle includes a completely controlled cascade of genes that can be expressed. The virion shape of CMV is similar to that of the herpes virus. There is evidence for the possibility that type 2 diabetes may be influenced by chronic CMV infection. According to the research of Koch *et al.* (2007), CMV may hasten immunosenescence by encouraging the growth of late-differentiated CD4+ and CD8+ T-cells that produce pro-inflammatory cytokines and so foster an environment that is more inflammatory.

1.2.3.2 Correlation between Graves' disease and type 2 diabetes mellitus.

T2DM is the most prevalent form of the disease, with an overall prevalence rate of 8.5% in adult participants (Song, *et al*., 2017; Zhao, *et al.,* 2018; Shi, *et al.,* 2018). According to Chen, *et al.* (2019), T2DM is caused by insulin resistance linked to insulin insufficiency, which can lead to carbohydrate dysregulation and hyperglycemia. According to Darwish , *et al.* (2018), long-term hyperglycemia is the primary cause of blindness, cardiovascular disease, renal failure, and even death. It can result in both acute and chronic consequences. It poses a significant difficulty not only in the therapeutic context but also poses a significant burden for public health. Thyroid hormones are essential for carbohydrate

metabolism (Gierach *et al*., 2014). Hyperthyroidism may affect the secretion, action, and clearance of insulin and many aspects of carbohydrate metabolism and thus lead to hyperglycemia (Roubsanthisuk *et al*., 2006; Maratou *et al*., 2010). Endocrinopathies such as thyroid dysfunction (TD) and diabetes mellitus (DM) are frequently co-present in clinical practice. Type 2 (T2DM) diabetes mellitus patients have a significant prevalence of TD (Gu *et al.*, 2017).

Thyroid hormone and insulin both influence cellular metabolism, have a role in the metabolic syndrome, and are connected to autoimmune diseases , interactions between multiple biochemical, genetic, and hormonal dysfunctions are thought to be the cause of the pathophysiological connection between T2DM and TD. Hyperthyroidism is associated with the overexpression of the hepatic glucose transporter type 2 gene (GLUT2) (Wang , 2013). Triiodothyronine (T_3) found inside cells may also affect how sensitive the body is to insulin (Ray *et al.*, 2016).

Research indicates that there may be a correlation between the two conditions, with individuals with Graves' disease having an increased risk of developing type 2 diabetes (Rong *et al.*, 2021).

Individuals with Graves' disease were found to have a significantly higher risk of developing type 2 diabetes than the general population. The study found that individuals with Graves' disease were 1.49 times more likely to develop type 2 diabetes than those without the condition. Another study by Chen *et al*. (2019) also reported a similar association between Graves' disease and type 2 diabetes.

The elevated levels of thyroid hormones in individuals with Graves' disease may play a role in the development of type 2 diabetes. Thyroid

hormones have been shown to affect glucose metabolism by increasing glucose absorption from the gut, promoting gluconeogenesis, and reducing glucose uptake in peripheral tissues (Wang , 2013). This may lead to insulin resistance and impaired glucose metabolism, which are key features of type 2 diabetes (Song *et al*., 2021).

In addition to the effects of thyroid hormones on glucose metabolism, other factors may also contribute to the correlation between Graves' disease and type 2 diabetes. For example, both conditions are linked to inflammation and oxidative stress, which can contribute to insulin resistance and impaired glucose metabolism (Jang *et al.*, 2018).

T2DM, the most common form of diabetes (~90%), is characterized by a systemic inflammatory disease accompanied by insulin resistance (IR) or decreased metabolic response to insulin in several tissues, including the adipose tissue, liver, and skeletal muscle, as well as reduced insulin synthesis by pancreatic beta cells (Daryabor *et al*., 2019; Makowski *et al*., 2020). Hyperglycemia impairs the normal functions of the circulatory system, gastrointestinal tract, pancreatic beta cells, liver, and skeletal muscles to boost systemic insulin resistance. A hyperglycemic environment also leads to immune cell dysfunction. It increases intestinal permeability, which subsequently enhances the risk of infection in T2DM patients. (Daryabor *et al*., 2020)

Both diabetes mellitus and thyroid diseases are very common in the fields of endocrinology and metabolism. The coexistence of diabetes mellitus and thyroid diseases is frequently experienced. The relationship between thyroid function and glucose intolerance, or type 2 diabetes mellitus, is complicated. Hyperthyroidism could be a risk factor for glucose intolerance (Nishi , 2018). Untreated thyroid dysfunction can

impair the metabolic control of diabetic patients, and this association can have important repercussions on the outcome of both of these disorders. (Biondi ,*et al*.,2019).DM and thyroid disease are two closely associated disorders. The NHANES III study reported a higher prevalence of TD in subjects in the United States with diabetes compared with those without diabetes, especially in patients with positive anti-thyroperoxidase (TPO) antibodies (Abs) (Hollowell, *et al*., 2002).

Research suggests that there may be a connection between Graves' disease and type 2 diabetes mellitus, with individuals with Graves' disease being at an increased risk of developing type 2 diabetes. The underlying mechanisms are not yet fully understood but may involve the effects of thyroid hormones on glucose metabolism. Healthcare providers should be aware of this association and consider screening individuals with Graves' disease for type 2 diabetes (Song, *et al*., 2021).

2.1 Study Design

2.2 Materials:

2.2.1. Laboratory Instruments and Equipment's

The laboratory instruments and equipment used in this study were listed in Table (2-1):

Table (2-1): Laboratory Instruments and Equipment

No.	Item	Company	Country
1	Biological safety cabinet	Thermo Scientific	Germany
2	Clot activating tube	Supreme	China
3	ELISA reader	Epson	USA
4	ELISA shaker	Pasture	France
5	ELISA washer	Biotec	USA
6	Filter paper	United	India
7	Incubator	Incucell	Germany
8	Laboratory centrifuge	Hettich	Germany
9	Mask	Citezhn	China
10	Micropipettes	Slamed	Germany
11	Multichannel pipette	Slamed	Germany
12	N95 mask	Citezhn	China
13	Oven	Heraeus	Germany

14	Printer	Epson	USA
15	Refrigerator	Concord	Italy
16	Timer	Citezhn	China
17	Tips (yellow and blue tips)	Plastilab	Lebanon
18	Vortex shaker	Stuart	Germany
19	Water Bath	Memmert	Germany

Table(2-2): Materials used by current study

Materials	Manufacturing	Origin
EDTA Tubes	AFCO	Jordan
Eppendrof tubes	Scharlau	Spain
Latex gloves	Enana	Malaysia
Test tubes	AFCO	Jordan

2.2 Diagnostic and research kits: are listed in table (2-3).

Table (2-3): The Immunological Kits

Kit	**Company-Country**
Human Tri-iodothyronine T_3 cobas e411 Kit	Elecsys-Germany
Human thyroxin T_4 cobas e411 Kit	Elecsys-Germany
Human thyroid stimulating hormone TSH cobas e411 Kit	Elecsys-Germany
Human Thyroid-Peroxidase Antibody TPO-cobas e411 Kit	Elecsys-Germany
Human Thyroglobulin Antibody TG-Ab cobas e411 Kit	Elecsys-Germany

Human Herpes Simplex Virus antibody IgG ELISA Kit	BT Lab-China
Human thyroid stimulating hormone receptor ELISA Kit	BT Lab-China
Human Cluster of differentiation 25 ELISA Kit	BT Lab-China
Human hemoglobin Alc HbA1c levels. kit	Abbott - United States,

2.3. Patients :

A cross-sectional study included (173) patients who were attending Marjan Teaching Hospital in the province of Babylon and Imam Al-Hussain Teaching Hospital in the province of Karbala from March 2022 to January 2023, with a set of (97) healthy individuals serving as the control group. Patients data was collected from the lab. report of the data included: name, sex, age, smoking habit, and other underlying disease (e.g. Diabetes mellitus type 2). Their ages ranged from (18 to 85) years. Divided into three groups (≤35, 36-60, ≥61) years old. There were more females 112 (66.7%) than males 61 (33.3%), Based on the findings of blood tests by the Cobas e411 uses patented ElectroChemiLuminescence (ECL) technology to assess T4, T3, TSH, TG.Ab., TPO.Ab., and TSH-R.Ab. By using an enzyme-linked immunosorbent assay (ELISA), the levels of HSV-1 and CD25 were determined, and Afinion measured HbA1c levels. A fully automated boronate affinity assay called HbA1c is used to measure human hemoglobin Alc levels.

2.3.1 Inclusion and Exclusion Criteria:

The inclusion criteria for the patient group involved in this study comprised any patient who had Graves' disease diagnosed by a specialist

in a hospital. TSH (mean levels were significantly lower while T_3 and T_4 increased) exclusion criteria are: any suspected patient who had no Graves' disease; any patient who took chemotherapy; and diseases such as lupus, Addison's disease, and multiple sclerosis (MS).

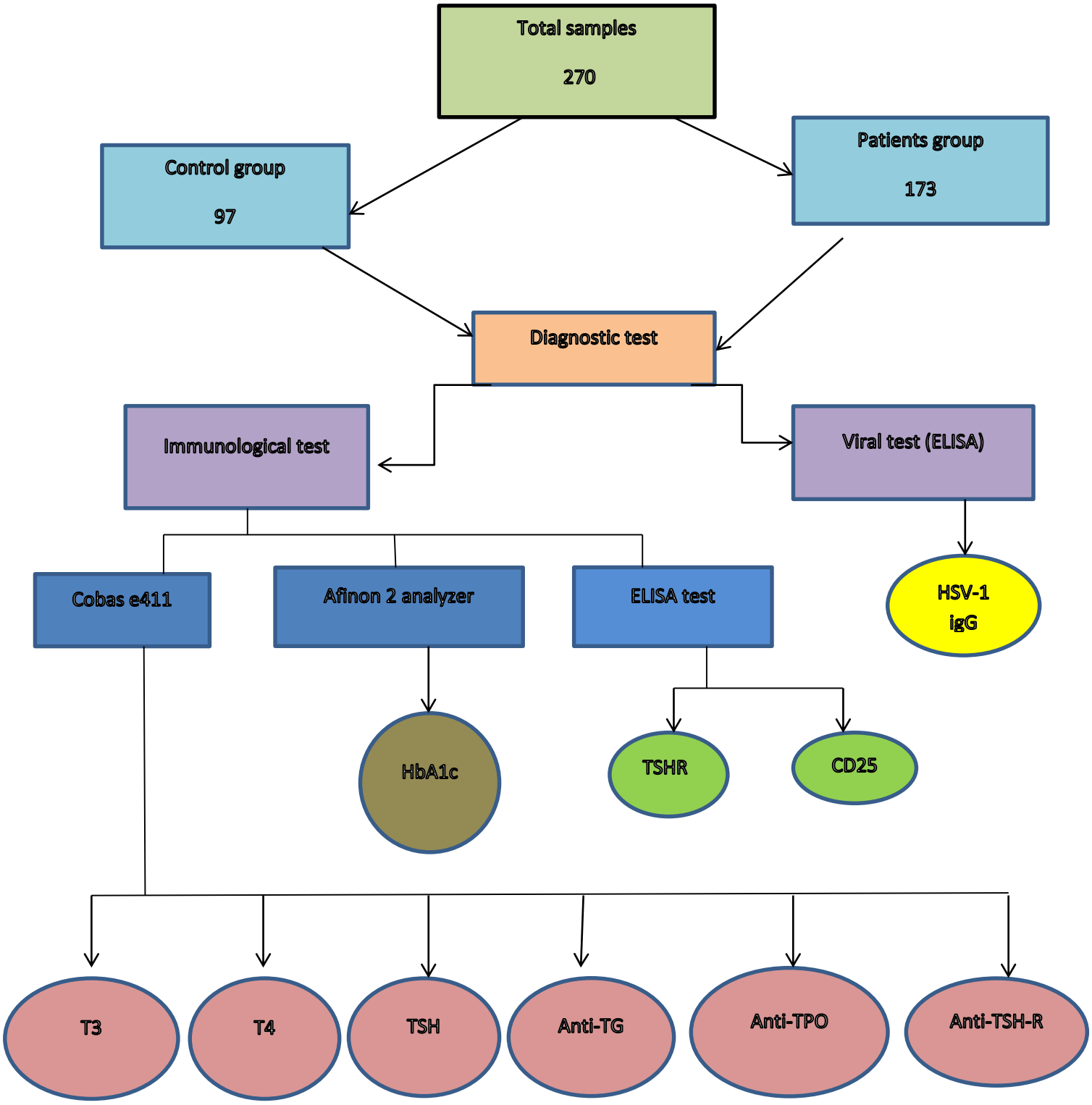
2.3.2 Ethical approval and consent :

This study was approved by the Ethics Committee of the Faculty of Medicine/Babylon University, Iraq, under the reference No.BMS/0231/016 and the Medical Ethics Committee of the Ministry of Health in Iraq and the Health Directorate in Babylon and Karbala. Patients' consent was taken, and health instructions and health safety conditions were followed when dealing with patients when blood was drawn.

2.3.3 Blood Sample Collection :

Each individual had five milliliters of venous blood drawn from them. Using an Abbott kit from the USA, 2 ml of blood was drawn for the Hb1Ac measurement. The remaining three milliliters of blood samples were left at room temperature for 15–30 minutes to clot before they were centrifuged at (3000 rpm) for (5 minutes) to remove the clot, and then the upper layer (serum) was separated by pipette, harvested into gel tubes, and centrifuged to start producing serum. The serum samples were transferred into sterile Eppendorf tubes, labeled with a serial number and the patient's name, and kept at -20°C until the Cobas e411 based on Elecsys-Germany was used to conduct this analysis. It uses patented ElectroChemiLuminescence (ECL) technology to assess T_3, T_4, TSH-R.Ab, TG Ab, TPO.Ab. and TSH-R Ab. By using an enzyme-linked immunosorbent assay (ELISA), the levels of HSV-1 IgG and CD25 (BT

Lab-China) were determined, and Afinion measured HbA1c levels. A fully automated boronate affinity assay kit called HbA1c, manufactured by Abbott in the United States, is used to measure human hemoglobin Alc HbA1c levels.



Figure(2-1).An illustrative diagram shows the procedures of the collected samples

2.4 Methods :

2.5 Hormonal Tests:

Thyroxin (T_4), triiodothyronine (T_3), and thyroid stimulating hormone (TSH) are fully automated analyzer tests for use on the Cobas e411 instruments that use a patented ElectroChemiLuminescence (ECL) technology for the determination of human T_4, T_3, and TSH in human serum immunoassay analysis using the Cobas e411.

Principle:

Test principle Competition principle Total duration of the assay: 18 minutes.

In the first step of the process, 15 µL of the sample is incubated with a T3-specific antibody and a T4-specific antibody, both labeled with a ruthenium complex. ANS is then used to release the bound T4 and T3 from the binding proteins in the sample.

In the second step, streptavidin-coated microparticles and biotinylated T_3 and T_4 are added to the mixture. The labeled antibodies then bind to the free T_3 and T_4, forming an antibody-hapten complex. The complex is captured by the solid phase through the interaction of biotin and streptavidin.

The mixture is then placed into the measuring cell, where the microparticles are magnetically captured onto the electrode's surface. Unbound substances are removed using I ProCell/ProCell M, and a voltage is applied to the electrode to induce chemiluminescent emission, which is then measured by a photomultiplier.

The results are determined by an instrument-specific calibration curve generated through a 2-point calibration and a master curve provided via the reagent barcode or e-barcode.

2.5.1 Determination of Triiodothyronine (T_3)

The determination of T_3 is utilized in the diagnosis of T_3-hyperthyroidism, the detection of early stages of hyperthyroidism, and for indicating a diagnosis of thyrotoxicosis factitia. The Elecsys T3 assay employs a competitive test principle with polyclonal antibodies specifically directed against T3. Endogenous T3, released by the action of 8-anilino-1-naphthalene sulfonic acid (ANS), competes with the added biotinylated T3 derivative for the binding sites on the antibodies labeled with the ruthenium complexa) .

Normal value: (1.3–3.1 nmol/L)

2.5.2 Determination of thyroxin (T_4)

The determination of T_4 can be utilized for the following indications: the detection of hyperthyroidism, the detection of primary and secondary hypothyroidism, and the monitoring of TSH suppression therapy. The Elecsys T_4 assay employs a competitive test principle with an antibody specifically directed against T_4. Endogenous T_4, released by the action of 8-anilino-1-naphthalene sulfonic acid (ANS), competes with the added biotinylated T_4 derivative for the binding sites on the antibodies labeled with the ruthenium complex. a) Tris (2,2-bipyridyl) ruthenium (II) complex (Ru (bpy) +)

Normal value: (66–181 nmol/L)

Principle:

The test is based on the sandwich principle and has a total assay time of 18 minutes.

In the first incubation step, a mixture of 50 µL sample, a biotinylated monoclonal antibody specific to TSH, and a monoclonal antibody specific to TSH labeled with a ruthenium complex forms a sandwich complex.

In the second incubation step, the addition of streptavidin-coated microparticles results in the complex being bound to the solid phase through biotin-streptavidin interaction. The reaction mixture is then moved to the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Any unbound substances are removed with ProCell / ProCell M. A voltage applied to the electrode leads to chemiluminescent emission, which is detected by a photomultiplier. Results are obtained by using an instrument-specific calibration curve generated through 2-point calibration, and a master curve provided through the reagent barcode or e-barcode.

2.5.3 Determination of Thyroid Stimulation Hormones (TSH)

The determination of TSH serves as the initial test in thyroid diagnostics. Even very slight changes in the concentrations of the free thyroid hormones bring about much greater changes in the TSH level. Accordingly, TSH is a very sensitive and specific parameter for assessing thyroid function and is particularly suitable for early detection or exclusion of disorders in the central regulating circuit between the hypothalamus, pituitary, and thyroid. The Elecsys TSH assay employs

monoclonal antibodies specifically directed against human TSH. The antibodies labeled with ruthenium complexa) consist of a chimeric construct from human-specific components. As a result, interfering effects due to HAMA (human anti-mouse antibodies) are largely eliminated. a) Tris (2,2'-bipyridyl) ruthenium (II) complex (Ru (bpy) +)

Normal value: 0.25–5.0 nmol/L)

2.6 Immunological Test:

2.6.1 Anti-TSH Receptor Antibodies Assay:

The Anti-Thyroid Stimulation Hormone Receptor Antibodies Assay is an automated quantitative test for use on the Cobas e411 instruments for the enzyme immunoassay determination of human TSH-R.Ab., TG.Ab., and TPO.Ab. in human serum using a patented ElectroChemiLuminescence (ECL) technology.

Principle:

Test principle: Competitive principle. The total duration of the assay is 27 minutes.

During the 1st incubation, 30 µL of serum sample are incubated with pretreatment buffer solution (ATSHR PT1) and pretreatment reagent buffer (ATSHR PT2), which contains a pre-formed immunocomplex of solubilized pTSHR and biotinylated anti-porcine TSH receptor mouse monoclonal antibody. TRAb in patients' sera interact with the TSHR complex.

In the 2nd incubation, TRAb further interact with the TSHR complex after the addition of buffer solution.

In the 3rd incubation, streptavidin-coated microparticles and a human thyroid-stimulating monoclonal autoantibody (M22) labeled with a ruthenium complex are added. Bound TRAb are detected by their ability to inhibit the binding of labeled M22. The entire complex becomes bound to the solid phase via the interaction of biotin and streptavidin.

The reaction mixture is aspirated into the measuring cell, where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are removed with ProCell II M. Application of a voltage to the electrode induces chemiluminescent emission, which is measured by a photomultiplier.

Results are determined via a calibration curve, which is instrument-specifically generated by 2-point calibration, and a master curve provided via the COBAS link.

2.6.2 Determination of Anti-TSH Receptor Antibodies

The development of a third-generation TRAb assay system utilizing a human thyroid stimulating monoclonal antibody (M22) has enabled the detection and discrimination of Graves' disease from other thyroid diseases with high reproducibility, sensitivity, and specificity. This new system performs similarly or better than established second-generation assays. The introduction of fully automated TRAb assays has reduced manual procedures and facilitated integration into routine laboratory analyzers. The Elecsys Anti TSHR assay uses solubilized porcine TSH receptor (pTSHR) immunocomplexed with a biotinylated mouse

monoclonal antibody to the porcine TSH receptor C terminus and a ruthenium-labeled human monoclonal autoantibody M22 as the assay ligand. The ruthenium complex used is Tris(2,2'-bipyridyl)ruthenium(II)-complex (Ru(bpy)).

TSH-R Ab. :

Negative < 1.8 IU/mI

Borderline 1.8- 2.0 IU/ml

Positive ≥ 2.0 IU/mI

2.6.3 Thyroid peroxidase antibodies TPO Abs assays.

Principle TPO.Ab

Competition principle Total duration of the assay: 18 minutes.

1st incubation: 20 µL of sample are incubated with anti -TPO - antibodies labeled with a ruthenium complexa) .

2nd incubation: After addition of biotinylated TPO and streptavidin - coated micro particles, the anti - TPO antibodies in the sample compete with the ruthenium - labeled anti - TPO antibodies for the biotinylated TPO antigen. The entire complex becomes bound to the solid phase via the interaction of biotin and streptavidin.

The reaction mixture is aspirated into the measuring cell, where the micro particles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell / ProCell

M. Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier.

2.6.4 Determination of Thyroid Peroxidase Antibodies TPO Abs :

Results are determined via a calibration curve, which is instrument-specifically generated by 2 - point calibration, and a master curve provided via the reagent barcode or e - barcode. a) Tris (2,2'-bipyridyl) ruthenium (II) complex (Ru (bpy) +)

Normal: < 35 IU/ml.

Borderline: 35 - 55 IU/ml.

Elevated: ≥ 55 IU/ml

2.6.5 Detections of Thyroglobulin Antibodies TG Abs.

Test principle

Competition principle Total duration of the assay: 18 minutes.

1st incubation: 10 µL of sample are incubated with biotinylated Tg, and the antibodies in the sample bind the antigen.

2nd incubation: After addition of anti - Tg antibodies labeled with ruthenium complexa) and streptavidin - coated microparticles, the immunocomplex produced becomes bound to the solid phase via the interaction of biotin and streptavidin.

The reaction mixture is aspirated into the measuring cell, where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell / ProCell M.

Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier.

2.6.6 Determination of Thyroglobulin Antibodies

Results are determined via a calibration curve, which is instrument-specifically generated by 2 - point calibration, and a master curve provided via the reagent barcode or e - barcode . a) Tris (2,2¹ -bipyridyl) ruthenium (II) complex (Ru (bpy) +)

Negative < 100 IU/mI

Positive ≥ 100 IU/mI

2.6.7 Human Thyroid Stimulating Hormone Receptor ELISA Kit

Assay Principle

This kit is an enzyme-linked immunosorbent assay (ELISA). The plate has been pre-coated with a human TSHR antibody. TSHR present in the sample is added and binds to antibodies coated on the wells. And then a biotinylated human TSHR antibody is added and binds to TSHR in the sample. Then streptavidin-HRP is added and binds to the biotinylated TSHR antibody. After incubation, unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added, and color develops in proportion to the amount of human TSHR. The reaction is terminated by the addition of an acidic stop solution, and absorbance is measured at 450 nm.

Table(2-4) Human thyroid stimulating hormone receptor ELISA Kit component

Components	**Specifications**
Standard Solution (48ng/mL)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution	6ml x1
Substrate Solution	B 6ml x1
Wash Buffer Concentrate	20ml x1
Biotinylated Human TSHR Antibody	1ml x1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

Assay Procedure:

1. All reagents, samples, and standard solutions are to be prepared as instructed and brought to room temperature before use. The assay is performed at room temperature.
2. The number of strips required for the assay is to be determined and inserted into the frames for use. Unused strips are stored at 2-8°C.
3. 50µl of the standard is added to the standard well. Biotinylated antibody should not be added to the standard well as it already contains biotinylated antibody.

4. 40µl of the sample is added to the sample wells, followed by 10µl of anti-TSHR antibody to each well. Then, 50µl of streptavidin-HRP is added to the sample and standard wells, but not to the blank control well. The contents are mixed well and the plate is covered with a sealer. It is incubated at 37°C for 60 minutes.

5. The sealer is removed and the plate is washed 5 times with the wash buffer. For automated washing, each well is aspirated or decanted and washed 5 times with the wash buffer. The wells are soaked with 300µl of wash buffer for 30 seconds to 1 minute for each wash. The plate is then blotted onto paper towels or other absorbent material.

6. 50µl of substrate solution A is added to each well, followed by 50µl of substrate solution B to each well. The plate is covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

7. 50µl of stop solution is added to each well. The blue color will immediately change to yellow.

8. The optical density (OD value) of each well is measured immediately using a microplate reader set to 450 nm within 10 minutes of adding the stop solution.

Calculation of Result: Create a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis. Draw a best-fit curve through the points on the graph. Use computer-based curve-fitting software for accurate calculation, and the best fit line can be determined by regression analysis.

2.6.8 Human Cluster of differentiation 25(CD25) ELISA Kit :

Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human CD25 antibody. CD25 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human CD25 Antibody is added and binds to CD25 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated CD25 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human CD25. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Table (2-5)Human Cluster of differentiation 25(CD25) ELISA Kits component

Components	**Specifications**
Standard Solution (80ng/ml)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate	20ml x1
Biotinylated Human CD25 Antibody	1ml x1
User Instruction	1

Plate Sealer	2 pics
Zipper bag	1 pic

Assay Procedure

1. The reagents, standard solutions, and samples should be prepared following the provided instructions. It is necessary to ensure that all reagents are at room temperature before use, as the assay will be performed at room temperature.
2. The number of strips required for the assay needs to be determined, and they should be inserted into the frames for use. Any unused strips should be stored at 2–8°C.
3. The standard well should be added with 50µl of the standard solution, being careful not to add biotinylated antibody to the standard solution since it is already present.
4. The sample wells should be added with 40µl of the sample, followed by 50µl of streptavidin-HRP to both the sample and standard wells (excluding the blank control well). The solution should be mixed well, and the plate should be covered with a sealer, then incubated at 37°C for 60 minutes.
5. The sealer should be removed, and the plate should be washed five times with wash buffer. For automated washing, each well should be aspirated or decanted and washed five times with wash buffer. The plate should be blotted onto paper towels or another absorbent material.
6. Substrate solution A should be added to each well, followed by 50µl of substrate solution B. The plate should be incubated, covered with a new sealer, for 10 minutes at 37°C in the dark.

7. Stop solution should be added to each well, and the immediate observation will be the change of the blue color to yellow.
8. The optical density (OD value) of each well needs to be determined using a microplate reader set to 450 nm within 10 minutes after adding the stop solution

Calculation of Results :

construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis. To determine the best-fit curve through the points on the graph, perform calculations using computer-based curve-fitting software and regression analysis.

2.6.9 Human Herpes Simplex Virus IgG ELISA Kit

Assay Principle

The kit employs a qualitative reverse-phase enzyme immunoassay technique, where the microtiter plate is pre-coated with a target antigen. Samples, positive or negative controls were added to the wells, and incubated. Antibodies present in the samples bind to the antigen on the plate, and unbound antibodies are washed away during a washing step. A horseradish peroxidase (HRP)-conjugated detection antibody is added, and incubated. Unbound HRP is washed away during a washing step.

TMB substrate is then added, and the resulting color change is measured at 450 nm after adding an acidic stop solution which changes the color to yellow. By comparing the OD of an unknown sample to the OD of the positive and negative controls, the presence of HSV-1 Ab IgG can be determined.

Table (2-6) Human Herpes Simplex Virus IgG ELISA Kits Component

Components	**Specifications**
Pre-coated Plate	12 * 8 well strips x 1
Positive Control	0.5ml × 1 vial
Negative Control	0.5ml × 1 vial
HRP Conjugated	6ml × 1 vial
Sample Diluent	6ml × 1 vial
Substrate Solution A	6ml ×1 vial
Substrate Solution B	6ml ×1 vial
Stop Solution	6ml ×1 vial
Wash Buffer (25x)	20ml × 1 vial
Plate Sealer	2 pcs
Zipper Bag	1
User Instruction	1

Assay Procedure

1. The instructions should be followed to prepare all reagents, standard solutions, and samples. It needs to be ensured that all reagents are at room temperature before use since the assay will be performed at room temperature.
2. The number of strips needed for the assay should be determined, and they should be inserted into the frames. Any unused strips should be stored at 4°C for up to a month.
3. A blank well should be set up with no solution.

4. Each negative control well should be added with 50µl of the negative control, and each positive control well should be added with 50µl of the positive control. Next, the sample well should be added with 40µl of sample diluent and 10µl of the sample. They should be mixed well.
5. The plate should be covered with a sealer and incubated for 30 minutes at 37°C.
6. The sealer should be removed and the plate should be washed five times with a wash buffer. For automated washing, all wells should be aspirated and washed five times with wash buffer, overfilling the wells with wash buffer. The wells should be soaked with at least 0.35 ml of wash buffer for 30 seconds to 1 minute for each wash. Then, the plate should be blotted onto paper towels or other absorbent material.
7. Each well (except the blank well) should be added with 50µl of HRP. They need to be covered with a plate sealer and incubated for 30 minutes at 37°C.
8. The sealer should be removed and the plate should be washed as described above.
9. Each well should be added with 50µl of substrate solution A and B. They should be mixed well. Then, the plate should be covered with a new sealer and incubated for 10 minutes at 37°C in the dark.
10. Each well should be added with 50µl of stop solution. The immediate observation will be the blue color changing into yellow.
11. The optical density (OD value) of each well needs to be measured using a microplate reader set to 450 nm within 15 minutes after adding the stop solution.

Calculation of Results

The results obtained from duplicate or triplicate samples should be averaged. To determine the valence of Human Herpes Simplex Virus IgG, the readings from the sample well should be compared with the control.

Quality Control

The average OD ≥1.00

The average OD negative ≤0.10

Results

Cutoff Value = average negative control value + 0.15

While ODsample < Cutoff Value: Negative

While ODsample ≥Cutoff Value: Positive

2.7 Afinion HbA1c Principle assay

The Afinion HbA1c assay is a boronate affinity test that automates the measurement of hemoglobin Alc in human whole blood. The Afinion Analyzer, together with the HbA1c Test Cartridge, has all the necessary reagents for the detection of HbA1c concentration. Using the integrated sampling device, blood samples were collected, and then the test cartridge is placed in the analyzer. The blood sample is automatically diluted and mixed with a solution that releases hemoglobin from red blood cells, causing precipitation. The resulting mixture is exposed to a blue boronic acid conjugate that binds to the cis-diols of glycated hemoglobin. This

mixture is then passed through a filter membrane where all precipitated hemoglobin (conjugate-bound and unbound, including glycated and non-glycated hemoglobin) remains. The excess conjugate is removed using a washing reagent, and the precipitate on the membrane is evaluated by the Afinion Analyzer. By measuring reflectance, the blue (glycated hemoglobin) and red (total hemoglobin) color intensities are assessed, and the ratio between them is proportional to the HbA1c percentage in the sample. The Afinion Analyzer displays the HbA1c concentration in mmol/mol, percentage (%), estimated average glucose (eAG), or any combination thereof.

2.7.1 The assay procedure for HbA1c measurement using the Afinion 2 analyzer from Abbott is as follows:

1. The Afinion 2 analyzer was turned on and waited for it to complete its startup self-test.
2. A capillary or venous blood sample was collected using standard procedures.
3. A test cartridge was inserted into the analyzer, ensuring it was securely seated.
4. 10 µL of the blood sample was added into the sample well of the test cartridge using a pipette.
5. The cartridge cover was closed and the assay was started by pressing the "Run" button on the analyzer.
6. The HbA1c measurement was performed by the Afinion 2 analyzer, and the results were provided in just 3 minutes.
7. The HbA1c result was read from the analyzer screen.
8. The test cartridge was removed and disposed of according to the instructions provided.

9. The Afinion 2 analyzer was turned off.

2.8 *Statistical Analysis*

This study was designed with a completely randomized design (CRD) that was used in the analysis of variance for data on TSH, T3, T4, anti-TSHR, anti-TPO, TSHR, and CD25 values by using a one-way ANOVA test and an independent *t*-test. Moreover, all frequency data was analyzed by Pearson's chi-squared test and Fisher's exact test.

The data were processed and analyzed using the statistical program Social Science (SPSS 22). Also, a prism was used, Moreover, GraphPad PRISM version 7.00 was used for visualization of data. and the results were expressed as Mean±SD or percentages. (McDonald , 2014).

3. Results and Discussion

3.1 Demographic characteristics of the selected groups:

In the present study, it was found that 36 (13.33%) cases of positive HSV-1 with TSH positivity [suppressed TSH levels] pointed to excessive thyroid hormone production (graves' disease). while T_4 and total T_3 can be measured when hyperthyroidism is of high suspicion, as it will improve the accuracy of the diagnosis. T_4, total T_3, or both are elevated, and serum TSH is below normal in Graves' disease. If the graves' disease is mild, only serum T3 may be elevated, and serum TSH can be low or may not be detected in the blood (Ross, *et al*., 2016). were classified as the "G+HSV+" group; 38 (14.07%) patients with a negative TSH but a positive HSV-1 positivity were classified as the "HSV+" group; and 99 (36.67%) patients with a positive TSH but a negative HSV-1 positivity were classified as the "G+" group, while Control: 97 (35.93%) of the total sample size. There was no noticeable difference between the patients and controls in terms of mean age between the third group, "HSV+," and the controls mean± SD (42.9 ± 14.5). Patients with Graves' illness who had HSV-1 had a Mean±SD(42.1 ± 11.6) compared to Mean±SD (45.1 ± 15.1) for those who did not and a control Mean±SD (42.9 ± 14.5).

Table 3-1: Analysis of N, Mean, and SD with Total Student Count and P Value.

Study groups	N	Mean	SD
Control	97	42.9485	14.51032
HSV+	38	42.4211	13.53001
G+	99	45.1313	15.16282
G+HSV+	36	42.1111	11.68828
Total	270	43.5630	14.26051
P value	0.576		

3.1.1 Age distribution of patients

In the present study, the majority of participants were in the 36–60 age group, with 156 participants (57.8%), followed by the ≤35 age group with 81 participants (30.0%), and the ≥61 age group with 33 participants (12.2%). The distribution of study groups within each age group was relatively consistent, with no significant differences observed. Additionally, the p-value of 0.822 suggests that there is no significant association between age and the distribution of study groups.

Table (3-2): Age and HSV-1 Abs Infection Distribution .

			Study groups				
			Control	HSV+	G+	G+HSV+	Total
Age	**≤35**	Count	30	13	27	11	81
		%	30.9%	34.2%	27.3%	30.6%	30.0%
	36-60	Count	55	21	57	23	156
		%	56.7%	55.3%	57.6%	63.9%	57.8%
	≥61	Count	12	4	15	2	33
		%	12.4%	10.5%	15.2%	5.6%	12.2%
Total		Count	97	38	99	36	270
		% of Total	35.9%	14.1%	36.7%	13.3%	100.0%
P value			0.822				

These findings coincide with earlier research on Graves' illness and HSV infection, which likewise found no discernible changes in these disorders' prevalence across various age groups (Babaei *et al*., 2021). Furthermore, research has shown that age may not be a reliable indicator of the onset or severity of Graves' disease or HSV infection (Kahaly *et al*., 2018 ; Zhang *et al*., 2022 ; Vargas-Uricoechea *et al.*, 2023). The study conducted indicated that most individuals diagnosed with Graves' disease were between the ages of 36 and 60 years. Although the disease can affect anyone, the prevalence is highest in

individuals aged 65 and older, which is consistent with Carlé *et al*. (2013) findings. Approximately similar to study of Al-Fatlawy (2014) observed a higher incidence of AITD "GD" in the 36–45 year age groups, which is in line with the current study's results. Elmugadam *et al.* (2014) reported that the onset of Graves' disease occurs between 20 and 58 years, while Hammoodi and Khuder (2015) found that hyperthyroidism tends to advance with age in patients between 40 and 50 years. Al-Bustany (2011) discovered that the average age of Graves' disease cases was approximately 43 years, with the disease being more common in females. Hussain *et al.* (2017) established that Graves' disease typically presents in patients aged 33–56 years, with a median age of 44, and Al-Gazally *et al.* (2013) reported that the age of patients with hyperthyroidism ranged from 15 to 61 years (35.8±9.7), indicating that thyroid disorders can occur at any age. The lower mean age of Iraqi patients with Graves' hyperthyroidism may be due to the shorter life expectancy of Iraqi patients compared to the European population, as well as the cumulative effects of various environmental, psychological, and stress factors. The study's finding of no significant correlation between age and the distribution of study groups suggests that age may not reliably predict the onset or severity of Graves' disease or HSV infection. These results are consistent with prior research and provide valuable insight into the factors that contribute to the development of these conditions.

3.1.2 Sex distribution of patients

The total number of participants in this study was 270, based on sex groups, i.e., female and male. with 180 (66.7%) females and 90 (33.3%) males. Among females, the highest percentage of participants belonged to the control group (70.1%), followed by the Graves+ (63.6%), Graves+HSV-1+ (69.4%), and HSV-

1+ (63.2%) groups. Among males, the highest percentage of participants belonged to the Graves+ (36.4%) group, followed by HSV-1+ (36.8%), Graves+HSV-1+ (30.6%), and control (29.9%) groups. The overall distribution of participants across study groups was 35.9% for control, 14.1% for HSV-1+, 36.7% for Graves+, and 13.3% for Graves+HSV-1+ groups.

Table (3-3) Distribution of the study sex groups (female and male).

			Study groups				
			Control	HSV+	G+	G+HSV+	Total
SEX	Female	Count	68	24	63	25	180
		%	70.1%	63.2%	63.6%	69.4%	66.7%
	Male	Count	29	14	36	11	90
		%	29.9%	36.8%	36.4%	30.6%	33.3%
Total		Count	97	38	99	36	270
		%	35.9%	14.1%	36.7%	13.3%	100.0%
P value			0.739				

The table (3–3) presents the distribution of study groups, including control, HSV-1+, Graves+, and Graves+HSV-1+. Thyroid dysfunctions are a prevalent global health issue, particularly affecting females. Various factors such as positive family history, smoking, infection, and iodine exposure increase the incidence of Graves' disease, which is more common in women than men (Wémeau *et al*., 2018). In Erbil Governorate, females were found to be approximately four times more affected than males (Al-Bustany, 2011). A survey conducted in Baquba-Diyala showed that out of 149 patients with hyperthyroidism, 125 were female and 24 were male.

The imbalance of sex hormones like estrogen, which tends to rise in females during puberty and pregnancy, as well as the impact of the X chromosome on the

immune system and thyroid function, may be to blame for the gendered differences in thyroid disease prevalence (Kjaergaard *et al.,* 2021). Hammoodi and Khudur (2015) did a study in the city of Baghdad. Hyperthyroidism is less prevalent in males, with only 31% of the sample affected, while females are more susceptible, with a prevalence rate of 69%, and 69% of them had a family history of the disease. Additionally, Kadhum and Hassan (2014) reported a higher incidence ratio in females than males across all age groups. Retrospective studies by Diker-Cohen *et al*. (2019) also confirmed that Graves' disease is more prevalent among women than men, with a frequency of 2.1:1. These findings suggest that sex hormones and genetic factors may contribute to the sex discrepancy in thyroid disease prevalence.

The female predominance in thyroid disease (Gessl *et al.*, 2012; Al-Gazally *et al.,* 2013; Hussain *et al*., 2017). In Hussain *et al*. (2017) study, females accounted for 79.8% of patients, with a crude ratio of 4:1 female to male. With a mean female-to-male ratio of 2:1, the gender distribution of GD patients in our study is comparable with other studies done in Iraq (Al-Gazally *et al.,* 2013). Both the majority of the hyperthyroidism patients and the control subjects in this study were female. To prevent any potential bias in the results caused by gender differences, the genders were statistically matched. The majority of thyroid illness research studies, such as Ladenson *et al.* (2000), González-Rodrguez *et al.* (2013), Olmos *et al.* (2015), Diab *et al.* (2019) and Chiovato *et al*. (2019) support this strategy.

3.1.3 Smoking distribution among patients

In the current study, according to smoking status, The data show that out of the total 270 participants, 65 were smokers (24.1%) and 205 were non-smokers (75.9%). Among smokers, the highest proportion was observed in the Graves+ (42.4%), followed by Graves+ group with HSV-1+ (38.9%), the HSV-1+ (7.9%)

and groups Control (6.2%) . Among non-smokers, the highest proportion was observed in the Control group (93.8%) followed by HSV-1+ (92.1%), the Graves+ with HSV-1+ (61.1%), and Graves+ group (57.6%).

The p-value of <0.001* indicates a statistically significant difference in smoking status among the study groups. This suggests that smoking may be associated with the development of Graves' disease and the presence of HSV-1 infection, as the highest proportion of smokers was observed in the Graves+ and Graves+ with HSV-1+ groups.

Table (3-4) Distribution of individuals based on their smoking status.

			Study groups				
			Control	HSV+	G+	G+HSV+	Total
Smoking	YES	Count	6	3	42	14	65
		%	6.2%	7.9%	42.4%	38.9%	24.1%
	NO	Count	91	35	57	22	205
		%	93.8%	92.1%	57.6%	61.1%	75.9%
Total		Count	97	38	99	36	270
		% within Smoking	35.9%	14.1%	36.7%	13.3%	100.0%
P value			<0.001*				

Cigarette smoking is a recognized risk factor for many chronic systemic diseases with inflammatory components, such as atherosclerosis, Crohn’s disease, rheumatoid arthritis, psoriasis, Graves’ ophthalmopathy (Sopori *et al*., 2002; Stampfli *et al*., 2009).Have confirmed a significant influence of smoking on Graves' hyperthyroidism and particularly on Graves' Here, smoking may increase the risk of disease development, reduce the effectiveness of treatment, and eventually induce relapse. The role of smoking in Hashimoto's thyroiditis is not as

well established as in Graves' disease. (Sawicka-Gutaj *et al.,* 2014). Smoking is a dose-dependent risk factor for Graves' hyperthyroidism. (Wiersinga *et al.,* 2013)

Smoking has been widely accepted as an important risk factor, and cigarette smoking cessation has been shown to improve the outcome and decrease the onset of GD. Despite many efforts to reduce its prevalence, approximately six million people worldwide die due to tobacco use each year (WHO, 2015).

The interaction between the immune system and herpes virus infections is crucial in determining the outcome of such infections. Smoking is a well-known environmental factor that can affect immune function by interfering with various immune pathways, but its impact on the incidence of herpes virus infections has not been thoroughly studied (Qiu *et al.,* 2017). This is particularly worrisome for individuals with severe psychiatric illnesses, who have higher rates of smoking and altered immune systems (Severance *et al.,* 2018). In previous research on individuals with schizophrenia and other severe psychiatric illnesses, shown that exposure to herpes viruses, such as Herpes Simplex Virus type 1 (HSV-1), Cytomegalovirus (CMV), and Epstein Barr Virus (EBV), can be linked to increased cognitive impairments and mortality rates, as evidenced by higher levels of virus-specific IgG antibodies in the blood (Dickerson *et al.,* 2003; Shirts *et al.,* 2008; Dickerson *et al.,* 2014). However, in the current study, found no significant difference in the prevalence of HSV-1 infection between smoking and non-smoking groups.

3.2 Hormone Study:

3.2.1 Evaluation of serum levels of TSH, T4, and T3

Tables 3-5 present a comparison of the mean levels of thyroid hormones, specifically TSH, T3, and T4, among four study groups: control, HSV+, Graves+, and Graves+HSV+. The results show that the mean TSH levels were not significantly different between the control and HSV+ groups (2.7±1.2 nmol/l vs. 2.8±1.3 nmol/l, respectively). However, the mean TSH levels in the Graves+ and Graves+HSV+ groups were significantly lower (0.05±0.02 nmol/l and 0.04±0.011 nmol/l, respectively) than those in the Control and HSV+ groups ($p<0.001$).

In terms of T_3 levels, the mean values were similar between the control and HSV+ groups (2.01±0.4 nmol/l and 2.1±0.4 nmol/l, respectively). However, the mean T3 levels in the Graves+ and Graves+HSV+ groups were significantly higher (6.56±1.8 nmol/l and 6.3±1.7 nmol/l, respectively) compared to those in the Control and HSV+ groups ($p<0.001$).

Similarly, the mean T_4 levels were not significantly different between the control and HSV+ groups (116.9±31.6 nmol/l and 116±31.4 nmol/l, respectively). However, the mean T_4 levels in the Graves+ and Graves+HSV+ groups were significantly higher (617.3±217.8 nmol/l and 592.54±139 nmol/l, respectively) compared to those in the Control and HSV+ groups ($p<0.001$).

These results indicate that the mean TSH levels are significantly lower, while the mean T_3 and T_4 levels are significantly higher in patients with Graves' disease and/or HSV infection compared to the control group. These findings suggest that both Graves' disease and HSV infection have a significant impact on thyroid

hormone levels. The mean values and standard deviations (SD) provide a measure of the central tendency and variability of the data, respectively, within each group.

Table (3-5) Comparison of the mean levels of thyroid hormones, specifically TSH, T3, and T4, among several study groups.

Parameter	Control	HSV+	G+	G+HSV+	P value
TSH(nmol/l)	2.7±1.2	2.8±1.3	0.05±0.02	0.04±0.011	<0.001*
T3(nmol/l)	2.01±0.4	2.1±0.4	6.56±1.8	6.3±1.7	<0.001*
T4(nmol/l)	116.9±31.6	116±31.4	617.3±217.8	592.54±139	<0.001*
N	97	38	99	36	

* represents a significant difference at $p<0.05$.

The study measured the serum levels of T_3, T_4, and TSH to establish how well thyroid hormones were functioning in the control and hyperthyroid groups. Serum T_3, T_4, and TSH levels were all within the normal range in the control group. In contrast, the hyperthyroid group displayed low TSH and high serum T_3 and T_4 levels, indicating that primary hyperthyroidism was the primary cause of the majority of cases. This is a sign of a thyroid disorder since it causes excessive T_3 and T_4 synthesis and inhibits the pituitary gland's ability to release TSH (Alina, 2019).

In primary hyperthyroidism, excessive production of thyroid hormones T_3 and T4 leads to the suppression of TSH secretion from the anterior pituitary through negative feedback inhibition. On the other hand, in secondary hyperthyroidism, excessive production of TSH by the anterior pituitary stimulates the thyroid follicular cells to secrete an excessive amount of thyroid hormones. The underlying cause of this excess production of T_3 and T_4, despite low levels of TSH, is believed to be the production of autoantibodies by immune-competent plasma cells. These autoantibodies bind to the TSH receptor (TSHR), triggering and enhancing the synthesis and production of T_3 and T_4, overriding the negative feedback

mechanism exerted by T_3 and T_4 on the pituitary and hypothalamic axes. (Pirahanchi *et al*., 2018).

Based on the results, the TSH levels of the control and HSV-1+ groups are comparable, while the G+ and G+HSV-1+ groups have significantly lower TSH levels. This finding is consistent with previous research linking Graves' disease to reduced TSH levels due to increased production of thyroid hormones. (Zoeller and Rovet, 2004). According to Diana *et al.* (2018), a suppressed (TSH) test was necessary to determine whether a patient had hyperthyroidism.

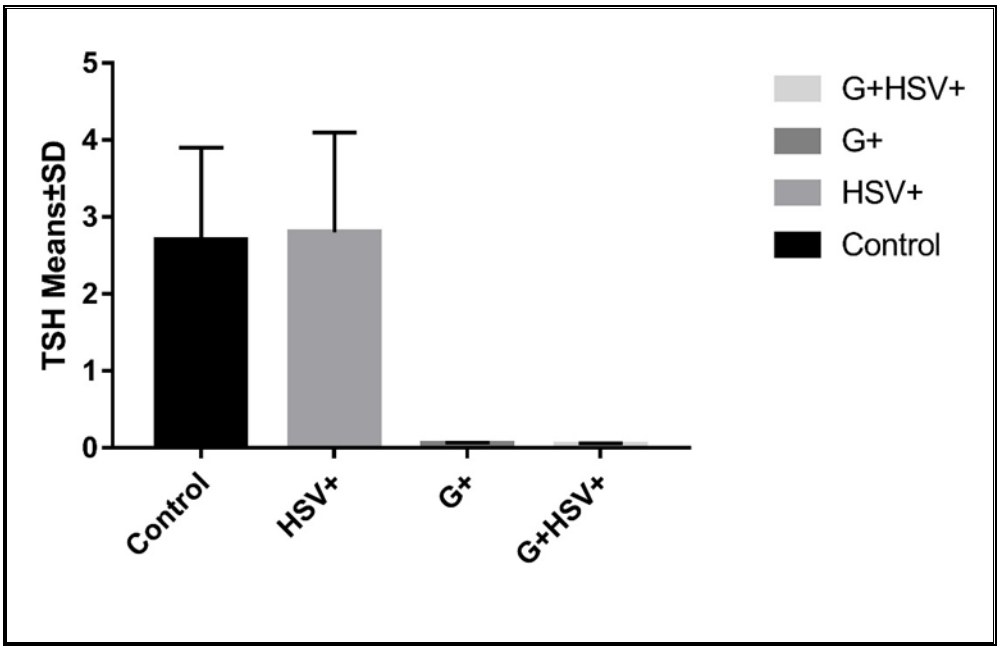


Figure 3-1: TSH level in serum among different study groups. Control, HSV+, Graves+, and Graves+ HSV+.

The results indicate a significant increase in T_3 levels in the G+ and G+HSV-1+ groups compared to the control and HSV-1+ groups. This finding is consistent with previous studies that have reported elevated T_3 levels in individuals with Graves' disease. (Karmisholt *et al.*, 2012). Confirmation of hyperthyroidism can be

achieved by the detection of T_3 (FT_3) in the presence of elevated levels of free T_4. (Koulouri *et al*., 2013)

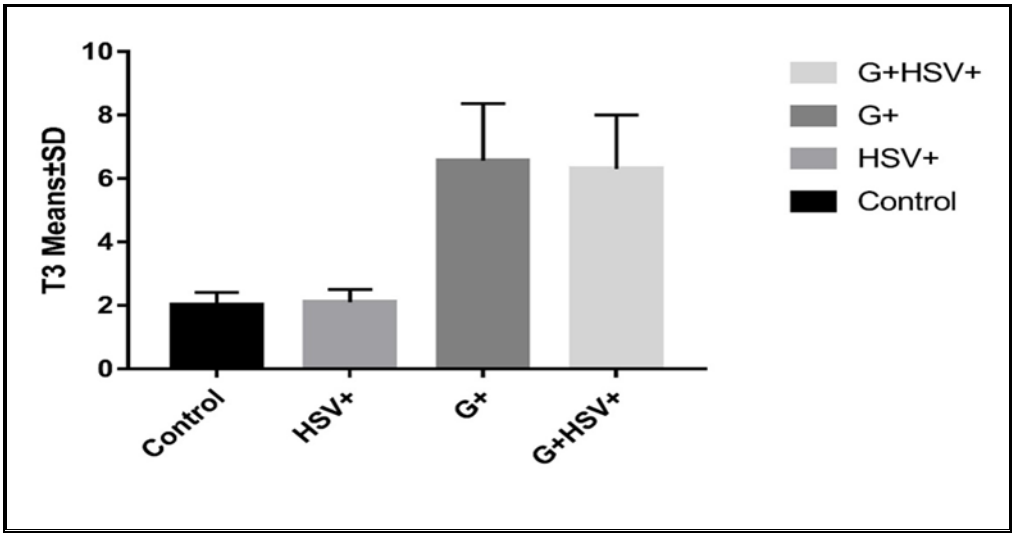


Figure (3-2): T_3 level in serum among different study groups. Control, HSV+, Graves+, and Graves+ HSV+.

The results demonstrate that the levels of T_4 are significantly higher in the G+ and G+HSV-1+ groups compared to the control and HSV-1+ groups. This observation is in line with the hypothesis that Graves' disease is an autoimmune disorder that stimulates excessive production of thyroid hormones, leading to hyperthyroidism. (Ross and Cooper, 2015). The level of T_4, which serves as a marker for the degree of thyrotoxicosis, is also associated with Th1/Th2 and proinflammatory cytokines in individuals with Graves' disease. (Shen *et al.*, 2015). According to Hüser *et al*. (2018), fluctuations in TSH, T_4, and T_3 levels are goiter indicators.

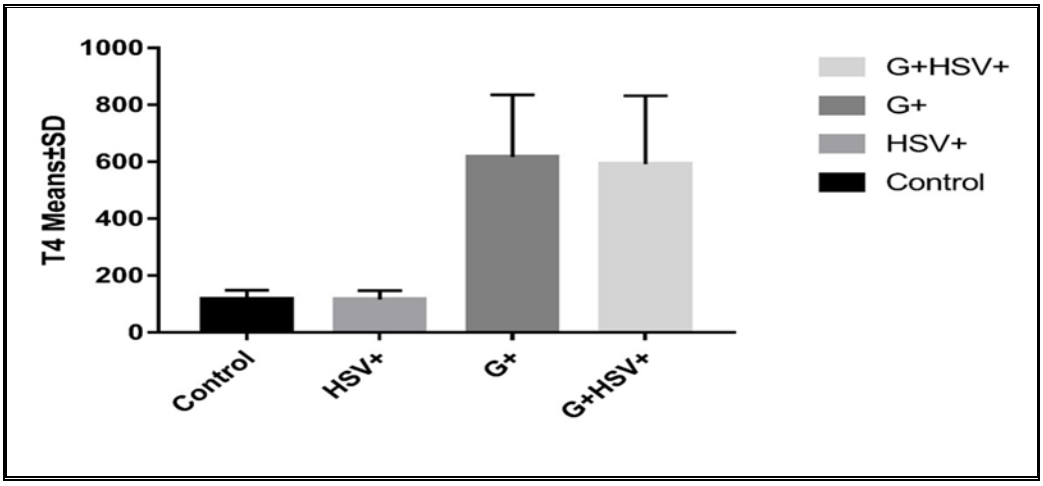


Figure 3–3: T_4 level in serum among different study groups. Control, HSV+, Graves+, and Graves+ HSV+.

As stated by Carlé (2013), a total T_3/T_4 ratio and TSH levels are useful criteria to quickly distinguish Graves' disease. Graves' disease is the most typical cause of hyperthyroidism, according to Shahid *et al.* (2018), increased efficiency of the thyroid gland tissue leads to hyperactivity in the synthesis of T3 and T4 hormones, resulting in hyperthyroidism. Although TSH, T3, and T4 hormones offer valuable insights, serum TSH levels remain the most reliable indicators of thyroid function due to the logarithmic/linear relationship between TSH and FT4. Any deviations from the genetically predetermined FT4 set point are noticed by the pituitary gland, leading to the initial diagnosis of thyroid function problems. Hyperthyroidism can arise from disorders of the thyroid gland, which cause excess T3 and T4 production alongside a compensatory decrease in TSH. Additionally, thyrotroph adenoma can contribute to unregulated TSH production, leading to increased T3 and T4 levels. Some conditions may also involve ectopic production of thyroid hormone, resulting in elevated thyroid hormones and a compensatory decrease in TSH levels..

The need for adequate reference ranges for TSH to identify mild hypothyroidism or hyperthyroidism stems from the fact that modest fluctuations in T_4 levels can result in considerable variations in serum TSH levels. Accordingly, Croker *et al.* (2021) highlighted that TSH measurement is typically preferable when choosing the hormone assay.

3.2.2 Evaluation of The Serum Levels of Anti-TG-Abs , Anti-TPO-Abs, And Anti-TSHR-Abs

Tables (3-6) present the mean levels of three types of thyroid hormone antibodies, including anti-TSHR, anti-TPO, and anti-TG, among different study groups, including control, HSV+, Graves+, and Graves+HSV+. The results showed that the mean levels of all three thyroid hormone antibodies were significantly different among the study groups.

In the case of anti-TSHR, the mean level was the highest in the Graves+ and Graves+HSV+ groups (5.73±2.4 IU/ml and 5.52±2.5 IU/ml, respectively), which were significantly higher than the control and HSV+ groups (0.84±0.18 IU/ml and 0.88±0.12 IU/ml, respectively). This finding is consistent with previous study that suggest that the production of anti-TSHR antibodies is a hallmark of Graves' disease(Diana *et al*., 2021).

Similarly, the mean levels of anti-TPO and anti-TG antibodies were also significantly higher in the Graves+ and Graves+HSV+ groups compared to the control and HSV+ groups. Specifically, the mean level of anti-TPO was 78.2±18.3 IU/ml and 74.4±15.4 IU/ml in the Graves+ and Graves+HSV+ groups, respectively, which were significantly higher than the control and HSV+ groups (35.7±8.15 IU/ml and 55.04±8.24 IU/ml, respectively). Likewise, the mean level of

anti-TG was 189.5±43.9 IU/ml and 338±48.5 IU/ml in the Graves+ and Graves+HSV+ groups, respectively, which were significantly higher than the control and HSV+ groups (62.2±17 IU/ml and 57.20±9.7 IU/ml, respectively).

These results indicate that the production of thyroid hormone antibodies, including anti-TSHR, anti-TPO, and anti-TG, is significantly higher in Graves' disease patients compared to healthy controls and those with only HSV infection. This finding suggests that the presence of these antibodies can serve as diagnostic markers for Graves' disease.

Table (3-6) display a comparison of the mean levels of thyroid hormone antibodies, specifically anti-TSHR, anti-TPO, and anti-TG. Among different study groups. Control, HSV+, Graves+, and Graves+ HSV+.

Parameter	Control	HSV+	G+	G+HSV+	P value
Anti-TSHR(IU/ml)	0.84±0.18	0.88±0.12	5.73±2.4	5.52±2.5	<0.001*
Anti-TPO(IU/ml)	35.7±8.15	55.04±8.24	78.2±18.3	74.4±15.4	<0.001*
Anti-TG(IU/ml)	62.2±17	57.20±9.7	189.5±43.9	338±48.5	<0.001*
N	97	38	99	36	

* represents a significant difference at p<0.05. Anti-TSH-R =anti-Thyroid Stimulating Hormone Receptor (TSH-R), Anti-TPO = anti-Thyroid Peroxidase (TPO), and Anti-TG = anti-Thyroglobulin were measured by using a one-way ANOVA test and an independent *t-test*, and the results were expressed as Mean±SD or percentages.

Graves' disease (GD) is an autoimmune disease, and it accounts for major cases of hyperthyroidism. Antibody against thyroid-stimulating hormone receptor (TSHR) (TRAb) is responsible for hyperthyroidism and is considered a diagnostic marker for GD (Zulkarnain *et al.*, 2022). Graves' disease (GD) affects nearly 0.5% of the general population, with a higher incidence among females relative to males. (Struja *et al.*, 2019; Zuhur *et al.*, 2021). Clinically, GD is characterized by the suppression of TSH levels, overstimulation of thyroid hormones, and the

production of antithyroid antibodies. (Liu *et al.*, 2008; Struja *et al.*, 2019) It is now well established that thyroid-stimulating hormone receptor autoantibodies According to earlier research (Fröhlich *et al.* 2017), the anti-TSHR antibody is high in Graves' disease, an autoimmune condition that results in hyperthyroidism. This conclusion is in line with that research. Fawzi *et al.* (2018) .The serological hallmark of GD is Graves' hyperthyroidism disease (GD), an autoimmune condition in which antibodies against the thyroid stimulating hormone receptor (TSHR) cause thyroid (TRAb). (Roggenbuck *et al.*, 2018), which is usually helpful in differentiating GD from other causes of hyperthyroidism. Additionally, the role of TRAb is not only in confirming GD diagnosis but also has potential for predicting the clinical course of GD, relapse risk, and treatment responses. (Liu *et al*., 2008; Barbesino *et al*., 2013; Struja *et al*., 2019)

Graves' hyperthyroidism, a condition in which an excessive amount of thyroid hormone is produced due to the presence of antibodies that target the thyroid stimulating hormone receptor (TSHR), has been extensively studied by Lane *et al.* (2020) and Bogusławska *et al.* (2022). The TRAB assay, a diagnostic test that detects TSHR autoantibodies, is a crucial tool in identifying Graves' disease when clinical features are inconclusive and is considered a dependable diagnostic marker, as stated by Bell *et al.* (2018).

Inaba *et al*. (2016) have further explored the mechanism behind Graves' disease, explaining how TSH binding to TSHR initiates signal transduction pathways. Among the various autoantibodies, TRAb is considered the most critical for inducing hyperthyroidism. Additionally, Kochman *et al*. (2021) have discovered a correlation between TSAb and oxidative stress in individuals with Graves' disease.

The effects of autoimmune stimulation of the thyroid stimulating hormone receptor on thyroid hormone production can significantly impact one's well-being, as highlighted by Cole *et al.* (2019). Managing Graves' hyperthyroidism, particularly in young patients, poses a challenge due to the more severe side effects of conventional treatments in this age group. Furthermore, the disease is unlikely to resolve spontaneously in the short to medium term, exacerbating the difficulty of managing this condition.

These findings suggest that there is a positive correlation between Graves' disease and elevated anti-TSHR levels, which is consistent with previous studies (Iitaka *et al*., 2004; Bahn, 2015). The presence of viral infections, such as HSV-1, has been shown to trigger or exacerbate autoimmune responses in individuals with autoimmune diseases, including Graves' disease (Wu *et al.*, 2012). however, the exact etiology of several autoimmune diseases is still unknown. Viruses have long been regarded as an important environmental trigger for autoimmune diseases in genetically predisposed patients (Arleevskaya *et al*.,2017 ; Lerner *et al*.,2017). They might activate some immunological responses through self-tolerance breakdown, which might overcome the immune regulating systems and induce autoimmune reactions. The most important known mechanisms in developing virus-induced autoimmunity are molecular mimicry between host self-antigens and microbial antigens, epitope spreading, bystander activation, and immortalizing infected B cells. Molecular mimicry plays a critical mechanism responsible for viruses-induced autoimmune disease. Conventionally, molecular mimicry applies to the similarity of antigens between viruses and self-antigens that can be recognized by immune systems and result in cross-reaction to self-antigens and viral antigens. The epitope spreading is another main mechanism responsible for the viruses-induced immune reaction in which viral infection results in more

discharge of self-antigens and novel autoreactive cells that subsequently target spared self-antigens (Klase *et al*.,2016).

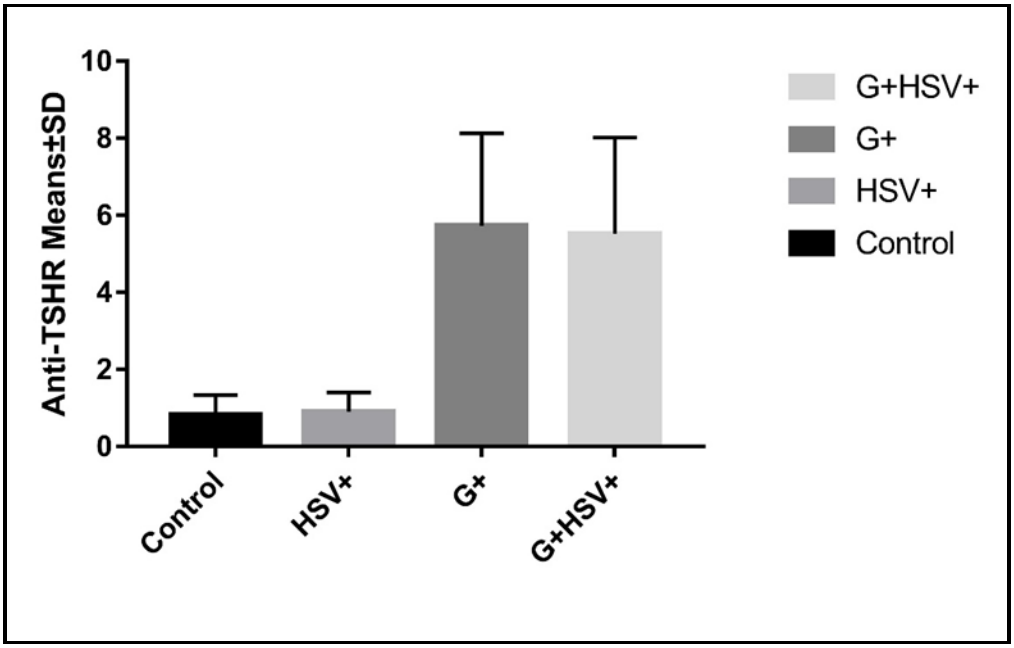


Figure (3-4) levels of anti-TSH-R in the serum among different study groups. Control, HSV+, Graves+, and Graves+ HSV+.

Similar to Anti-TPO, the mean values in the Graves+ (78.2±18.3) and Graves+HSV-1+ (74.4±15.4) groups were substantially higher than those in the control (35.7±8.15)and HSV-1+ (55.04±8.24) groups ($p<0.001$). The major cause of hyperthyroidism, autoimmune thyroid disease, is correlated with a rise in anti-TPO antibodies (Siriwardhane *et al.,* 2019).

Previous studies have also reported a significant association between the presence of anti-TPO and autoimmune thyroid diseases such as Graves' disease (Prummel , 2004 ; Effraimidis *et al.,* 2014). In a study by Kahaly *et al.* (2016), the prevalence of anti-TPO was significantly higher in patients with Graves' disease compared to healthy controls.

It is believed that anti-TPO antibodies play a crucial role in the pathogenesis of autoimmune thyroid diseases such as Graves' disease by damaging thyroid cells and leading to thyroid dysfunction (Mazokopakis, *et al.*, 2007). Therefore, the higher levels of anti-TPO observed in the Graves+ and Graves+HSV-1+ groups in the present study may indicate a greater risk of thyroid dysfunction in these individuals.

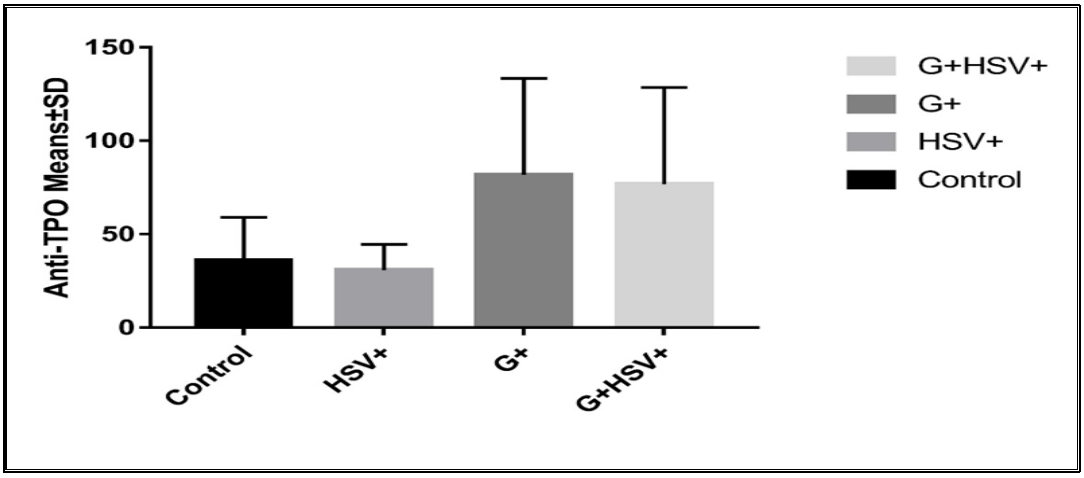


Figure (3-5): anti-TPO level in serum among different study groups. Control, HSV+, Graves+, and Graves+ HSV+.

The present study investigated the levels of anti-thyroglobulin (anti-TG) antibodies in different groups, including control, HSV-1+, Graves+, and Graves+HSV-1+. The results showed that there was a significant difference in the mean values of anti-TG antibodies among the four groups ($p < 0.001$). Specifically, the mean value of anti-TG antibodies was found to be higher in the Graves+ group (189.5±43.9) compared to the control (62.2±17) and HSV-1+ groups (57.20±9.7). Furthermore, the mean value of anti-TG antibodies was found to be highest in the Graves+HSV-1+ group (338±48.5) among all groups. These findings are consistent with previous studies that have reported a higher prevalence of anti-TG antibodies

in patients with autoimmune thyroid diseases, particularly Graves' disease (Brix *et al.,* 2000; Marwaha , 2012).

Several studies have suggested that viral infections, such as HSV-1, may trigger or exacerbate autoimmune thyroid diseases by stimulating the immune system. A study by Desailloud and Hober (2009) reported that viral infections are one of the environmental factors that can trigger autoimmune thyroid diseases.

The present study highlights the importance of monitoring anti-TG antibody levels in patients with Graves' disease and considering the potential role of viral infections in the development and progression of autoimmune thyroid diseases.

In comparison to the control (62.2±17) and HSV-1+ (57.20±9.7) groups, the mean values of anti-TG were markedly higher in the Graves+ (189.5±43.9) and Graves+HSV-1+ (338±48.5) groups ($p<0.001$). This result is in line with other research that showed higher anti-TG levels in autoimmune thyroid illnesses such as Graves' disease (Khamisi *et al*., 2021).

particularly in Graves' disease patients. A similar finding was reported by a study conducted by Desailloud and Hober (2009), which revealed a significant correlation between HSV-1 infection and the development of AIT.

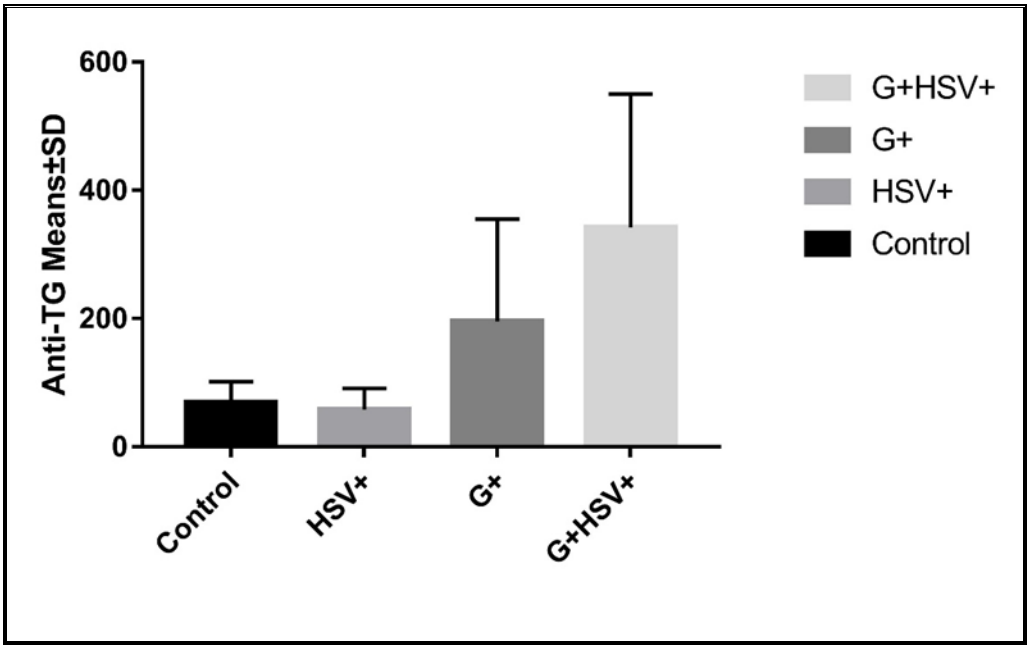


Figure (3-6): anti-TG level in serum among different study groups. Control, HSV+, Graves+, and Graves+ HSV+.

3.3. Immunological Study:

3.3.1 Evaluation of serum levels of CD25 and TSHR in patients and healthy groups

T-cell activation is indicated by CD25, and TSHR is a thyroid-stimulating hormone receptor. the results of a study comparing the mean levels of CD25 and TSHR in different study groups, which include control, HSV+, Graves+, and Graves+HSV+.

The results show that the mean levels of CD25 were significantly different among the study groups, with the highest levels observed in the G+HSV+ group (27.25±9.5 ng/ml) and the lowest levels in the HSV+ group (5.46±0.93 ng/ml). The control group had a mean CD25 level of 7.34±1.5 ng/ml, while the Graves+ group had a mean level of 15.16±6.5 ng/ml.

Similarly, the mean levels of TSHR were also significantly different among the study groups, with the highest levels observed in the Graves+HSV+ group (14.46±6.5 ng/ml) and the lowest levels in the Control and HSV+ groups (10.07±3.09 ng/ml and 10.09±3.2 ng/ml, respectively). The Graves+ group had a mean TSHR level of 11.40±4.7 ng/ml.

Table (3-7): Comparison of CD25 and TSHR means among study groups (control, HSV+, Graves+, and Graves+ HSV+)

Parameter	Control	HSV+	G+	G+HSV+	P value
CD25 ng/ml	**7.34±1.5**	**5.46±0.93**	**15.16±6.5**	**27.25±9.5**	**<0.001***
TSHR ng/ml	**10.07±3.09**	**10.09±3.2**	**11.40±4.7**	**14.46±6.5**	**<0.001***
N	**97**	**38**	**99**	**36**	

* represents a significant difference at $p<0.05$. **CD25 =** Cluster of Differentiation, and **TSHR** =Thyroid Stimulating Hormone Receptor were measured by using a one-way ANOVA test and an independent *t-test*, and the results were expressed as Mean±SD or percentages.

The findings of the study are consistent with previous research on the relationship between CD25 and Graves' disease. Several studies have found that Graves' disease patients have higher levels of CD25 than healthy controls (Furmaniak *et al.,* 2012; French *et al.,* 2017). The current study adds to the body of knowledge by demonstrating that the presence of HSV infection in Graves' disease patients increases CD25 expression even more.

The percentage distribution of CD4+, CD4+CD25+, and CD4+ CD25+ CD127+ regulatory T cells was similar in active GD patients and control subjects. The number of CD25+ and CD4+ CD25+ CD127 cells was similar in GD patients and control subjects (Pan *et al.,* 2009).

Elevated CD25 levels in Graves' disease patients may reveal an imbalance in the regulatory T cell population, which results in the onset of autoimmunity. (Dejaco *et al.,* 2006)

Studies have shown that CD4+CD25+ Tregs are significant in various autoimmune diseases (Bacchetta *et al.,* 2005), and abnormalities in CD4+CD25+ Tregs may contribute to the occurrence and progression of hyperthyroidism (Esfahanian *et al.,* 2013).

Studies have demonstrated that the removal of CD4+CD25+ Tregs in mice made them more prone to hyperthyroidism, indicating that these Tregs play a crucial role in regulating immune responses (Saitoh *et al*., 2006; Nakano *et al.,* 2007). However, the exact mechanism by which CD4+CD25+ Tregs regulate hyperthyroidism is not yet fully understood, and previous studies have primarily focused on investigating changes in the percentage of CD4+CD25+ Tregs in the PB of patients with hyperthyroidism. Some studies have suggested that the percentage of CD4+CD25+ Tregs is decreased in patients with hyperthyroidism (Hansen *et al*., 2023), while others have found no significant alterations in the percentage of these Tregs (Chen *et al.,* 2014).

Another study by (Jiang *et al*.,2018) The current study found that CD4+CD25+ Treg function was reduced in hyperthyroid patients and that this non-proportional decrease may be linked to the beginning and development of hyperthyroidism.

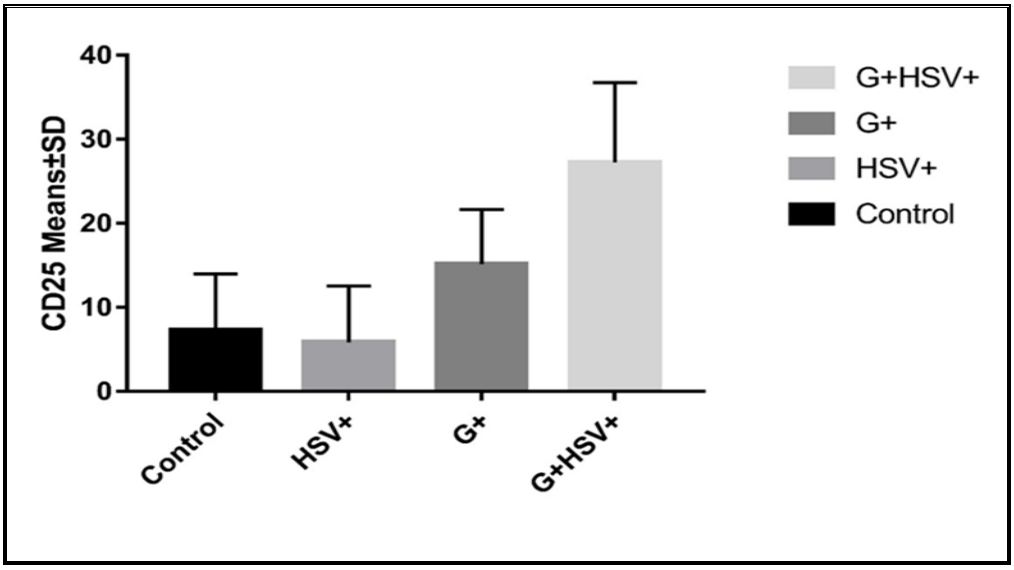


Figure 3–7: CD25 level in serum among different study groups. Control, HSV+, Graves+, and Graves+ HSV+.

Similarly, there is a well-established link between TSHR and Graves' disease (Rapoport *et al.*, 2018). TSHR antibodies are a defining feature of Graves' disease and play an important role in thyroid hyperactivity. The current study confirms that TSHR expression is higher in Graves' disease patients than in healthy controls, and it also shows that HSV infection increases TSHR expression. Woo *et al.* (2015) discovered that TSHR levels were significantly higher in Graves' disease patients compared to controls. TSHR, on the other hand, is the autoantigen in Graves' disease, and raised TSHR levels may reflect disease severity (Smith, 2016).

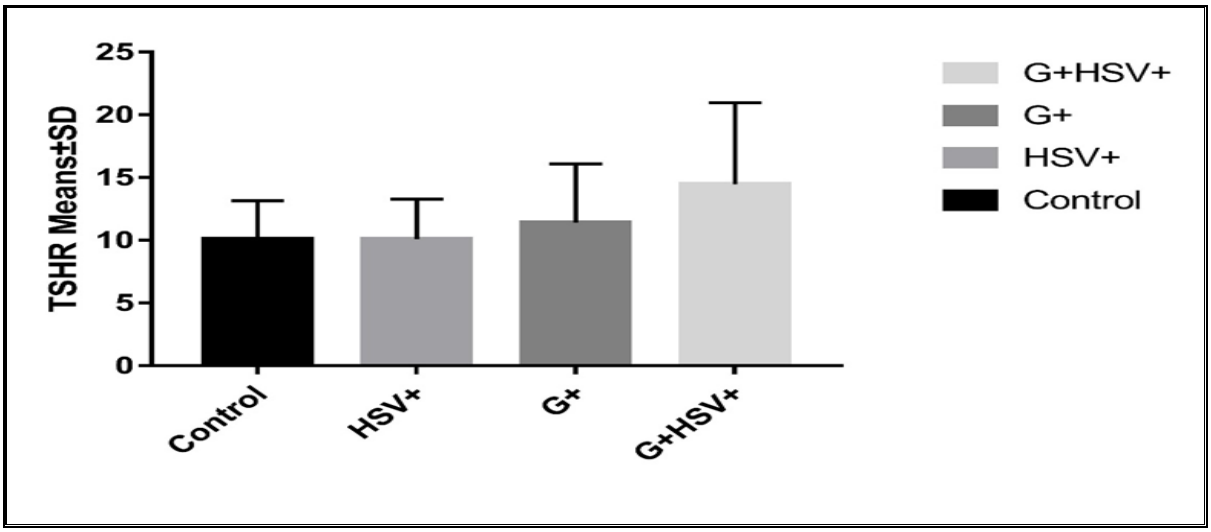


Figure 3–8: TSHR level in serum among different study groups. Control, HSV+, Graves+, and Graves+ HSV+.

The current study shows that Graves' disease patients have higher levels of CD25 and TSHR expression than healthy controls and that HSV infection exacerbates this effect. These results correspond to previous research and support the role of CD25 and TSHR in Graves' disease pathogenesis. Some limitations of the study include the cross-sectional design and a lack of information on the duration of HSV infection in the study groups. More research is required to confirm these results and investigate the underlying mechanisms.

3.3.2 Evaluation of serum levels of HbA1c in patients and healthy groups

The table shows the mean values of HbA1c levels in four study groups (control, HSV-1+, Graves+, and Graves+HSV-1+) and the P value that indicates the statistical significance of differences among groups.

The results show that the mean HbA1c levels are significantly different among the four study groups ($P<0.001$). The control group has the lowest mean HbA1c

level of 6.5±0.73, while the Graves+HSV-1+ group has the highest mean HbA1c level of 7.70±1.19. The Graves+ group has a higher mean HbA1c level of 7.77±1.008 compared to the other two groups, while the HSV-1+ group has a mean HbA1C level of 6.72±0.81.

Table 3–8: Comparison of HbA1c means among study groups (control, HSV+, Graves+, and Graves+ HSV+)

Parameter	Control	HSV+	G+	G+HSV+	P value
HbA1c	6.5±0.73	6.72±0.81	7.77±1.008	7.70±1.19	<0.001*
N	97	38	99	36	

* represents a significant difference at p<0.05.

1. Control group: The mean HbA1c level in the control group was 6.5±0.73, which is within the normal range for HbA1c levels (less than 6.5%) recommended by the American Diabetes Association (ADA) for diagnosing diabetes (American Diabetes Association, 2021). This suggests that the control group did not have diabetes or impaired glucose tolerance.
2. HSV-1+ group: The mean HbA1c level in the HSV-1+ group was 6.72±0.81, which is slightly higher than the control group but still within the normal range. Several studies have suggested that HSV-1 infection may affect glucose metabolism and increase the risk of developing diabetes (Antrim,2022). However, more studies are needed to confirm this association.
3. Graves' disease group: The mean HbA1c level in the Graves+ group was 7.77±1.008, which is significantly higher than the control and HSV-1+ groups (P<0.001). Graves' disease is an autoimmune disorder that affects the thyroid gland and is associated with an increased risk of glucose intolerance and diabetes (Duntas, 2018; Ogbonna, 2019). This finding is consistent with previous studies that have reported higher HbA1c levels in Graves' disease patients compared to

healthy controls (Kalra *et al.,* 2019). Poor glycemic regulation, including hyperglycemia and insulinopenia, is linked to excessive thyroid hormones circulating in hyperthyroidism. A little more than 2% to 3% of healthy individuals who acquire hyperthyroidism also develop overt diabetes (Ray *et al.,* 2016). According to Ray *et al.* (2016), about 50% of people with Graves' illness have some degree of glucose intolerance. Improved glycaemic management is shown in diabetic people with hyperthyroidism. Diabetes problems, including diabetic ketoacidosis, can be triggered by thyroid dysfunction (Hage *et al.*, 2011).

4. Graves' disease and HSV-1 co-infection group: The mean HbA1C level in the Graves+HSV-1+ group was 7.70±1.19, which is similar to the Graves+ group and significantly higher than the control and HSV-1+ groups ($P<0.001$). This suggests that the co-infection of Graves' disease and HSV-1 may have a synergistic effect on glucose metabolism and increase the risk of developing diabetes. However, more studies are needed to confirm this finding. According to Ray, and Ghosh, (2016), almost half of the individuals diagnosed with Graves' disease have some level of glucose intolerance. Additionally, hyperthyroidism in diabetic patients can lead to poorer control of blood sugar levels. Hage *et al.* (2011). also suggests that thyrotoxicosis can trigger diabetic complications, such as diabetic ketoacidosis.

The results of this study shows that Graves' disease and HSV-1 infection are associated with higher HbA1c levels, which may indicate impaired glucose metabolism and an increased risk of developing diabetes. These findings highlight the importance of monitoring glucose metabolism in patients with Graves' disease and HSV-1 infection and implementing appropriate interventions to prevent or manage diabetes.

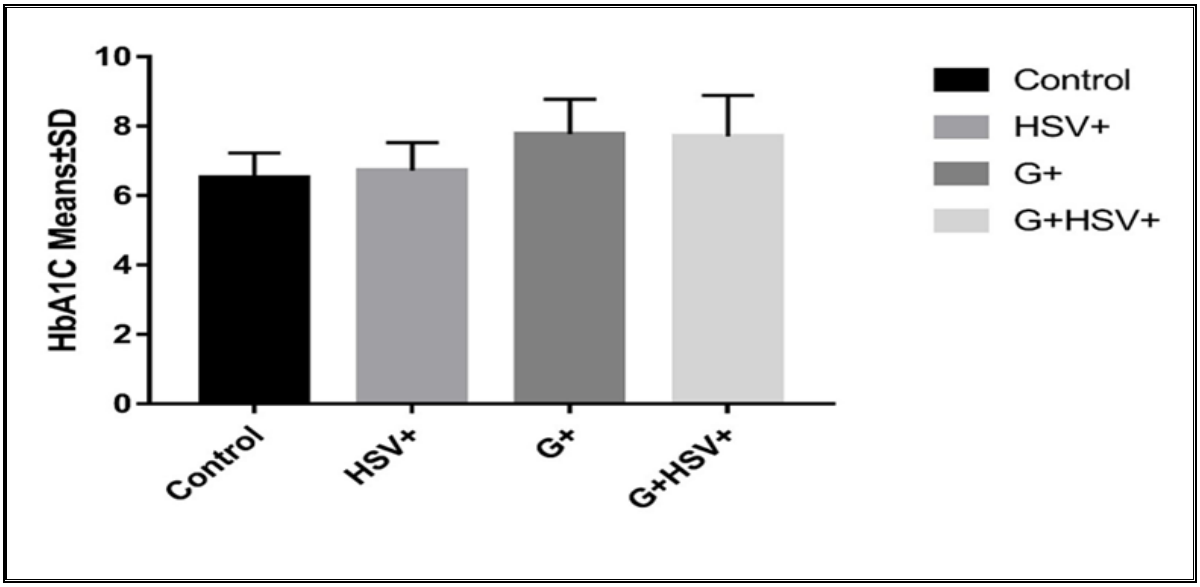


Figure 3–9: HbA1C level in serum among different study groups. Control, HSV+, Graves+, and Graves+ HSV+.

4.1. Conclusions:

The findings of the current study can be concluded as follows:

1:Thyroid dysfunction is a disease that affects both sex. Hyperthyroidism (Graves' disease) is more frequent in female than male patients .

2: Patients with G.D. with HSV-1 infection, with the majority of participants in the 36–60 age group, are more susceptible to G.D than other age groups.

3: TSH-receptor Abs can aid in the diagnosis of Graves' disease in situations where clinical features alone are not sufficient for an initial diagnosis.

4: The levels of anti-TSHR, anti-TPO, and anti-TG antibodies were significantly elevated in patients with Graves' disease who had HSV-1 infection when compared to the control group, according to this study.

5: The study shows that Graves' disease patients have higher levels of CD25 and TSHR expression than healthy controls, and it also shows that HSV-1 infection raises CD25 and TSHR expression.

6: A high concentration of inflammatory CD25 plays a crucial role in the pathogenesis of HSV-1 that leads to G.D., is associated with an autoimmune thyroid disease (Graves' disease), and is positively correlated with the level of TSHR expression.

7: This study suggests that Graves' disease and HSV-1 infection are associated with higher HbA1C levels.

8: Patients with type 2 diabetes mellitus are exhibiting an elevated occurrence of GD.

4.2 Recommendations:

1: Detecting and investigating other viruses(members of herpesviridae) other than HSV-1 that infect the G.D. patients

2: Further of the HSV-1 infection and it is relationship to the efficiency of human TSH

3: Some limitations of the study include the cross-sectional design and a lack of information on the duration of HSV infection in the study groups. More research is required to confirm these results and investigate the underlying mechanisms.

4: A molecular study about HSV-1 also GD cases with a large sample size