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جامعة بابل/ كلية الطب

فرع الكيمياء والكيمياء الحياتية

تأثيرتعدد الأشكال الجينية لجين الريسيستين على مقاومة الأنسولين ولمرضى السكري النوع الثاني في المرضى العراقيين / محافظة بابل.

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الخلاصة

مرض السكري النوع الثاني يشكل 90% من جميع حالات مرض السكري.في هذا المرض , استجابة الخلية للأنسولين تقل, وهذا يشار اليه كمقاومة للأنسولين .في هذه المرحلة انتاج الانسولين يزداد لمواجهة هذه المقاومة للحفاظ على ثباتيه السكر, بعد انقضاء فترة من الزمن , انتاج الانسولين يقل , وهذا يؤدي الى النوع الثاني لمرض السكر. السمنة هو عامل محفز لمرض السكري من النوع الثاني المرتبط بمقاومة الأنسولين. مقاومة الانسولين هو على الاغلب مرض مكتسب مرتبط بزيادة في دهون الجسم و اسباب جينية.

الدراسة الحالية تم تصميمها لتقييم دور بروتين الريسيستين في مقاومة الانسولين وتاثير التغييرات الجينية من خلال اثنين من تعدد اشكال النوكليوتيدات المفردة (rs34861192) و (NG023447) على مستويات الريسيستين (لمرضى السكري من النوع الثاني للسمنة والوزن الطبيعي) ومقارنتهم مع الاصحاء. وايضا اختبار التعبير الجيني لجين الريسيستين (420 C>G-) لهذا البروتين في كلا المجموعتين.

الدراسة شملت 120 مشارك(60 مرضى السكري نوع الثاني و 60 اصحاء) وباعمار تتراوح بين (42 – 58) سنة لمرضى السكري من النوع الثاني واعمار تتراوح بين (59-41) سنة للاصحاء. اعتمادا على مؤشر الكتلة , كل مجموعة قسمت وبالتساوي الى (30 سمنة و30 وزن طبيعي مرضى السكري من النوع الثاني) و (30 سمنة و30 وزن طبيعي اصحاء) مع مؤشر كتلة الجسم (30- 38.6 Kg/m^2) سمنة (19- 24.6 Kg/m^2) وزن طبيعي (30 -34.2 Kg/m^2) سمنة (19.2- 24 Kg/m^2) وزن طبيعي بالتتابع. الدراسة الحالية قدرت مستويات الانسولين, الريسيستين, مقاومة الانسولين , سكر الصائم , الهيموكلوبين السكري وتم فحص كل من النمط الجيني لأثنين من تعدد اشكال النوكليوتيدات المفردة (rs34861192) و (NG023447) والتعبير الجيني لجين الريسيستين .علاوة على ذلك الدراسة اختبرت انتقائية وحساسية الريسيتين كمحدد لبداء مرض السكري من النوع الثاتي. مستويات الانسولين تم قياسها بواسطة الممتز المناعي المرتبط بانزيم, بينما سكر الصائم تم قياسه بواسطة Roche COBAS c311 , الهيموكلوبين السكري بواسطة COBAS INTEGRA© 400 plus. اما فيما يخص التحليل الجيني تم تنفيذه لدراسة اثنين من تعدد اشكال النوكليوتيدات المفردة والتعبير لجين الريسيستين بواسطة استخدام تحليل ذوبان عالي الدقة وبنظام تضخيم الطفرة الحرارية لتفاعل سلسلة البلمرة.

اشارت النتائج الحالية الى زيادة ملحوظه (P<0.05) في المستويات الهرمونية والمعلمات البايوكيميائية لمرضى السمنة المصابين بمرض السكري النوع الثاني عندما تمت مقارنتهم ب المجاميع الاخرى. النتائج التي عرضت بصيغة (mean ranks difference) للسمنة المصابين بمرض السكري النوع الثاني ومقارنتهم مع المرضى المصابين وغير المصابين بمرض السكري النوع الثاني ذي الاوزان الطبيعية والسمنة غير المصابين بمرض السكري النوع الثاني كانت للأنسولين (74.68-) ,(24.9-) ,(55.08),الريسيستين (33.46-) ,(70.58-) ومقاومة الانسولين (52.9-) ,(86.51-) (27.4), وسكر الصائم (53.06-),(75.4-) والهيموكلوبين السكري (51.8-),(76.1-) على التوالي. مستويات الريسيستين كانت مرتبطة ايجابيا مع الانسولين(r= 0.947, P< 0.05) ,سكر الصائم(r=0.8, P< 0.05) , مقاومة الانسولين(r=0.983, P<0.05) و مؤشر كتلة الجسم ,r= 0.988) P<0.05) للسمنة وغير السمنة للمرضى المصابين بالسكري النوع الثاني. تحاليل ال ROC (AUC= 0.873) الانتقائية والحساسية للريسيستين كانت (76% , 90%) على التوالي.

التحليل الجيني اشير الى انه rs34861192 كانت مرتبطة وبشكل ملحوظ (P< 0.01) في الصفات السائدة والمتنحية والسائدة المشتركة لمرضى السكري من النوع الثاني , بينما فرط السائدة لم تكن مرتبطة[P=0.75, OR=0.8, CI (0.23-2.8)]. النمط الجيني AA ل rs34861192 اظهر اختلافية عالية ملحوظة (P< 0.001) في مرضى السكري النوع الثاني السمنة والوزن الطبيعي مقارنة ب الاصحاء, بينما لاتوجد اختلافية ملحوظة للمجاميع الاخرى, ولكن اظهر اختلافية ملحوظة (P< 0.05) في مرضى السكري النوع الثاني فقط للسمنة والوزن الطبيعي فقط. ايضا النمط الجيني GG ل NG023447 اظهر اختلافية عالية ملحوظة (P< 0.001) في مرضى السكري النوع الثاني السمنة والوزن الطبيعي مقارنة ب الاصحاء, بينما لاتوجد اختلافية ملحوظة للمجاميع الاخرى, ولكن اظهر اختلافية ملحوظة (P< 0.05) في مرضى السكري النوع الثاني فقط للسمنة والوزن الطبيعي فقط. النمط الجيني AA المتنحي ل rs34861192 كان لديه ارتباطيه عالية(P<0.05) مع مستوى الريسيستين فى مرضى السكري النوع الثاني السمنة وغير السمنة, بينما النمط الجيني GG ل NG023447 المتنحي لم يكن لدية مثل هذه الارتباطية (P>0.05) مع مستوى الريسيستين فى مرضى السكري النوع الثاني السمنة وغير السمنة. التعبير الجيني للريسيستين (420 C>G-) كان عالي في المرضى مقارنة ب الاصحاء, حيث كانت قيمة تغير الانطواء يقيم ((mean± SD) and (17.66±156.80) للمرضى .(6.56±19.69) للأصحاء. ايضا كانت هنالك علاقة ايجابية (P<0.004) بين النمط الجيني AA المتنحي ل rs34861192 وتغير الانطواء

في الاستنتاج, ريسستين ممكن ان يكون لديه رابط قوي مع السمنة, مقاومة الانسولين من خلال خلل في طريق الاشارة التابع للأنسولين, في النهاية يؤدي الى مقاومة الانسولين وتطور مرض السكري من النوع الثاني. تعدد الشكل الجيني للريسيستين(rs34861192) يلعب دور مهم في تطور مرض السكري من النوع الثاني. عند المستوى الجيني المتنحي AA (rs34861192) يلعب دور مهم في زيادة مستوى الرسيستين في الدم والذي يعتبر عامل خطر في تطور مرض السكري من النوع الثاني. التعبي الجيني لريسيستين يعتبر محدد مهم لمرضى السكري النوع الثاني السمنة والذين يملكون النمط الجيني AA ل (rs34861192) , بينما (NG023447) ليس كذللك.

Decision of discussion committee

We, the examiner committee, certify that we have read the thesis entitled (**Impact of Resistin Gene Polymorphism on Insulin Resistance and Type 2 Diabetes in Iraqi Patients / Babylon Governorate.**) and have examined the student (**Zaid Adnan Abdul Hameed Abdulhameed**) in its contents, and that in our opinion it is accepted as a thesis for **Degree of Doctor of Philosophy in Science/ Clinical Biochemistry**.

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Summary

Type 2 diabetes mellitus (T2DM) constitutes 90% of all cases of diabetes. In T2DM, the responsiveness of the cell to insulin is reduced, and this is referred to as insulin resistance. During this state, the production of insulin increases in an attempt to face this resistance and to maintain glucose homeostasis, after a period of time, insulin production diminishes, leading to T2DM. Obesity is a provoking factor for T2DM related to insulin resistance(IR). Insulin resistance which is primarily an acquired condition is connected to both high body fats and genetic reasons.

The present case control study was designed to evaluate the role of resistin protein in insulin resistance and the effect of gene polymorphism through two SNP_S(rs34861192 and NG023447) on resistin levels in (obese and non obese T2DM patients). Also, to examine the expression of *resistin* gene (– 420 C/G) for this protein and to determine its role in developing of T2DM.

The study was involved 120 participants (60 T2DM patients and 60 control with an age range between 42-58 year for T2DM patients and the age 41-59 years for control. Dependent on BMI , each group subdivided equally into (30 obese and 30 non obese T2DM patients) and (30 obese and 30 non obese controls) with BMI range (30 – 38.6 Kg/m^2) obese (19 – 24.6 Kg/m^2) non obese and (30–34.2 Kg/m^2) obese (19.5 – 24 Kg/m^2) non obese respectively. The current study was estimated the levels of insulin, resistin, insulin resistance, fasting blood sugar and HbA1c, also both the genotyping of the two SNP_S(rs34861192 , NG023447) and the expression of *resistin* gene. Moreover, the study determined the specificity and sensitivity of resistin, as a sign for the development of T2DM. Insulin and resistin levels were measured by enzyme linked immune assay (ELIAS), while fasting blood sugar was measured by

using the Roche COBAS c311 and HbA_1c by using COBAS INTEGRA© 400 plus. Genetic analysis was performed by using high resolution melting and amplification refractory mutation PCR.

The results of present study showed significant ($P<0.05$) rise in levels of the hormonal and biochemical markers of the obese patient with T2DM when compared with other studied groups. The results that showed as (mean ranks difference) for obese with T2DM in comparison with non obese without T2DM, non obese with T2DM and obese without T2DM were insulin(-74.68), (-55.08), (-24.9), resistin (-70.58), (-33.46), insulin resistance (-86.51), (-52.9),(-27.4), fasting blood sugar(-75.4),(-53.06), HbA_1c(-76.1),(-51.8) respectively. Resistin levels were positively correlated with insulin($r= 0.947$, $P=< 0.05$), fasting blood glucose ($r= 0.8$, $P=< 0.05$), IR($r=0.983$,$P<0.05$) and body mass index($r= 0.988$, $P<0.05$) in obese and non obese T2DM patients. Receiver operating characteristic analysis results of resistin were (area under curve=0.873) with specificity and sensitivity (90%,76%) respectively.

Gene analysis was indicated that rs34861192 was associated significantly ($P<0.01$) with T2DM in dominant, recessive, and co-dominant models, while the over dominant model was non significant [$P>0.05$, OR=0.8, CI (0.23-2.8)]. The rs34861192 AA genotype was showed the highly significant difference ($P<0.001$) in non obese and obese T2DM compared to control, while no significant different between other groups, but it was significant ($P<0.05$)difference in non obese and obese of T2DM patients only. Also there was highly significant difference ($P<0.001$) of GG genotype in non obese patients as compared with control subjects, while no significant different between other groups and between patients and control for obese only. Mutant genotype (AA) of rs34861192 was significantly ($p<0.05$) associated with retn level in obese and non obese T2DM patients, while genotype(GG) of NG023447 was non significantly ($p<0.05$) associated with retn level in obese

and non obese T2DM patients. The expression of *resistin* gene (– 420 C/G) was high in patients group when compared with control, where the values of folding change (mean± SD)were (156.80±787.66 for patient) and (19.69±36.56 for control).Also there was positive correlation (P<0.004) between mutant genotype AA of rs 34861192 and folding change.

In conclusion, resistin might be consider as a strong link between obesity, and IR through derangement of the signalling pathway of insulin, eventually this may lead to insulin resistance and development of T2DM. Also polymorphisms of *resistin* gene (rs34861192G>A) play an important role in development of T2DM at the level of genotypes mutant AA of rs34861192. Resistin expression is a good indicator for obese individuals with T2DM that have AA genotype of *resistin* gene rs34861192 ,while NG023447 is not.

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Abbreviations

Abbreviation	Details
ACC	Acetyl-CoA carboxylase
ACACA	Acetyl-CoA Carboxylase Alpha
ADP	Adenosine diphosphate
AGEs	Advanced glycation end products
Akt	Protein kinase B
ALDH-2	Aldehyde dehydrogenase-2
ANG2	Angiotensin II
ANOVA	Analysis of Variables Test
AS-160	Akt substrate(Protein kinase B substrate)
BMP	Bone morphogenetic protein
CAP	Catabolite activator protein
CBL	Cellular homologue of Cas NS-1 oncogene
CCL CC	Chemokine ligand CC
CXCL C-X-C	Motif chemokine ligand
CVD	Cardiovascular Disease
DPP-4	Dipeptidyl peptidase-4 inhibitors
FASN	Fatty acid synthase
G6Pase	Glucose 6-phosphatase
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon like peptide one
GLUT-1	Glucose transpoter-1

GLUT-2	Glucose transpoter-2
GSH	Glutathione
GSK-3β	Glycogen synthase kinase-3 beta
GYS-1	Glycogen synthase-1
X^2	Chi – Square
DAG	Diacylglycerol
DCM	Diabetic cardiomyopathy
DM	Diabetes mellitus
DME	Diabetic macular edema
DHD	Diabetic heart disease
DNA	Deoxyribonucleic acid
DPN	Diabetic peripheral neuropathy
DR	Diabetic retinopathy
DNL	De novo lipogenesis
4E-BP1	A multifactor regulated multifunctional protein
ERK	Extracellular regulated protein kinases
ESRD	End stage renal disease
ER	Endoplasmic Reticulum
FAO	Fatty acid oxidation
FGF	Fibroblast growth factor
FFA	Free fatty acid
FOXO	Forkhead box protein O
GRB-2	Growth factor receptor-bound protein2

GWAS	Genome wide association studies
Gly	Glycine
HbA1c	Glycated hemoglobin
HIF-1α	Hypoxia inducible factor-1 alfa
HRM	High resolution melt
HRP	Horseradish Peroxidase
IAAP	Islet amyloid polypeptide
ICAM-1	Intercellular Adhesion Molecule 1
IP_2	Inositol 1,3-bisphosphate
IP_3	Inositol 1,4,5-Trisphospate
IRS	Insulin receptor substrate
IR	Insulin resistance
L-VGCCs	L-type voltage-gated Ca^{++} channels
ME	Malic Enzyme
MAPK	Mitogen-activated protein kinase
MCP-1	Membrane cofactor protein-1
MIF	Macrophage migration inhibitory factor
MIP-1	Macrophage inflammatory protein-1
M-CSF	Macrophage colony-stimulating factor
MODY	Maturity-onset diabetes of the young
mTOR	Mammalian target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

NF-κB	Nuclear factor kappa B
NLPR3	NLR family pyrin domain containing 3 inflammasome
OGTT	Oral glucose tolerance test
PEPCK	Phosphoenolpyruvate carboxykinase
PDK1	Phosphoinositide dependent kinase 1
PGC-1 α	Peroxisome-proliferator-activated receptor-γ coactivator-**1** alpha
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PLC	Phospholipase C
P2X	Purinergic receptor 2 X
P2Y	Purinergic receptor 2 Y
PDGF	Platelet-derived growth factor
PKC	Protein Kinase C
PPAR-ɣ	Peroxisome Proliferator-Activated Receptor-gamma
PP2A	Protein phosphatase 2A
PTEN	Phosphatase and tensin homolog
PTP1B	Protein-tyrosine phosphatase 1B
RAS	Rat sarcoma
RAGE	Receptor of advanced glycation end products
RAF	Rapidly accelerated fibrosarcoma
RAAS	Renin-angiotensin-aldosterone system
Retn	Resistin.
RNA	Ribonucleic acid

ROC	Receiver operating characteristic.
ROS	Reactive oxygen species.
RS	Reference of single nucleotide polymorphisim
RYR	Ryanodine receptor channel
SCD	Stearoyl-CoA Desaturase
S6	Ribosomal protein
S6K1	Ribosomal protein S6 kinase beta-1
SERCA	Sarco-endoplasmic reticulum Ca^{2+}-ATPase
SGLT2	Sodium dependent glucose transporters 2
SHC	Src homology and collagen
SNPs	Single Nucleotide Polymorphisms
SOD-1	Super oxide dismutase -1
SOS	Son of sevenless
TBC1D1	Tre-2/BUB2/cdc 1
TC10	GTP-binding protein TC10
TGFβ	Transforming growth factor-β
TLR4	Toll-like receptor 4
TLR-4	Toll-Like Receptor 4 (TLR4)
TRIB3	Tribble homolog 1
TSC1	Tumour suppressor control 1
TWEAK	Tumor necrosis factor-like weak inducer of apoptosis,
UKL-1	Uridine/cytidine kinase
UPR	Unfolded protein response.

VAT	Visceral adipose tissue
VAP-1	Vascular adhesion protein-1
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VSM	Vascular smooth muscle.
vWAT	Visceral white adipose tissue
WAT	White adipose tissue
WHO	World health organization.

Republic of Iraq
Ministry of Higher Education
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Impact of Resistin Gene Polymorphism on Insulin Resistance and Type 2 Diabetes in Iraqi Patients / Babylon Governorate.

A thesis

Submitted to the Council of the College of Medicine University of Babylon in Partial Fulfillment for the Requirements for the Degree of Doctor of Philosophy in Science/ Clinical Biochemistry

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2023A.D. **1445 A.H.**

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

﴿ وَلَوْلَا فَضْلُ اللَّهِ عَلَيْكَ وَرَحْمَتُهُ لَهَمَّت طَّائِفَةٌ مِّنْهُمْ أَن يُضِلُّوكَ وَمَا يُضِلُّونَ إِلَّا أَنفُسَهُمْ ۖ وَمَا يَضُرُّونَكَ مِن شَيْءٍ ۚ وَأَنزَلَ اللَّهُ عَلَيْكَ الْكِتَابَ وَالْحِكْمَةَ وَعَلَّمَكَ مَا لَمْ تَكُن تَعْلَمُ ۚ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيمًا ﴾

صَدَقَ اللهُ العَلِيُّ العَظيم

[سورة النساء أية ١١٣]

"Supervisor Certification"

"We certify that this thesis entitled **(**Impact of resistin gene polymorphism on insulin resistance and Type 2 diabetes in Iraqi patients / Babylon province**)"** has been "prepared under our supervision at the Department of chemistry and Biochemistry", College of Medicine, University of Babylon, in partial requirements for the Degree of Doctor of Clinical Biochemistry.

Professor

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Date: / /2023

Professor

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Date: / /2023

In the view of the available recommendation, I forward this thesis for debate by the Examination Committee.

Professor

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Date / / 2023

College of Medicine/ University of Babylon

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I would like to express sincere gratitude to my friends and family for their" "support and assistance me"

Zaid

Dedication

I dedicate this work to :

To soul of my father……

My mother ,MY brother, MY sister

My wife for Supporting and Encouraging

My kids with my love.

1. Introduction:

Diabetes mellitus (DM) depicts a combination of metabolic disorders recognized and identified by the presence of hyperglycemia, in the absence of the treatment which comprises defects in insulin secretion, insulin action or both and deranges of carbohydrate, fat and protein metabolism[1]. Type 2 DM (T2DM) is caused by either insulin resistance (IR) with relative insulin deficiency or secretory defect with IR[2]. IR is identified by abnormal response of target tissues to insulin stimulation, leading to increase insulin production and hyperinsulinemia[3]. IR which is primarily an acquired condition is connected to both high body fats and genetic reasons[4].

Resistin(retn) is encoded by *retn* gene that is located on 19p13.2 [5]. Retn is a polypeptide hormone produced by adipocytes and macrophages[6,7].Its expression is increased with adipose differentiation[8]. It resists insulin action and impairs glucose homeostasis, resulting in the development of T2DM. Increased levels of serum retn is related with many metabolic diseases such as obesity insulin resistance and T2DM. Indeed, single nucleotide polymorphism (SNPs) of *retn* gene such as rs-34861192, was largely significantly correlated with the serum resistin concentration [9,10,11].

1.1 Diabetes mellitus:

Diabetes mellitus (DM) is a chronic hyperglycemia resulted from a collection of metabolic diseases. The main cause ranges from absolute deficiency to relative with insufficient action of insulin or both[12]. Both dysfunction and destruction of β cells of the pancreas are the common characteristics of all types of diabetes. The underlying mechanisms that cause both defect include genetic and epigenetic abnormalities, an

autoimmune diseases, insulin resistance, inflammation, illnesses and environmental factors. These mechanisms lead to decrease in function and/or mass of β cells. The decline is due to incapable of the pancreas of regenerating of these cells after the age of 30 [13].

Diabetes mellitus can be defined according to world health organization (WHO) based on laboratory findings as shown in Table (1-1) [14].

Table (1-1): Diagnostic criteria for diabetes.[14]

Measurement	Diagnostic cut-off values	Comments
Fasting venous or capillary plasma glucose.	≥7.0 mmol/L (126 mg/dL)	Least costly but difficulties with ensuring a fasting state.
2-hour post-load venous plasma glucose.	≥11.1mmol/L (200 mg/dL)	Cumbersome and costly, difficulties with ensuring a fasting state.
2-hour post-load capillary plasma glucose.	≥12.2mmol/L (220 mg/dL)	Cumbersome and costly, difficulties with ensuring a fasting state.
Random plasma glucose.	≥11.1mmol/L (200 mg/dL)	To be used only in the presence of symptoms.
HbA1c.	≥ 6.5% (48mmol/mol)	• Less intra-individual variability than plasma glucose. • Does not require the fasting state but substantially more costly than glucose measurements. • Is an indirect method. • Can be inaccurate in some conditions (haemoglobinopathies, renal failure, some anaemias, conditions with rapid red blood cell turnover).

1.1.1 Classification of Diabetes Mellitus:

All types of diabetes have common features that is hyperglycemia, but they differ in aetiology, pathogenic mechanism, natural history and treatment. DM can be classified into the following categories:

1-Type I DM is caused by an autoimmune disease, leading to β cell destruction and insulin deficiency, including Latent autoimmune diabetes of adulthood (slow autoimmune β-cell destruction that can occur in adults leading to a long duration of marginal insulin secretory capacity.

2-Type 2 DM is due to relative decrease insulin secretion and insulin resistance.

3-Diabetes Mellitus due to other disease such as pancreatic disease (pancreatitis, cystic fibrosis) or due to drugs (glucocorticoids) or due to monogenic syndrome (maturity onset diabetes of the young).

4-Gestational DM is diagnosed during pregnancy in the second or third trimester(it can be distinguished from other types of DM after performing an OGTT test and the diagnosis is made if any of the following values are met or exceeded:

* Fasting: 92 mg/dL (5.1 mmol/L)

* 1 h: 180 mg/dL (10.0 mmol/L)

*2 h: 153 mg/dL (8.5 mmol/L) [15,16].

1.1.2. Type 2 Diabetes Mellitus:

Type 2 Diabetes Mellitus is a metabolic disease and constituted 90% of all cases of DM. Actually, Patients with T2DM are older than 40 years, but it is also frequently seen in children, adolescents and adults of younger age because of increasing in obesity, physical inactivity, diets containing energy [17].Two factors are responsible on its development: defective insulin secretion and unresponsiveness of the target tissues to insulin. So that both secretion and action mechanism of insulin must be

tightly regulated to meet metabolic demand and any defect of these mechanisms can lead to the development of T2DM[18].

1.2. Epidemiology:

The rate of prevalence is increased rapidly because of hereditary factors, unhealthy diet routine, high average age, sedentary lifestyle and obesity. The most important reasons of morbidity and mortality in the world is DM and their complications[19]. The mortality rate was 3% between 2000- 2019 and this rate was increased to 13% in low-middle income countries . About 1.5 million were dead and 48% of all death is due to patients with diabetes aged before 70 years in 2019.[20] In the same year, 463 million of people aged 20 – 79 years lived with DM in worldwide with prevalence 9.3%.By 2045, this number may increase to 700 million with prevalence10.9% with greatest percent in the low-middle income countries [21]. In study that is a comparative cross-sectional study was carried out at Babylon Diabetic Tertiary Center in Babylon Governorate, Iraq. It showed that for 18 months, out of a total of 253 children and adolescents diagnosed with diabetes, 16 patients had Type 2 DM, (the prevalence of Type 2 DM in young people is 6.3%. This prevalence was similar to that documented in the USA (7% of all new cases), less than that documented in Egypt (13.3%),in Kuwait (11.5%) , and much less than that documented inTaiwan (54.2%) [22].

1.1.2.3 Etiology and risk factors:

Type 2 diabetes mellitus is a multifactorial disease and so named because it is linked to genetic , environmental and epigenetic risk factors [23,24,25]. Moreover, the effect of T2DM varies by population, relying on some variables such as (age, race, ethnicity, geography, and socioeconomic status) [26].These factors play an important role in the

function and activity of insulin, resulting in the development of the disease [27].

●Obesity is a major risk factor for T2DM and can increase the risk by 90 fold in overweight or obese patients. Body mass index (BMI) is positively correlated with the obesity and the risk is increased when BMI above 30[28].The risk can be increased to more than double by increasing the deposition of fat in ectopic regions of the body specially visceral fat [29].In obesity ,the increment in white adipose tissue (WAT) which is called visceral depot (vWAT), induces the secretion of adipokines and associates with metabolic disease (obesity, insulin resistance, T2DM). This is due to vWAT adipocytes diminishes the sensitivity to insulin, angiogenic potential, and increases lipolytic activity. Moreover, visceral adipose tissues have negative effect that is associated with the mobilization of visceral fat. These effects underlying causes for increasing of the transporting of free fatty acids (FFA) to the liver ,resulting in insulin signaling inhibition [30].

● Type of food and sedentary life style are the main reason of T2DM and both factors are responsible for the epidemic of obesity which is associated with prevalence of T2DM. Diet low in fiber with high glycemic index is greatly correlated with higher risk of T2DM and specific diet containing fatty acids such as saturated fat may influence on insulin resistance. Other life style factors physical inactivity, smoking, alcohol consumption are greatly important factors that participating in the progression of this disease [31].

● Physical activity has protective effect on the development of T2DM. There are a number of biological mechanisms for the protective effect of physical activity. First, it increases the sensitivity of the cells to insulin by

improving abnormal glucose tolerance when the cause is due to insulin resistance other than when the cause is due to deficiency of circulating insulin. Second, during initial stages and before insulin therapy, physical activity has beneficial and synergistic effect with insulin. This effect is appeared during skeletal muscle contraction that increases both blood flow and glucose uptake into the cells. Third, it reduces intra-abdominal fat, which is considered as risk factor for insulin resistance [32].

● Genetics have substantial role in both TIDM and T2DM. This role is more observed in T2DM than TIDM [33]. In T2DM , the risk of mono zygotic twins is higher (approximately 70%) compared with TIDM(30-50 %) and indeed, the risk for developing T2DM with one affected parent is 40% and 70% if both are affected. Also the risk from dizygotic twins with T2DM is 20 -30% and are higher than TIDM(10%). The reason that leads to increase risk of developing T2DM for the first degree relative patients is due to those patients and their relatives share the same diets, life style, and genesis [28].

Various genes are involved in the molecular mechanism and participate in the progression of the disease. This role is directly or indirectly affected by overall genetic background associated to the family or population that are interacted with variety of environmental factors [34].

1.1.2.4 pathophysiology

Development of T2DM may be underlying two mechanisms

1.1.2.4.1 β cells dysfunction

The pathophysiology of the T2DM includes a disturbance in the feedback loop between insulin action and insulin secretion that results in increase in the glucose concentration in the blood [35].Normal insulin secretion dependent on normal β cell physiology as in figure (1-1A). Consequently, When the abnormality in the β cells, the secretion of insulin is low, resulting in inability of the body to maintain physiological glucose levels. In the case of insulin resistance, this will cause increase glucose production by the liver and decrease glucose uptake by muscle, liver and adipose tissues. In both abnormality, hyperglycemia will be amplified, leading to the progression of T2DM [36].

The dysfunction of βcells is related to β cells death [37].However,βcell dysfunction in T2DM may be due to interfere between environmental and different molecular pathways in the cell [38]. Hyperglycemia and hyperlipidemia are often founded in the excess of nutritional status similar to that found to in the obesity and this is favored IR and chronic inflammation. So that β cells loss there integrity due to exposure to inflammation, inflammatory stress, IR, metabolic, oxidative stress and amyloid stress [37]. An excess of FFA and hyperglycemia induce endoplasmic reticulum stress (ER stress) through the activation of the apoptosis unfolded protein response (UPR) pathway causing β cell dysfunction [39]figure (1-1B).

In obesity, lipotoxicity, glucotoxicity, gluculipotoxicity induce metabolic and oxidative stress, leading to β cells damage [38]. Several mechanisms can activate the UPR pathway result from stress obtained from high level of saturated fatty acids, these include inhibition of the sarco/endoplasmic reticulum Ca^{++} ATPase (SERCA) charging of ER

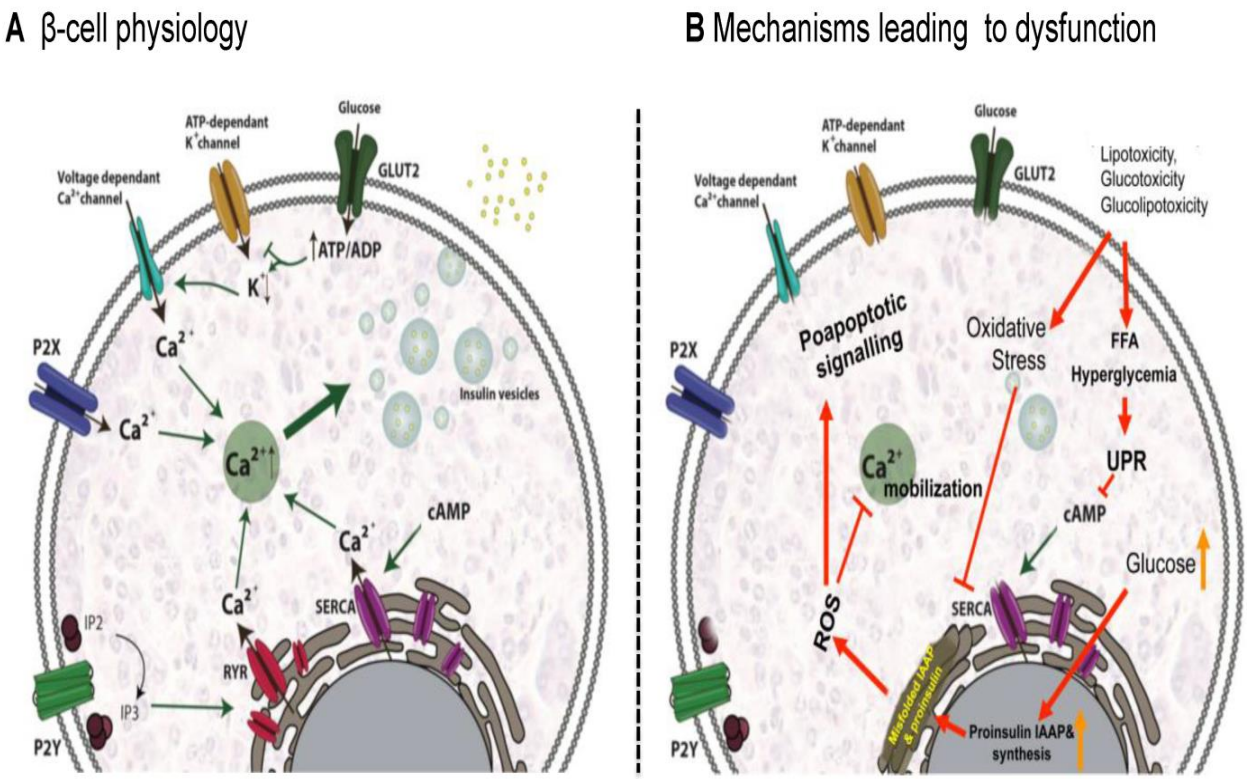


Figure (1-1): (A) Insulin secretion signaling pathways in β-cells under physiological conditions. (B) mechanisms of β-cells dysfunction. cAMP(cyclic adenosine mono phosphate), FFA(free fatty acid), GLUT2(glucose transporter 2), IAAP(Islet amyloid polypeptide), IP_2(Inositol 1,3-bisphosphate), IP3(Inositol 1,4,5-Trisphospate), P2X(Purinergic receptor 2 X), P2Y(Purinergic receptor 2 Y), RYR(ryanodine receptor channel), ROS(Reactive oxygen species), SERCA(Sarco-endoplasmic reticulum Ca2+-ATPase), UPR(Unfolded protein response.).

Ca^{++} mobilization, activation of IP3 receptors. Also increase pro-insulin biosynthesis and islet amyloid polypeptide (IAAP) in β cells due to sustained high glucose levels resulting in the accumulation of misfolded insulin and IAAP and raising in oxidative protein folding mediated reactive oxygen species production (ROS) [39]. Eventually ER Ca^{++} mobilization is altered under all these effects favoring pro-apoptotic signals, pro-insulin m RNA degradation and enhance interleukins (IL)-1B release that recruit macrophage and induce local islet inflammation

[38]. All above mechanisms can lead to disruption of integrity ,organization, cell to cell communication of islet cells, insulin / glucagon release and finally magnified the hyperglycemia. Impaired insulin or any insulin precursors synthesis and secretion mechanism can lead to β cells failure and T2DM [40].

1.1.2.4.2 Insulin resistance:

The skeletal muscle, liver and adipose tissues are primarily target tissues that have impaired biological response to insulin stimulation. This can lead to increase in insulin production and hyperinsulinemia [41-43].Hyperglycemia and eventually T2DM are the primary consequence of IR. IR is occurred prior to the development of T2DM by 10–15 years [44-45].

IR is either an acquired condition associated with excess body fat or genetic condition. The majority of cases fall in the acquired categories. Acquired is happened due to high impairment in the function of adipose tissues, aging, imbalance of nutritional status, physical inactivity, glucose and lipid toxicity (high FFA in the circulation) and drugs such as (glucocorticoids, anti-adrenergic, protease inhibitors and some exogenous insulin. while genetic type is caused by Myotonic dystrophy, Ataxia telangiectasia, Alstrom syndrome, Rabson-Mandenhall syndrome, Werner syndrome, lipodystrophy, PCOS and type A,B insulin resistance. There is a third classification of IR concerning the site of insulin receptor defect, these include: pre-receptor, Receptor and post receptor[46].

As mentioned above, skeletal muscle, liver and adipose tissues are main sites which resist insulin action. IR is assumed to start in muscle tissues with changing in the immune mediated inflammatory process and

increasing in FFA that lead to ectopic lipid deposition[47]. These mechanism describe below:

- Adipose Tissue

Insulin inhibits lipolysis process and the process become more sensitive in the case of hyperinsulenemia. During insulin-resistant in adipose tissue especially visceral adipose tissue (VAT), insulin fails to suppress lipolysis which leads to increase circulating FFAs. High circulating FFAs directly influence the action of insulin in both liver and muscle which further triggering of insulin resistance[48].

●MuscleTissue

About 70 % of glucose is up taken by muscle which is considered as primary site for glucose disposal after taking of a caloric meal. With high caloric loads, the amount of glucose exceeds muscle capacity and the high amount of glucose turns back to the liver ,leading to trigger de novo lipogenesis (DNL). Both triglyceride and FFA levels are increased from increasing DNL process, resulting in ectopic fat accumulation in the muscle, liver and adipose tissues. As a result, insulin resistance raises the production of inflammatory markers. Other factors that enhance insulin resistance in muscle tissue include physical inactivity and genetic risk [49].

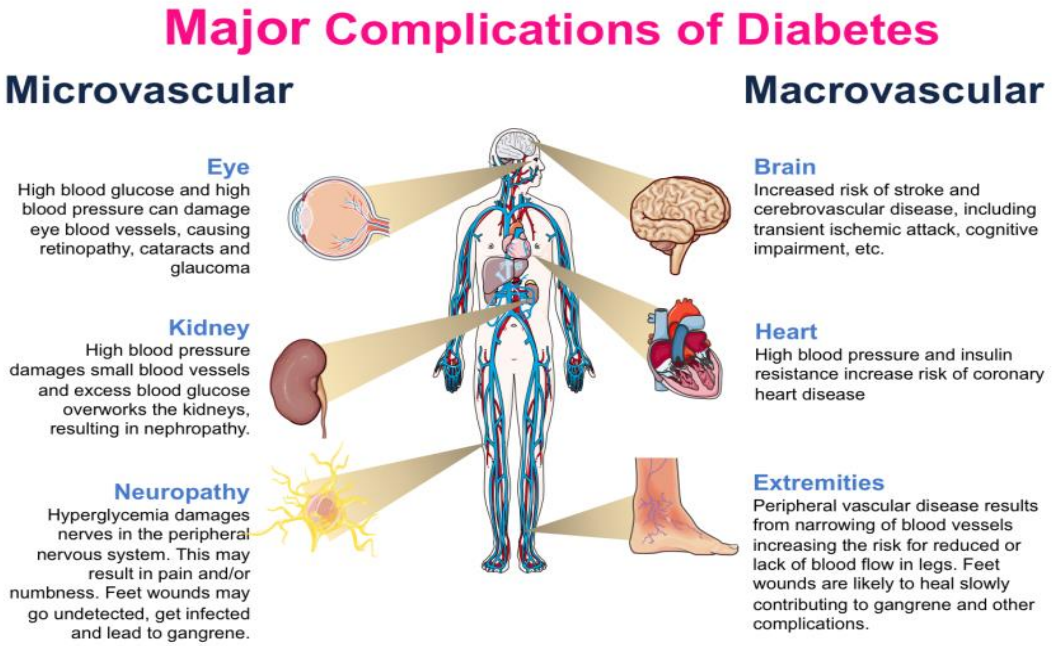
●The liver:

As a consequence of insulin resistance in muscle, more glucose is delivered to the liver, leading to DNL, ectopic lipid deposition and inflammation. Lipolysis is also increased in adipose tissue due to IR, leading to high circulating FFA , steatosis and IR in muscle tissue. In normal state and after taking of a caloric meal, hepatic glucose production

is reduced via inhibition of glycogenolysis by insulin, lowering of the rise in glucose in postprandial state. However, in IR, this feedback mechanism is disturbed, and the production of glucose continues to rise by the liver, causing Glucotoxicity that further contributes to insulin resistance[48].

1.1.2.5 Complications of T2DM:

Diabetes mellitus will Participate to a plenty of dangerous health problems if left untreated, affecting on both smaller (micro-vascular) and large (macro-vascular) vessels. Micro-vascular complication affect the kidney , the most costly complication with DM, with continuous renal failure (nephropathy) and nerve trauma (neuropathy) increasing the probability with diabetic foot ulcers and/or amputations. moreover, eye trauma (retinopathy) can due to blindness. Macro-vascular disease includes (coronary heart failure, peripheral artery disease , and stroke) which can lead to blindness [50].Figure [1-2][51-53].



Figure(1-2): complications of hyperglycemia[51-53].

1.1.2.5.1 Retinopathy:

Some of Diabetic eye disease such as diabetic retinopathy (DR), diabetic macular edema (DME), cataract, and glaucoma are a group of eye diseases that affect people with DM [54].DR is a micro-vascular complication and can lead to vision loss and blindness [55]. The loss of Vision can be due to either DME which causes swelling of the retinal macula or proliferative diabetic retinopathy. Prolonged duration of diabetes and blood pressure control are factors which are strongly associated with DR.[56,57]. The hyperglycemic-mediated pathways that can lead to DR are: activation of both the protein kinase C and the polyol pathway; glycated end products deposition, and increased hexosamine flux. The effect of these pathways is to induce retinal blood flow, promote vascular permeability, activate multiple growth factor receptors, loss of pericytes ,increase the thickness of capillary basement membrane[58].figure(1-3) [59].

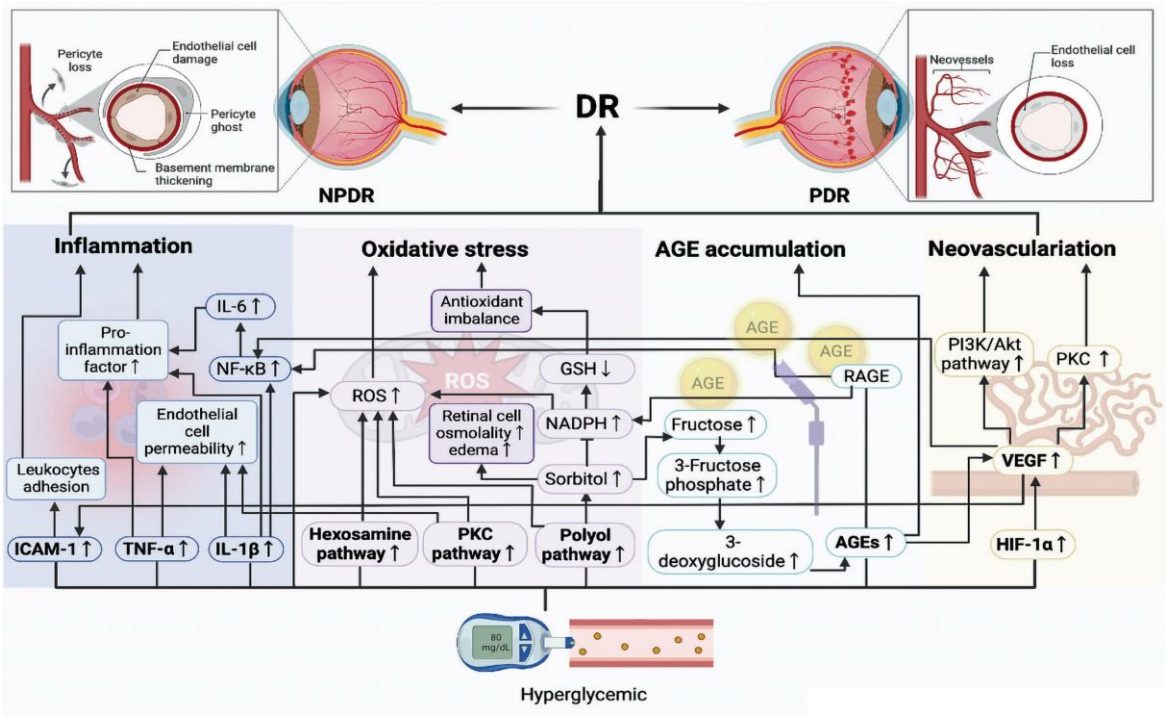


Figure (1-3): hyperglycemic-mediated pathways.[59]

1.1.2.5.2 Nephropathy:

Formerly called diabetic nephropathy, diabetic kidney disease (DKD) is characterized by high ratio of urine albumin-to- creatinine ≥30 mg/g , reduced glomerular filtration rate (<60 mL/min/1.73 m2), or both [60]. It may lead to end stage renal disease (ESRD) which affect (20–30%)diabetes patients [61]. It affects 10–40% of T2DM patients who ultimately experience from kidney failure [62]. The damage of kidney is caused by DM and the mechanism is complex and may include hemodynamic, metabolic, and inflammatory pathways and targets [63]. Figure (1- 4) [59].

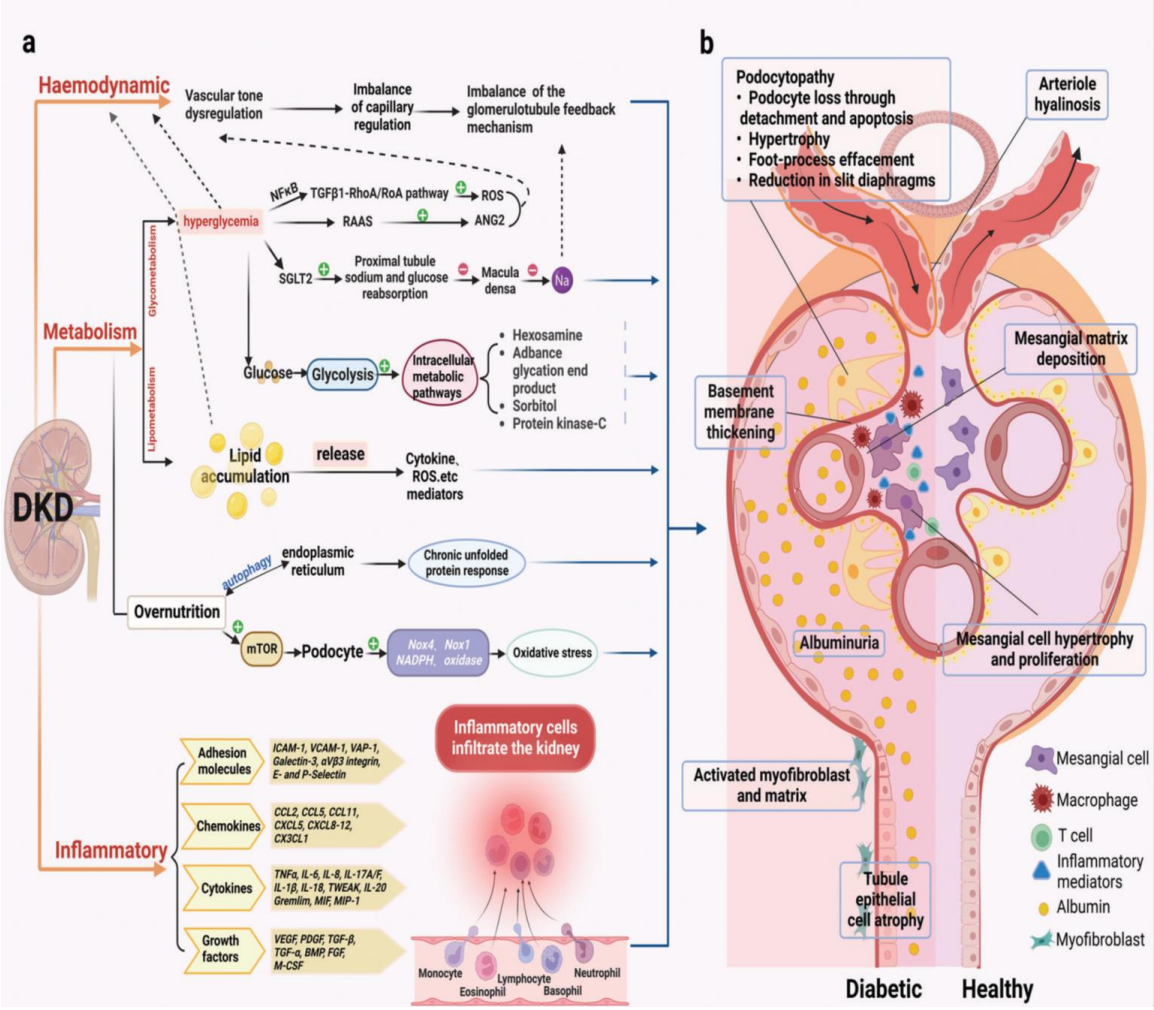


Figure (1-4): Pathology of the glomerulus and tubules in DKD[59].

The disturbance of hemodynamic state can result in dysregulation of tubulobulbar feedback balance[64,65]. This can be due to hyperglycemic state that influences the pathways such as TGFβ1-RhoA/Roa, RAAS, reabsorption of sodium and glucose in proximal tubular, and intracellular metabolism [66-68]. The releasing of mediators such as cytokines and ROS from abnormal lipid metabolism can cause injury of the vascular tubulointerstitial [69,70]. Endoplasmic reticulum autophagy process and mTOR formation due to over nutrition lead to both a chronic unfolded protein response and disturbs the podocytes respectively, resulting in generation of oxidative stress [71-73]. The releasing of inflammatory mediators such as adhesion molecules, chemokines, cytokines, and growth factors due to inflammatory process will cause renal infiltration.[74]All above abnormality promote changes in the structure of glomeruli and tubules in the diabetic patients figure(1-4 b)[59].

1.1.2.5.3 Neuropathy:

Neuropathy comprises both peripheral and autonomic nerves. it affects 50% of all diabetic patients. Hyperglycemia-induced polyol pathway, injury from AGEs, and enhanced oxidative stress have been implicated in its pathogenesis [75,76]. Diabetic peripheral neuropathy (DPN) is the most common form in patient with diabetes. The distal nerves of the feet is mostly affected by DPN. Lower-limb amputation is more common in patients with T2DM than TIDM and is higher by 10 to 20 times than those without diabetes [77,78]. DPN in diabetes appears in several forms relying on the site, manifesting as sensory, focal/multifocal, and autonomic neuropathies.[79] DPN in T2DM patients is positively associated with age, duration of diabetes, body weight, gender, history of foot ulcers, and patient's education [80]. Nevertheless, most of the

developed DPN prediction models focused on predicting DPN severity degree among T2DM patients who were already pre-diagnosed with DPN [81–83].

1.1.2.5.4 Cardiovascular disease:

The classical types of cardiovascular disease (CVD) related to diabetes are coronary heart disease, peripheral artery disease , congestive heart failure and cerebrovascular disease. In T2DM, cardiovascular complications are associated with arteriosclerosis incidence, and the chance of occurrence in patient with diabetes is greater than those without it. [84] High blood glucose concentration, insulin resistance, low-grade inflammation, and promotion of the coagulation cascade increases the incidence of CVD in T2DM[85,86], which is collectively considered as the leading cause of both morbidity and mortality [87].

The mechanisms of diabetic cardiomyopathy (DCM) are due to abnormal cardiac metabolism, glycotoxicity , lipotoxicity, and oxidative stress causing by inflammation.[88] The myocardium uses both Fatty acid oxidation(FAO) and aerobic oxidation process of glucose as the main sources of energy. In the presence of T2DM, lipid synthesis in the liver and lipolysis in adipocytes are increased due to IR, resulting in high circulating fatty acids and triglyceride levels. So that the accumulation of lipids and fatty acids promotes lipotoxicity leading to abnormal myocardial FAO processes. Consequently, this will induce endoplasmic reticulum (ER) stress, autophagy, and apoptosis, eventually cause ventricular remodeling [89,90] Advanced glycation end-products (AGEs) are metabolites of diabetic glycotoxin and are involved in the pathogenesis of DCM. AGEs bind to its receptor (RAGEs) that promote

reactive oxygen species (ROS), nuclear factor kappa-B (NF-κB), and cytokines such as interleukin (IL)-1β, IL-6, IL-18, tumor necrosis factor-alpha (TNF-α).These proinflammatory cytokines increases intracellular ROS production and activates inflammation cascade [91,92] The end of these pathways causes changings in the structure of the myocardium. Also $AGE_S/RAGE_S$ pathway induces inflammatory signals on extracellular macrophages and smooth muscle cells, leading to increased ROS and decreased production of nitric oxide synthesis, thus triggering the development of DCM [93,94]. Hyperglycemia, excess of ROS and AGEs stimulate Protein kinase C (PKC) to impair VSM function. This leads to vascular remodeling and accelerated development of Diabetic heart disease [95,96]. Other mechanism synergistically act for both impairing the structural function of the heart and triggering the development of Diabetic heart disease. Figure(1- 5) [59].

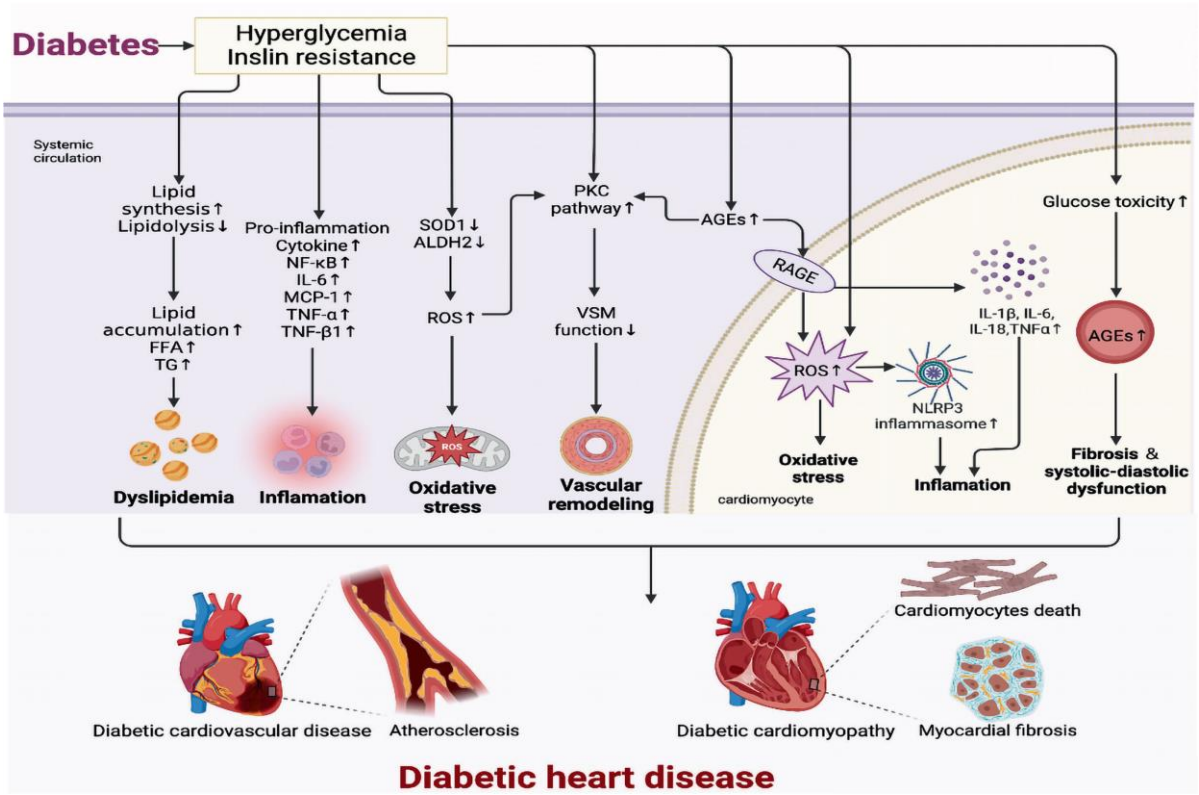


Figure (1-5): Pathology and molecular mechanisms of diabetic heart disease [59].

1.1.2.6 The diagnosis of T2DM:

The diagnosis of T2DM is made when fasting plasma glucose is ≥7.0mmol/L (126 mg/dl) or random plasma glucose is ≥11.1 mmol/L (200 mg/dl) in the presence of diabetes symptoms such as frequent urination, thirst(polydipsia) and unexplained weight loss (Box 1). The oral glucose tolerance test (OGTT) can also be established as a diagnostic tool, where this test is made and the diagnosis can be confirmed if a plasma glucose level of ≥11.1mmol/L after two hours from the ingestion of a 75g glucose solution[97]. The OGTT has been substituted by the HbA1c test and the OGTT is now mainly used in the diagnosis of gestational diabetes. HbA1c cutoff for diagnosing diabetes is 48mmol/mol (6.5%). Repeating both HbA1c or plasma glucose test is recommended in an asymptomatic person for confirming the diagnosis, preferably using the same test. If both HbA1c or plasma glucose measurements are in diabetic range, a diagnosis can be made [98].

Box 1**:** The International Diabetes Federation and World Health Organization.[97.98]

Presence of diabetes symptoms (e.g. frequent urination, thirst, unexplained weight loss) and one of the following abnormal test results:

A fasting plasma glucose concentration of ≥7.0mmol/L;
A random venous plasma glucose concentration of ≥11.1mmol/L;
A plasma glucose concentration of ≥11.1mmol/L two hours after 75g anhydrous glucose in an oral glucose tolerance test;
An HbA1c level of ≥48mmol/mol (6.5%).

In the absence of diabetes symptoms, two abnormal test results are required for confirmation (preferably the same test. However, there are patient groups in whom HbA1c is inappropriate for diagnosis, including:

Children; pregnant women; people who are taking medicines that can cause an acute glucose rise (e.g. steroids or antipsychotics); people with acute pancreatic damage; people with haematological conditions that may influence HbA1c and its measurement (e.g. haemoglobinopathies, decreased erythropoiesis/administration of erythropoietin, erythrocyte destruction, alcoholism, chronic kidney disease and chronic opioid us.

Urinary glucose should not be used as a diagnostic test owing to its low sensitivity[99]. Diagnosis should be further investigated in people diagnosed with T2DM who failed to respond to oral antihypoglycaemic agents. Additional diagnostic tests are often required, such as 'GAD' autoantibody tests or C-peptide tests, to distinguish between TIDM and T2DM. Other types of diabetes mellitus must also be excluded, such as maturity-onset diabetes of the young (MODY), which is characterized by impaired insulin secretion with minimal or no defects in insulin action resulting from genetic defects in β-cell function[100].

1.1.2.7 Treatment of T2DM:

Patients with T2DM are initially treated by changing of lifestyles such as diet, weight reduction, and increased physical activity[101]. Dietary interference should include both low caloric intake and reduction of high carbohydrates diets. Physical activity improves both calorie expenditure and insulin sensitivity in muscle tissue [102]. Sulphonylurea drugs such as gliclazide, glipizide, glibenclamide or glimepiride stimulate insulin secretion. Metformin which is a biguanides , can also be used and is especially useful in obese patients. Metformin decreases both glucose absorption and gluconeogenesis in the intestine and liver respectively. It increases the sensivity of tissues to insulin. It accumulates lactic acid by inhibiting oxidative phosphorylation under certain circumstances. Acarbose hinders glucose absorption after a meal by inhibiting a-glucosidase. Other drugs such as rosiglitazone and pioglitazone increase the transcription of nuclear proteins that control free fatty acid and tissue glucose uptake and decrease insulin resistance through activation of g-peroxisome proliferator-activated receptors [103].

The glucagon like peptide one (GLP-1) receptor agonists increases insulin release from the pancreas by stimulating the GLP-1 receptors and inhibiting glucagon secretion in the pancreas. It also increases weight loss which may decrease IR. Dipeptidyl peptidase-4 inhibitors**(** DPP-4) prevent the breakdown GLP-1 and gastric inhibitory polypeptide(GIP) [104,105].

1.2 Biochemical markers in diabetes mellitus:

1.2.1 Insulin:

Insulin is a peptide hormone with a molecular weight of 5808 dalton produced by the β cells of the pancreas, and it is responsible for regulating the metabolism of glucose.[106] Insulin is consisted of two chains, the A chain with 21 amino acids and the B chain with 30 amino acids is linked by disulfide bridges figure (1-6) [107,108]. The pro-insulin which is a precursor of insulin, contains 74 amino acids with molecular weight of 5802 Da[109]. This precursor is secreted by the β cells and is relatively inactive under biological conditions, but it becomes active after cleavage to produce (A and B chains) and the biologically inactive C peptide [110]. The precursor proinsulin and its products(insulin and C-peptide) are stored in granules in the β cells. These granules release their continent to the capillaries of the islet cells under stimuli [111]. The most important stimuli is the glucose concentration in circulation[112], other stimuli are fatty acids, amino acids, several hormones, and keto acids secreted by the gastrointestinal tract[113]. it is inhibited by somatostatin and by sympathetic activation of the nervous system(fight or flight response)[114].

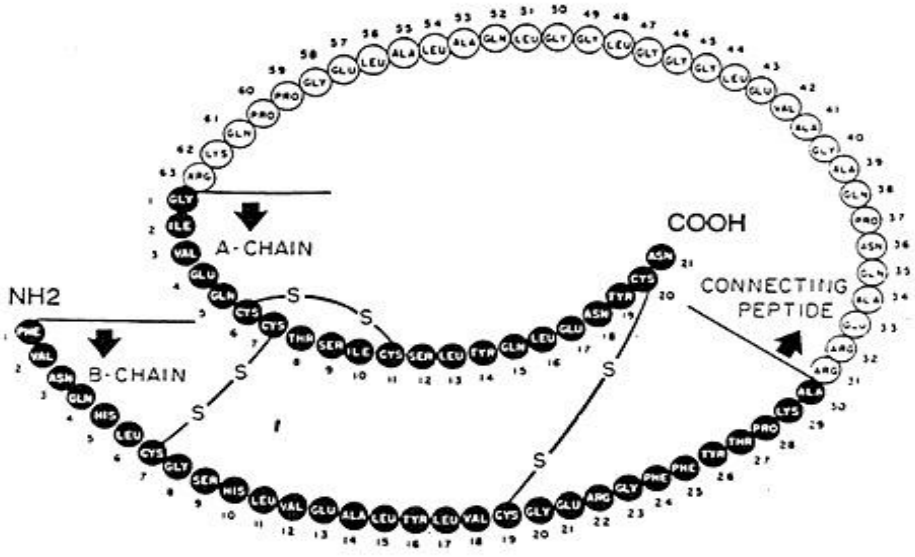
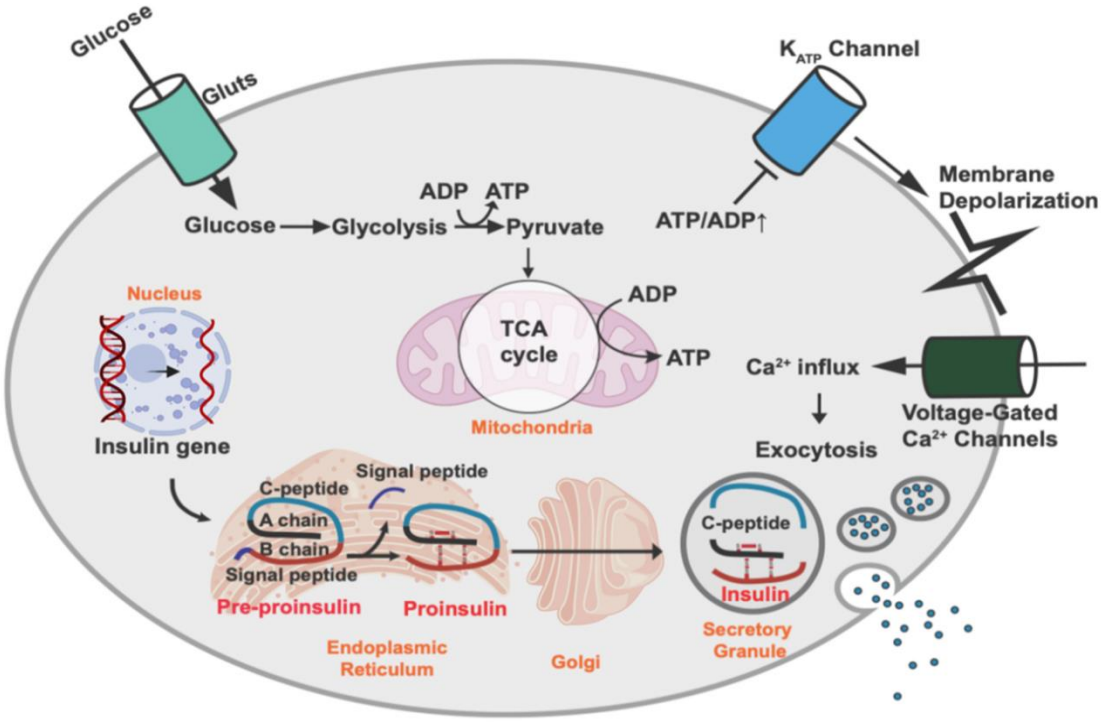


Figure (1-6): the structure of insulin[107].

The biosynthesis of Insulin is began in the nucleus where insulin gene is transcribed and mRNA is translated into pre-proinsulin figure (1-7). The latter is translocated into the ER and the signaling peptide is removed, resulting in creating proinsulin. Both folding and stabilizing of proinsulin is occurred by a disulfide bond in the ER and then transported into the secretory granules, where proinsulin is cleaved to C-peptide and insulin. The major stimulus of insulin secretion is glucose which is transported across the β-cell plasma membrane via the glucose transporter GLUT1. Then, glucose is metabolized to pyruvate via glycolysis. The pyruvate is further metabolized to produce adenosine triphosphate (ATP) through oxidative phosphorylation in the mitochondria. The increment in the ratio of ATP/ADP causes closure of the ATP-sensitive K^+ channels and membrane depolarization, resulting in the opening of L-type voltage-gated Ca^{++} channels (L-VGCCs). The extracellular calcium ions influx into the β cells which induce the exocytosis of secretory vesicles with insulin[115].



Figure(1-7): Insulin biosynthesis and secretion by pancreatic β-cells[115].

Insulin is an anabolic hormone that binds with specific receptors on the outer membrane of target cells to trigger the translocation of glucose transporters to the cell membrane from within the cell and stimulates lipogenesis, glycogenesis, glucose uptake, and protein synthesis for skeletal muscle and fat tissues through the kinase tyrosine receptor pathway [116,117].

1.2.1.1 Insulin receptor

Insulin receptor which is a transmembrane ligand activated receptor and belongs to tyrosine kinase family, is consisted of two α and two β subunits. Insulin binds to its receptor and causes conformation changes of the insulin receptor, leading to induce auto-phosphorylation of tyrosine residues in the β subunit [118]. This auto-phosphorylation stimulates tyrosine phosphorylation of intracellular substrate proteins called insulin-

responsive substrates (IRS).This process continues to include other signaling molecules and induces downstream substrates, leading to recruitment of glucose transporter 4 (GLUT4) to the cell membrane, which allows glucose uptake. Two different pathways result from The binding of insulin to its receptor: the phosphatidylinositol-3-kinase (PI3K)-protein kinase B (Akt or PKB) pathway and mitogen-activated protein kinase (MAPK) pathway. The AKt pathway is responsible for most of the metabolic effects of insulin, while the MAPK pathway is involved in the regulation of gene expression, cell growth and differentiation control figure (1-8) [119].

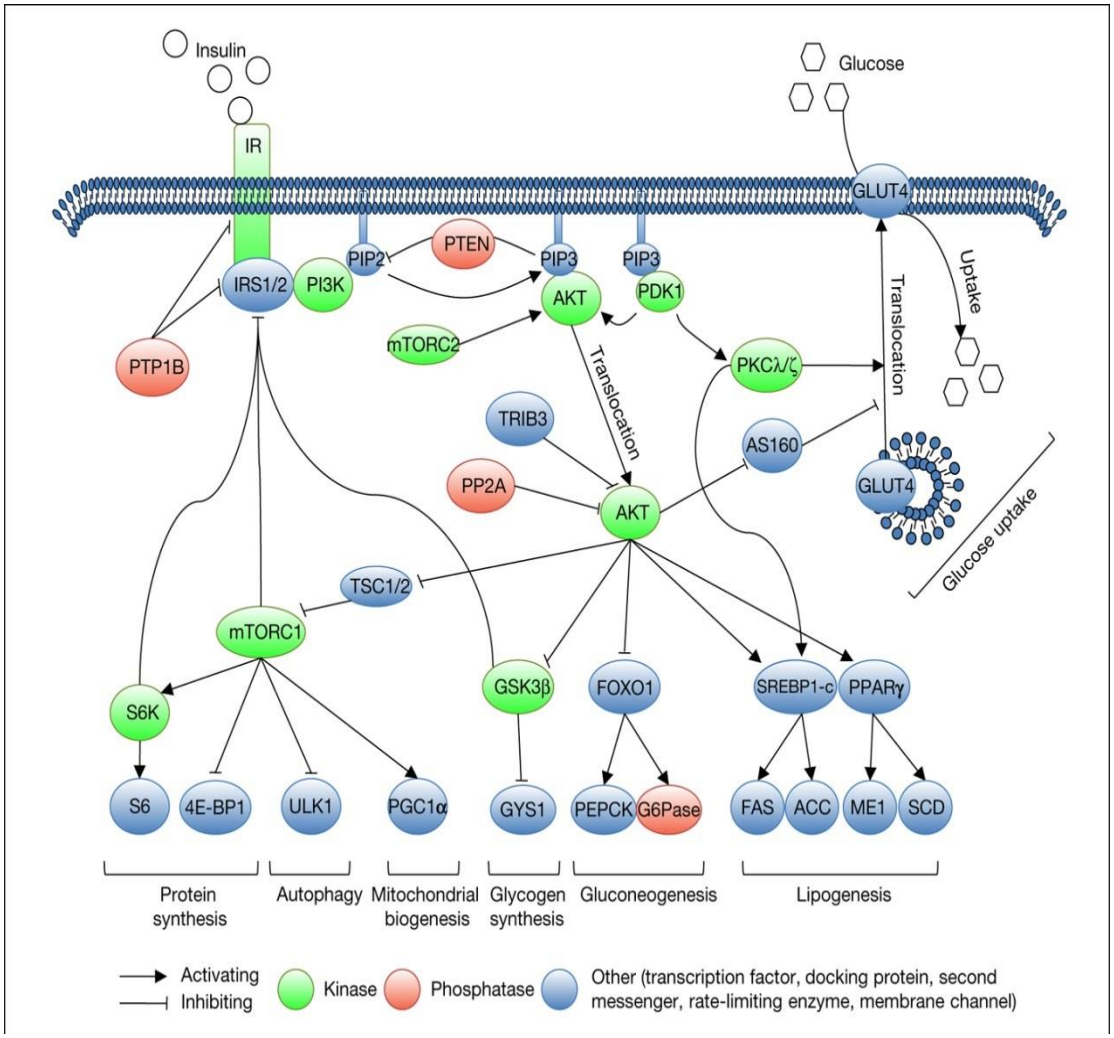
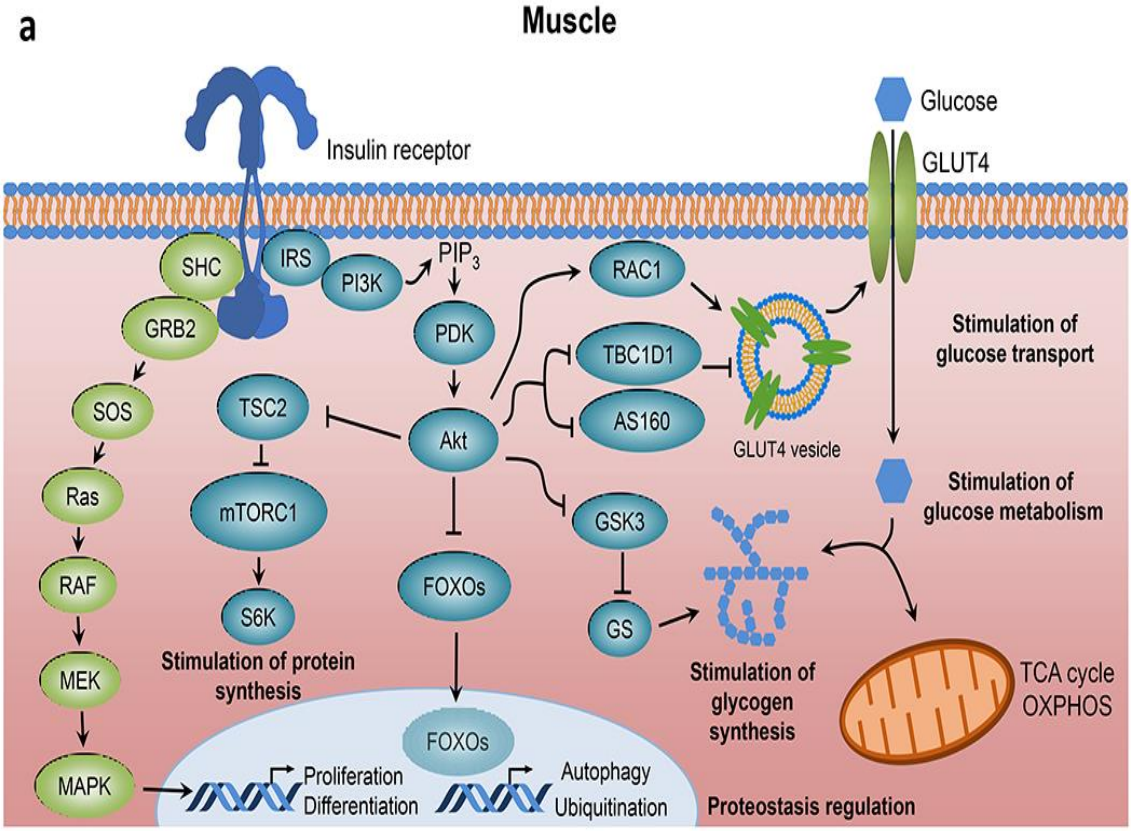
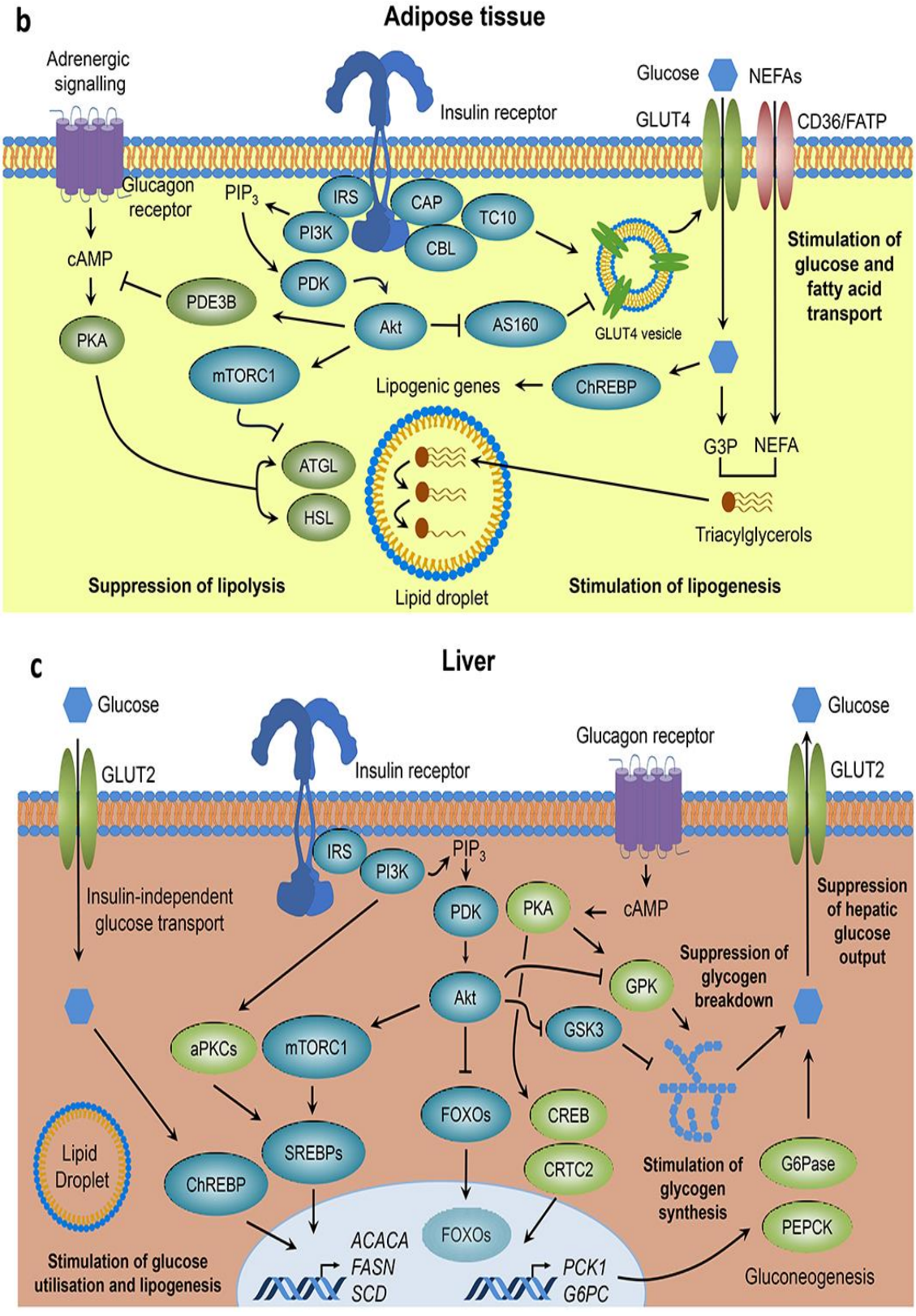


Figure (1-8): PI3K/AKt signaling pathway.

The biological outcomes from either activation or disruption of insulin signal transduction are highly based on the cell type and physiological context figure(1-9). In skeletal muscle, insulin stimulates glucose transport and utilization, glycogen synthesis and inhibits protein catabolism Figure (1-9 a). Insulin induces glucose transport , lipoenesis and inhibits lipolysis in adipose tissues figure (1-9 b). In liver, insulin acts to inhibit glucose production and fatty acid oxidation and promotes glycogen synthesis and lipogenesis figure(1-9 c). Insulin can also regulate metabolism indirectly. For example insulin inhibits both lipolysis in fat and protein catabolism in muscle, reduces substrate supply for gluconeogenesis in the liver [120].





Figure(1-9): Insulin signalling in classical tissues. (**a**) skeletal muscle, (**b**) adipose tissue and (**c**) liver.

1.2.1.2 Insulin and insulin resistance:

Some type of cells resist normal insulin levels so that these cells require more insulin levels to maintain their normal functions, this physiologically called IR. Insuin inhibits gluconeogenesis , lipolysis, glucose uptake and glycogenesis. At normal levels, This normal functions are not seen in insulin resistant tissues [121]. Glucose disposal is occurred in skeletal muscle which is considered as a central tissue for this process and liver and adipose tissue are the sensitive sites for glucose-induced insulin signaling, these tissues are main site for the understanding of the mechanisms responsible for insulin resistance as following below:

1.2.1.2.1 Skeletal muscle

The consumption of glucose is largely relied on skeletal muscle by inducing its uptake under insulin stimulation [122].The GLUT4 is the most important transporter isoform in the skeletal muscle and about 80% is lacated in GLUT4 storage vesicles (GSV). GLUT4 is translocated from GSV to cell surface of the muscle through insulin stimulation or exercise [122,123]. The translocation of GLUT4 to the cell surface is disrupted in T2DM. [124] In T2DM, GLUT4 translocation and glucose transporting are triggered by hypoxia or exercise through (AMPK)-mediated regulation of GSV translocation. So that, the suggestion that the abnormality in the glucose transporting in insulin resistance is due to impairment in the signaling pathway of insulin rather than impairment in the transporting system itself. Indeed, the defect at the proximal level of insulin signaling for example, in the activities of IRTK, IRS1, PI3K, and AKT due to insulin resistance in skeletal muscle contributes to this abnormality. Moreover, the tyrosine kinase activity of IRTK , IRS1

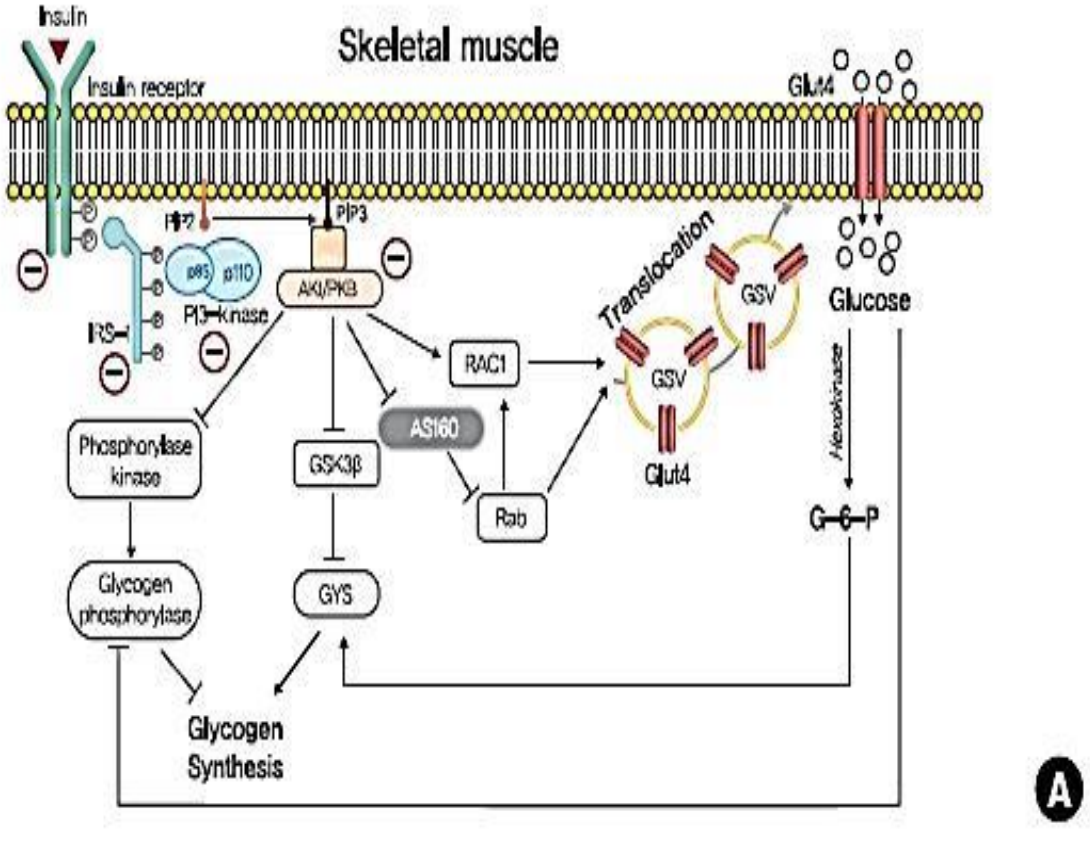
tyrosine phosphorylation and IRS1-associated PI3K activity is reduced in skeletal muscle of obese mice and obese/diabetic humans, which supports this suggestion figure(1-10A) [125].

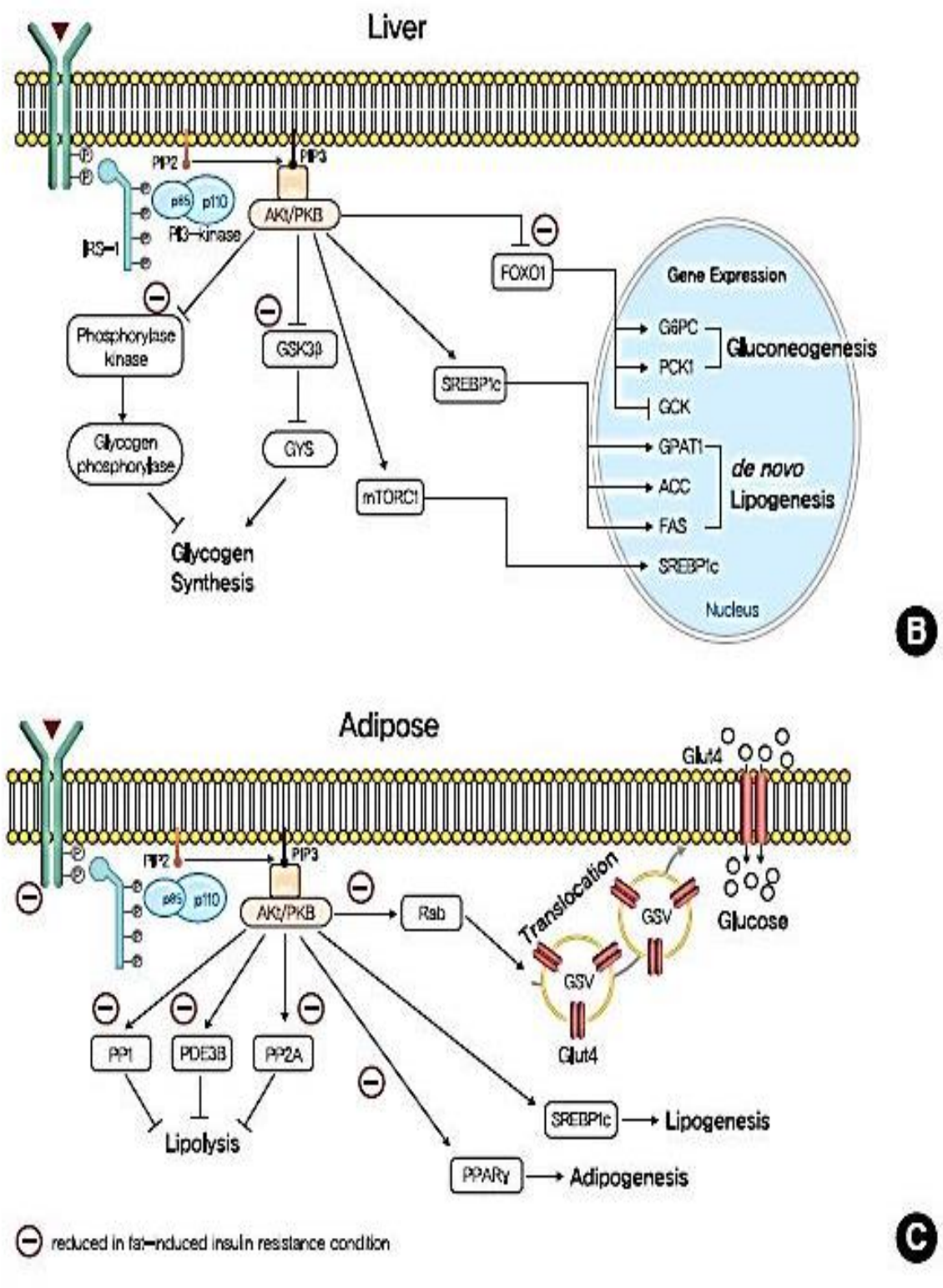
1.2.1.2.2 Insulin resistance in liver and adipose tissue

In the liver , the levels of postprandial carbohydrate is controlled by inhibiting of hepatic glucose production and promoting the synthesis of glycogen from glucose. In fasting state , the liver is the primary source of glucose production [126]. Glycogenesis and gluconeogenesis cannot be regulated by insulin and fasting hyperglycemia in T2DM is primarily due to gluconeogenesis in the liver [127,128]. Defect in the suppression of the hepatic gluconeogenesis is predominantly related to lipolysis defects in adipose tissue and the de-suppression of forkhead box protein O1 (FOXO1) transcription factor in liver [126].There are several proposal mechanism of selective insulin resistance that have been suggested. One of these mechanism involve the differences in the substrate specificities of AKT phosphorylation between gluconeogenesis and lipogenesis [129].The phosphorylation of Akt at ser473 may stimulate some of AKT substrates associated with gluconeogenesis, such as FOXO, and these stimulation might be inhibited in a case of insulin resistance. Other substrates, such as glycogen synthase kinase 3 beta (GSK3β) and tuberous sclerosis complex 2 (TSC2), which require the phosphorylation of AKT at Thr308, might not be disrupted [130,131].The second mechanism involves activation of Sterol regulatory element-binding proteins (SERBP-1c) and inhibition of gluconeogenesis, which both functions require specific insulin levels [132,133]figure(1-10 b). In white adipocyte tissue, insulin suppresses lipolysis which in turn inhibites

hepatic glucose production by decreasing gluconeogenic substrates figure(1-10c) 1C) [134].

The mechanism of insulin-induced lipolysis suppression is mediated by phosphodiesterase 3B (PDE3B) through reduces the activity of cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) [135]. furthermore, protein phosphatase 1(PP1) and protein phosphatase-2A (PP2A) mediate PI3K-dependent insulin-induced lipolysis suppression through the dephosphorylations of lipolytic regulatory proteins [136,137]. Insulin also promotes lipogenesis by stimulating SREBP-1c, inducing the signal of the translocations of glucose or fatty acid transport proteins (FATPs), activating fatty acid esterification [138], and promoting adipogenesis through the transcription factor peroxisome proliferator-activated receptor-γ (PPARγ) [139]





Figure(1-10) : Role of insulin signaling in A- skeletal muscle. B-liver and C- adipose tissue[125].

1.2.2 Resistin:

Resistin (Retn) is a hormone with high cysteine residues which is mainly produced by macrophages and white adipocytes. Structurally, it has different forms in the blood circulation , mainly as trimers, hexamers, and also polymers [140]. Retn is encoded by *retn* gene that is located on 19p13.2 figure (1-11) [141]. Retn is a pre-polypeptide precursor with a low molecular weight (12.5 kDa) and composed of 108 amino acids that includes a signal peptide, a variable region, and a conserved C-terminus. In the circulation, it is presented as a dimeric protein consisting of two 92-amino acid polypeptides that are connected by a disulfide bridge forming both high- and low-molecular weight complexes (Figure 1-12). Retn has a common feature that is the existence of a motif (10–11 cysteine-rich) at the carboxyl terminus that could support the globular domain of the retn monomer via the formation of 5 disulfide bridges. Also the formation of dimer, trimer and hexamer structure of circulating retn is based on disulfide and non-disulfide bonds figure(1-12) [142].

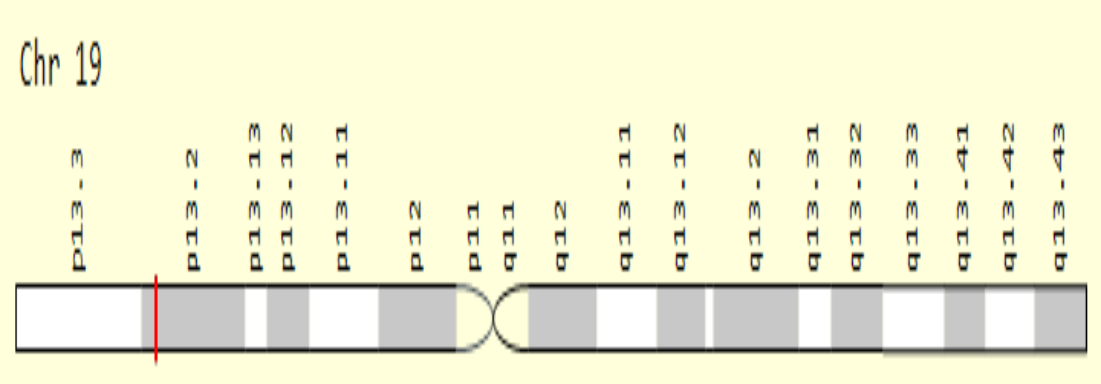


Figure (1-11): *Retn* gene location.

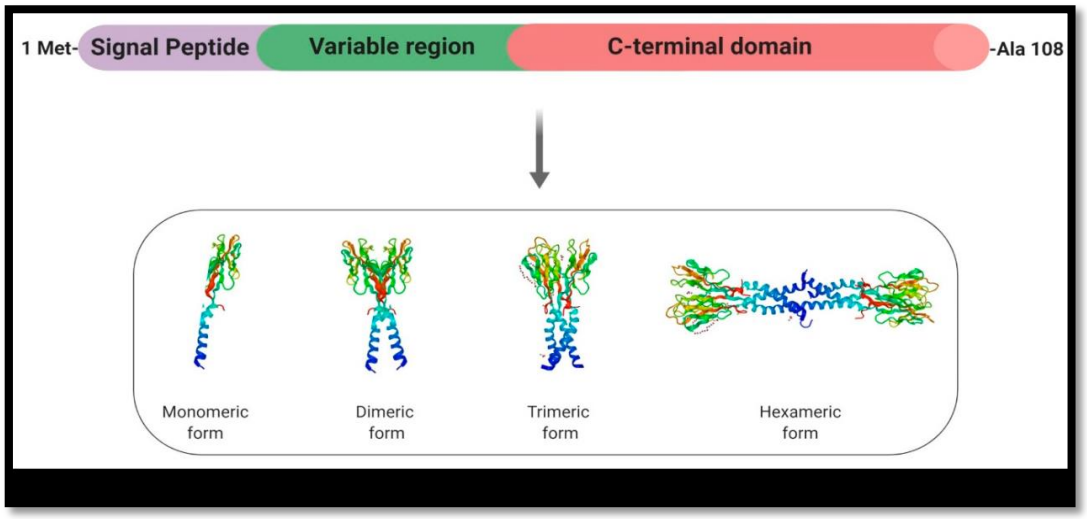


Figure (1-12): Structure and specific forms of human retn.

1.2.2.1 Resistin receptor and function:

Resistin plays a pivotal role in inflammation process because of its expression and secretion from human macrophages is promoted by various pro-inflammatory stimuli [143], a variant of decurin (Δ-DCN) is one of retn receptor that was mainly presented in white adipose tissue, lung and bone marrow, and its production is increased in obese individuals to regulate white adipose tissue expansion [144,145]; the Toll-Like Receptor 4 (TLR4) is another retn receptor that stimulate signaling pathways of retn [146]; and the third receptor suggested is the Adenylyl cyclase associated protein 1 (CAP-1), that could promtes NF-κB gene expression which is mediated by PKA, leading to the expression of pro-inflammatory cytokines [143]. The presence of high cysteines residues in the sequence of retn explain the interaction of retn with these receptors so that retn can be a potential target. Retn can be regulated at the endocrine level to exert therapeutic preventive effect in their functions such as regulating of glucose,lipid metabolism, pituitary somatotropin cells, and the satiety from the hypothalamus[147,148].

The variant of decurin receptor is associated with WAT [144,145]. So that, the adipose tissue can be expanded, leading to hypertrophy of adipocyte and releasing of adipokines that promote increased mobilization of M1 macrophages from the circulation. This can leads to shifting of anti-inflammatory macrophages (M2 phenotype) to pro-inflammatory macrophages (M1 phenotype) in cellular composition surrounding WAT. Consequently this will increase cytokine production and promote adipose tissue dysfunction and disturb of glucose tolerance [149]. When retn concentration is increased as a result of increasing of WAT and macrophages; the effect of TLR4 is appeared; Retn and TLR4 interaction can participate to produce insulin resistance [146].The interaction between Adenylyl cyclase associated protein 1 (CAP-1) and retn can be occurred through endocytotic process in cultured monocytes, leading to increase the pro-inflammatory cytokines [150,151]. The changes above can result in increased lipolysis, FFA , altered adipokine secretion and finally may participate in insulin resistance Figure(1-13) [152,153].

1.2.3 Insulin, insulin resistance, obesity and retn in T2DM:

Obesity is defined as abnormal accumulation of fat in the adipose tissue caused by persistent over nutrition or low physical activity or hereditary causes [154]. Obesity induces the risky of T2DM and other diseases. About 80% of diabetic individuals are Obese which explain the closely relationship between both diseases. Obesity is a common finding in T2DM. In obese patients, the insulin sensitivity is impaired in the peripheral tissues such as liver, muscle and fat cells (IR). There are at least three distinct mechanisms that have been suggested to connect

obesity with insulin resistance and lead to T2DM: 1. High levels of adipokines and cytokines such as tumor necrosis factor-α, retn, and

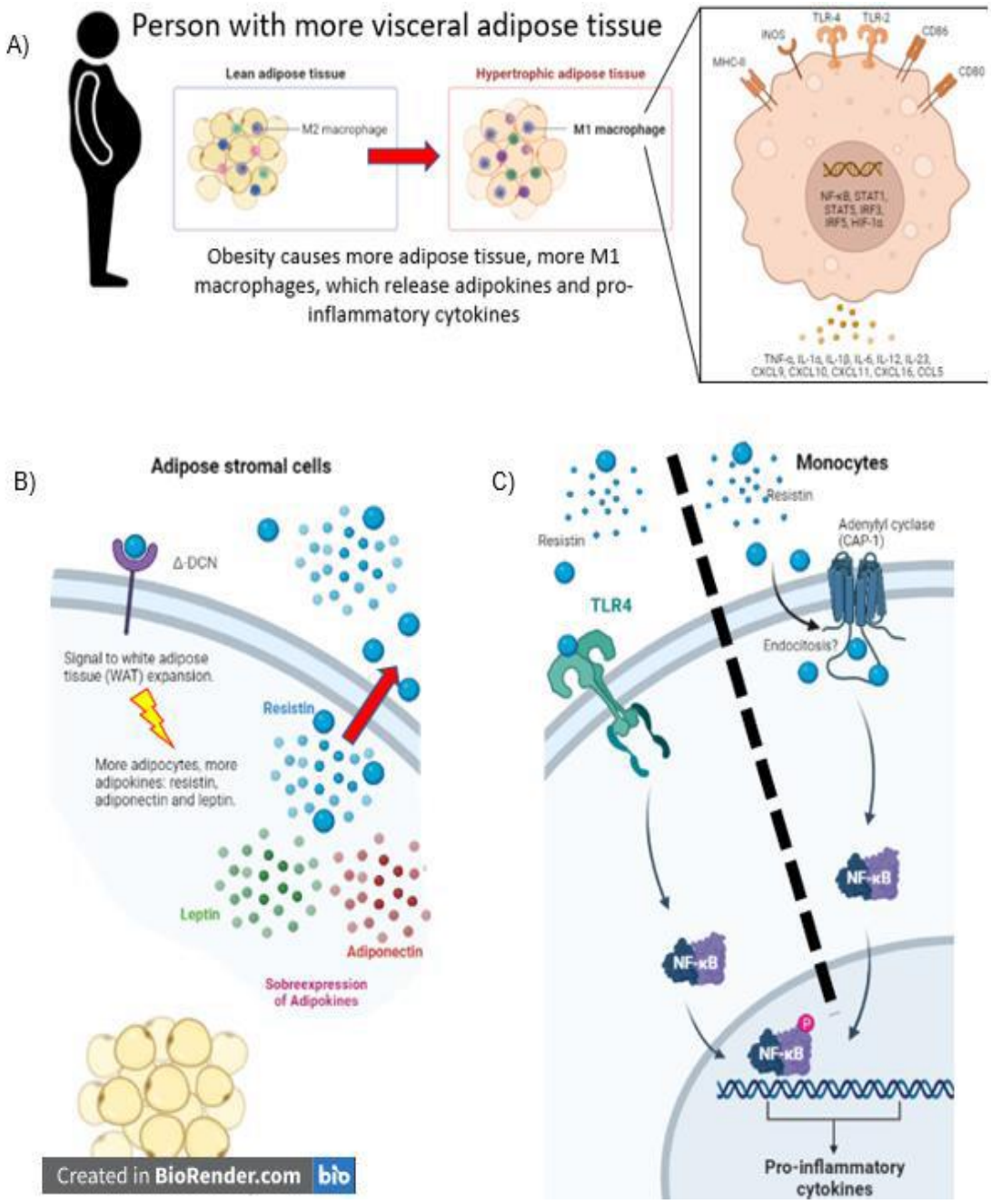


Figure (1-13)**:** Resistin levels are increased by different ways. (**A**) obesity causes an increased of adipose tissue and recruitment of M1 macrophage, leading to the releasing of both adipokines and pro-inflammatory cytokines (**B**) In stromal cells of adipose tissues, DCN receptor promote the WAT expansion , leading to increase the secretion of adipokinesș (**C**) TLR4 and CAP-1 receptor in monocytes stimulate the releasing of pro-inflammatory cytokines [152-153].

retinol binding protein, that participate in IR as well as low levels of adiponectin ; 2. the deposition of fat in ectopic sites, especially in liver and in skeletal muscle; and 3. mitochondrial dysfunction, manifested by both low mitochondrial mass and/or function[155]. The most important impairment of the above mechanism is mitochondrial dysfunction that links obesity to diabetes by lowering both insulin sensitivity and compromising β-cell function[156]

Despite its low-grade nature, inflammation of adipose tissue negatively affects the function of remote organs, a phenomenon that is considered to cause the complications of obesity [157]. The cell-cell interactions that take place under the stress of obesity are mediated by intracellular contact and cytokine production and constitute a complicated network that drives phenotypic alterations in immune cells and perpetuates a loop of metabolic decline [158].

Insulin resistance is a common feature of obesity and T2DM and comprises dysfunctional adipose tissue, lipotoxic insulin signaling followed by glucotoxicity, oxidative stress, and low-grade inflammation [159]. Insulin resistance is manifested primarily by unoxidized glucose disposition in response to insulin, as well as reduced suppression of lipolysis and hepatic glucose production. Although insulin resistance often leads to T2DM, it initially develops as an adaptive physiological response to obesity, resisting the anabolic pressure of insulin to reduce excessive nutrient storage [160,161].

1.2.4 Polymorphism and expression *retn* gene in T2DM:

In the serum of mammals, retn is detectable and its function may be for regulating body fat mass through both negative feedback inhibition and peripheral signaling. According to İrem Bilgetekin etal a study, the levels of retrn in the plasma have been associated with metabolic and inflammatory factors a [162]. A single nucleotide polymorphisms which is a genetic factors are thought to participate in the increasing incidence of DM through insulin resistance. In the *retn* gene, these SNPs which is encoded the retn protein have been documented to play a role in causing abnormalities of glucose and lipid metabolism in the circulation, so that disturbances in the expression of *retn* gene are assumed to be connected to insulin resistance. *Retn* gene SNPs may require interaction with other factors or genes to induce insulin resistance or act by indirect glucose–fatty acid metabolic cycle mechanisms [163]. SNPs in the *retn* gene have been linked to insulin resistance indices [164]. Insulin resistance which can be caused by the *retn* gene, can negatively impact how insulin works. Numerous both in vivo and in vitro studies have demonstrated that the *retn* gene can impair glucose tolerance and cause insulin resistance. Therefore, it is believed that the *retn* gene represents a key link between obesity and T2DM [165].

Aims of the Study

The current study aims to the following :

1. Estimate the level of retn and its association with insulin resistance, BMI, and FBS in T2DM patients.
2. Evaluate the association between *retn* gene polymorphisms and *retn* gene expression in T2DM.
3. Estimate the relationship between *retn* gene expression and retn level in T2DM development.

2. Materials and Methods

2.1. Instruments and equipment for laboratories.

The instruments and equipment used in this study are shown in table(2-1) :

Table (2-1): Instruments and equipment for laboratories.

No.	Instruments	Company	Country
1.	Autoclave	Haramaya	japan
2.	Centrifuge 5418 R	Eppendorf	Germany
3.	COBAS INTEGRA®400plus	COPAS	Germany
4.	Cooling centrifuge 5424 R	Eppendorf	Germany
5.	Deep Freeze	GFL	Germany
6.	Disposable syringes (5 mL)	Medical jet	Syria
7.	Distillater	O-Purite	UK
8.	EDTA tube (5mL)	AFCO	Jordan
9.	Electronic sensitive balance	Kern	UK
10.	Electrophoresis system	Mupid one	Japan
11.	ELISA system	Bio-tech	USA
12.	Hood	DWYER	USA
13.	Incubator	Memmert	Germany
14.	Micropipettes (0.1-2.5),(0.5-10),(2-20),(20-200),(100-1000) µl	Eppendrof	USA
15.	Microwave	LG	Korea
16.	Nano drop 2000	thermo scientific	USA
17.	Pipette tips (1,10,100,1000 µL)	Dolphin	Syria
18.	Plane tube Centrifuge	CAPP	Denmark
19.	Thermo cycler Rotor gene Q	Qiagen	Germany
20.	Roche COBAS c311	Roche	Germany
21.	Thermo cycler TC5000	Techne	UK
22.	UV transilluminator	Quantum Vilber lourmat	France
23.	Vortex(eppendorf tube)	Heidolph	Germany
24.	Vortex centrifuge (PCR tube)	ExiSpin	Korea
25.	Water bath	GFL	Germany

2.2 Chemical and biological materials used in this study.

Table (2-2): Chemical and biological materials

No.	Chemicals and biological materials	Country	Country
1.	Absolute ethanol 99%	Haymankimia	UK
2.	Agarose	Fisher bioreagents	UK
3.	DDW	O-Purite	UK
4.	DNA Ladder Marker 100bp	Promega	USA
5.	DNA loading dye	Promega	USA
6.	Ethanol 70 %	Haymankimia	UK
10.	Isopropanol 70%	Chem-lab NV	Belgium
11.	Medical cotton	Kardelen	Turkey
12.	Nuclease free water	HIMEDIA	India
13.	Primers	Macrogen	Korea
14.	Proteinase K	Promega	USA
15.	Red safe 1ml	BDH	England
16.	Resistin kit	Biotn	China
17.	TBE buffer(10x)	Intron	Korea

2.3 Marketable kits.

The commercial kits were used in this study are listed in Table (2-3)

Table (2-3): Marketable kits used in the study.

No.	Commercial kits	Company	Country
1.	COBAS HbA_1c kit	Roche	Germany
2.	DNA extraction Favorgen/Favor prep	Biotech CORP	Taiwan
3.	Glucose kit	Roche	Germany
4.	GoTaq G2 green Master Mix	Promega	USA
5.	HbA_1c kit	Roche	Germany
6.	Insulin ELISA Kit	Biont	China

No.	Commercial kits	Company	Country
7.	Resistin ELISA Kit	Biont	China
8.	Reverse Transcription	Biosharp	China
9.	RNA extraction Genezol/TriRNA Pure	Geneald	Taiwan
10.	Real time PCR smart mix (SolGent 2x)	SolGent	China
11.	SYBR Green qPCR Mix Fluorescence quantification PCR	Biosharp	China
12.	Universal SYBR qPCR Master Mix Universal Real-Time PCR	Biosharp	China

2.4 Study groups

The current study has involved 120 participants (60 patients have T2DM and 60 as control groups. Dependent on BMI , each group subdivided equally into (30 obese and 30 non obese) T2DM patients and (30 obese and 30 non obese) controls with BMI range (30 – 38.6 Kg/m^2) obese (19 – 24.6 Kg/m^2) non obese and (30 – 34.2 Kg/m^2) obese (19.5 – 24 Kg/m^2) non obese respectively. The blood samples were collected during the period from 1/3/2022 to 20/9/2022. All patients in this study were referred and diagnosed in the Diabetic and Endocrine Center in Marjan Medical City, while control were selected from medical staff and their relatives as in Figure(2-1). The practical side of the study was performed at the laboratory of chemistry and Biochemistry Department/College of Medicine and College of Science for women /Biology Department/ University of Babylon .

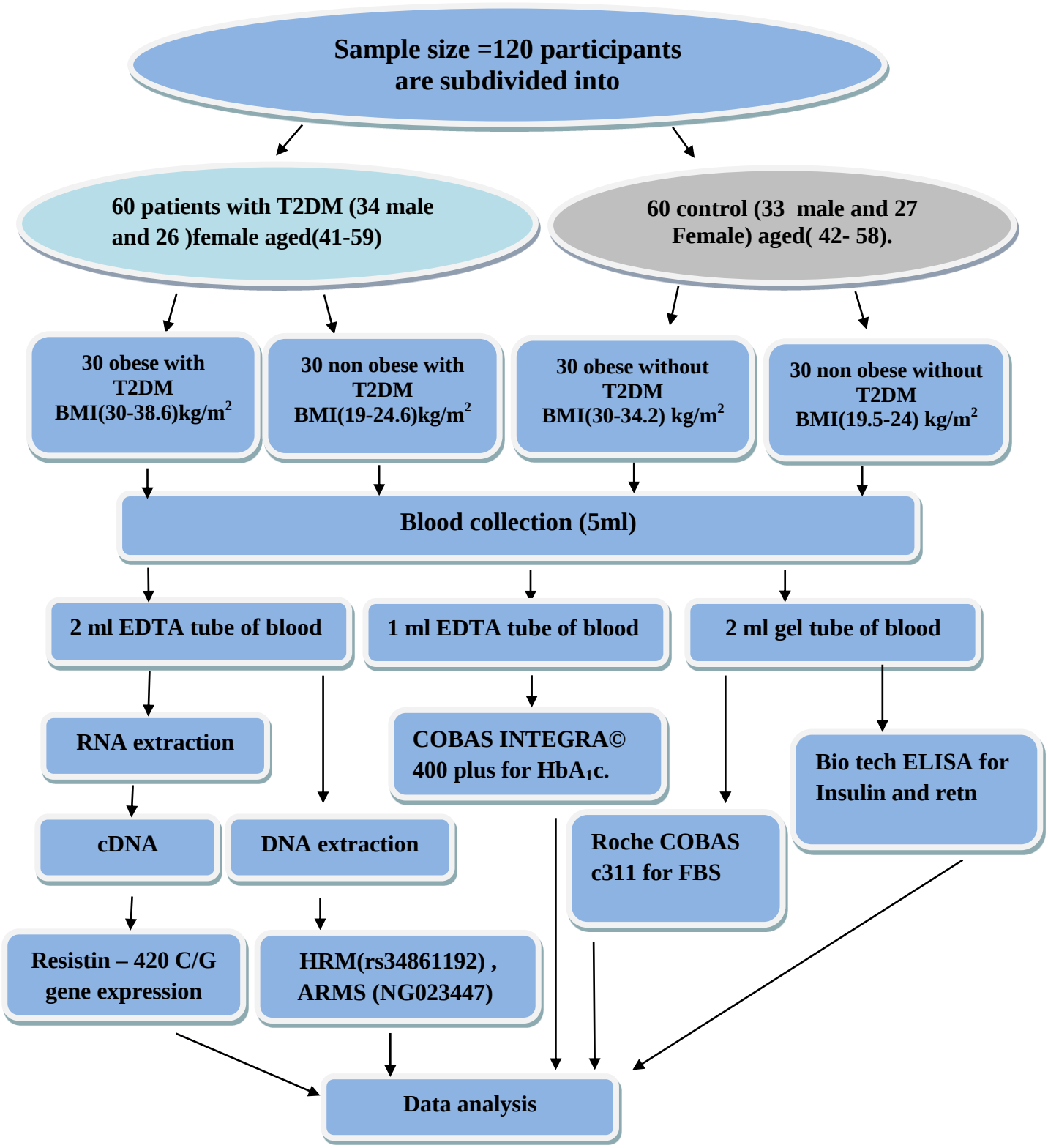


Figure (2-1): Study design.

2.5 Studied Design

Case control study.

2.5.1 Control group

This group included 60 participants(30 non obese and 30 obese without T2DM) (33 male, 27 female) have been enrolled in this study.

2.5.2 Type 2 Diabetes Mellitus group

This group included 60 participants(30 non obese and 30 obese with T2DM) (34 male, 26 Female) have been enrolled in this study.

2.6 Ethical approval

Depends on the following:

a- Approval of scientific committee College of Medicine ,University of Babylon, Iraq and the Biochemistry Department in the same college.

b- Approval of scientific committee of Marjan Medical City in Hilla city.

c- The objectives and methodology of this study were explained to all participants in the current study to gain their verbal acceptance.

2.7 Selection criteria

The inclusion and exclusion criteria for this study were as follows:

- Inclusion criteria:

1-T2DM(for patients group)without complication.

2-Obesity included in half number of participants.

- Exclusion Criteria:

1. Type 1 diabetes mellitus.
2. Malignancy.
3. Auto-immune diseases,
4. Hypertension.

5-Overweight (BMI 25-29.9), underweight (BMI<18.5).

6- Pregnant women.

2.8 Study requirement

a-Questionnaire

The socio- demographic characteristics that composed of age, sex, family history and duration of DM. The questionnaire is show in appendix (1).

b-Anthropometric Measurement

Including:

Measurment of body mass index:

Weight (kg) and height (m), were measured practically. BMI was calculated as weight (in kilograms) divided by the square of height (in meters)[166], weight and height are measured on the same scale for all members of the sample.

$$\mathbf{BMI(Kg/m^2)} = \frac{\mathbf{Weight\ (Kg)}}{\mathbf{(Hight)^2\ m^2}}$$

2.9 Sample collection

Five milliliters of blood were drawn by vein puncture from all individuals participated in this study after taking the consent. The collected blood was divided into three parts:

1. A volume of 2ml of blood was taken that used for gene analysis, and collected in EDTA(ethylene diamine tetra acetic acid) containing tube stored at -20 °C used for DNA and RNA extraction.

2. A volume 1ml of blood was taken and placed in EDTA containing tube for analyzing HbA_1c test .

3. A volume 2ml of blood was drained into gel tube for serum preparation, which would be used in measurement of insulin, retn and FBS.

2.10 Determination of insulin Concentration

Insulin concentration is measured by enzyme linked immunosorbent assay kit .

A. Assay Principle

This ELISA kit uses sandwich method for the accurate quantitative detection of insulin. The plate has been pre-coated with human insulin antibody. Insulin present in the sample is added and bound to antibodies coated on the wells. And then biotinylated human insulin antibody is added and bound to insulin in the sample. Then streptavidin-HRP is added and bound to the biotinylated insulin antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color developed in proportion to the amount of human insulin. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm [167].

B.Reagents Preparation

- **Wash Buffer** :A volume of 20ml of concentrated Wash Buffer was diluted into 480ml of distilled water to yield 500 ml of Wash Buffer.

- **Standard**

A volume of 120ul of the standard.(80mIU/L) was reconstituted with 120ul of standard diluent to generate a 40 mIU /standard stock solution. The standard was allowed to sit for 15 mins with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (40 mIU/L) 1:2 with standard diluent to produce 20 mIU/L, 10 mIU/L, 5 mIU/L and 2.5mIU/L solutions.

Dilution of standard solutions suggested are as follows: (80, 40, 20, 10, 5, 2.5 mIU/L).

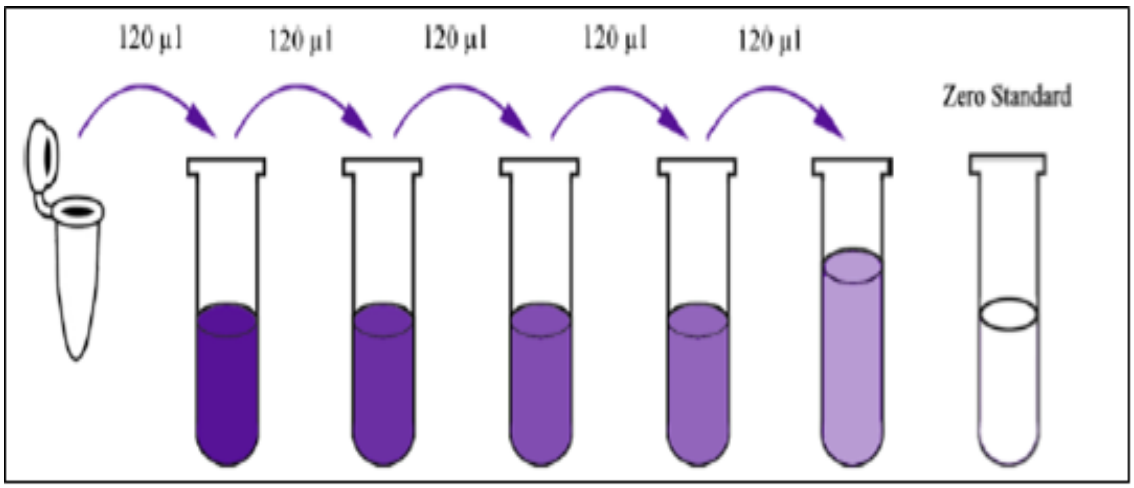


Figure (2-2): Preparation of standard concentration.

C-Assay Procedure

1-All reagents, Standard solutions and samples were prepared as instructed. All reagents were brought to room temperature before use.

2-A volume of 50ul Standard. was added to Standard well**.**

3-A volume of 40ul sample was added to sample wells and then a 10ul human lgals3 antibody was added to sample wells,

4- A volume of 50ul streptavidin-HRP was added to sample wells and Standard. wells. Mixing well. Covering the plate with a sealer. Incubation 60 minutes at 37°c.

5-The sealer was removed and the plate washed 5 times with wash buffer. Wells soaked with 300ul wash buffer for 30 seconds to 1 minute for each wash.

6-A volume of 50ul substrate solution (tetra methyl benzyl) was added to each well and then a volume 50ul substrate solution buffer was added to each well.

7-Plate covered with a new sealer and incubated for 10 minutes at 37°c in the dark.

8-A volume of 50ul stop solution was added to each well, the blue color will change into yellow immediately.

9-Absorbance in each well was read at 450 nm in a micro – plate reader.

10-The results was read within 15 minutes of adding the stop solution. The standard curve is depicted in Figure 2-3.

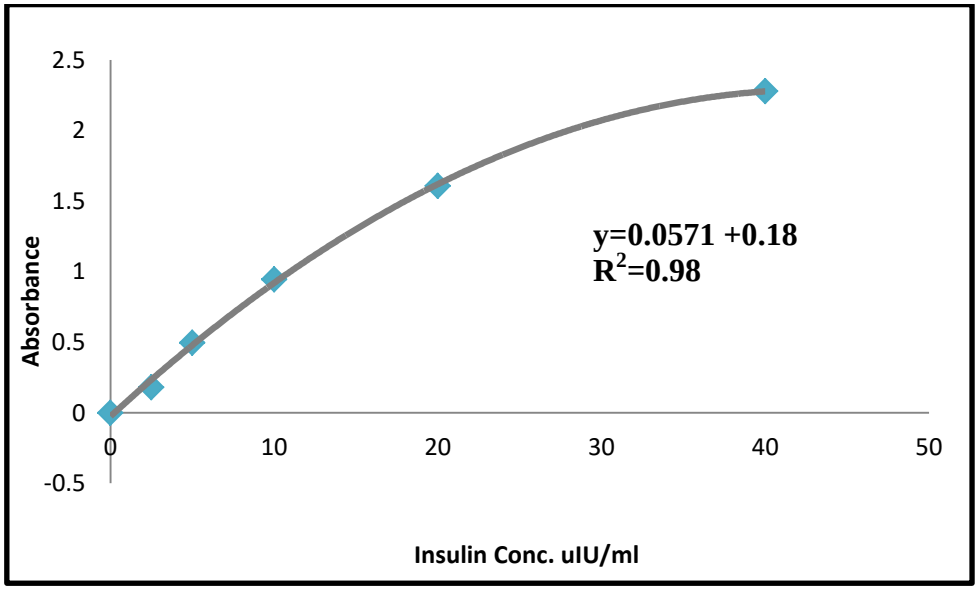


Figure (2-3**):** Standard curve for insulin concentration by ELISA.

2.11 Determination of retn concentration:

Resistin concentration was measured by enzyme linked immune -sorbent assay kit .

A. Assay Principle

This ELISA kit uses sandwich method for the accurate quantitative detection of retn**.** The plate has been pre-coated with human retn antibody. Retn present in the sample is added and bound to antibodies coated on the wells. And then biotinylated human retn antibody is added and bound to retn in the sample. Then Streptavidin-HRP is added and bound to the biotinylated retn antibody.

After incubation, unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color developed in proportion to the amount of human retn. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm[168].

B-Reagents Preparation

Wash buffer: A volume of 20 ml of concentrated wash buffer was diluted into 480 ml of distilled water to yield 500 ml of washing buffer.

Standard

A volume of 120ul of the standard.(6400ng/L) was reconstituted with 120ul of standard diluent to generate a 3200ng/ standard stock solution. The standard. was allowed to sit for 15 mins with gentle agitation prior to making dilutions. Duplicated standard points were prepared by serially diluting the standard stock solution (3200ng/L) 1:2 with standard diluent to produce 1600ng/L, 800ng/L, 400ng/L and 200ng/L solutions as shown in Figure (2-4).

Dilution of standard solutions suggested are as follows: (6400,3200, 1600, 800, 400, 200 ng/L)

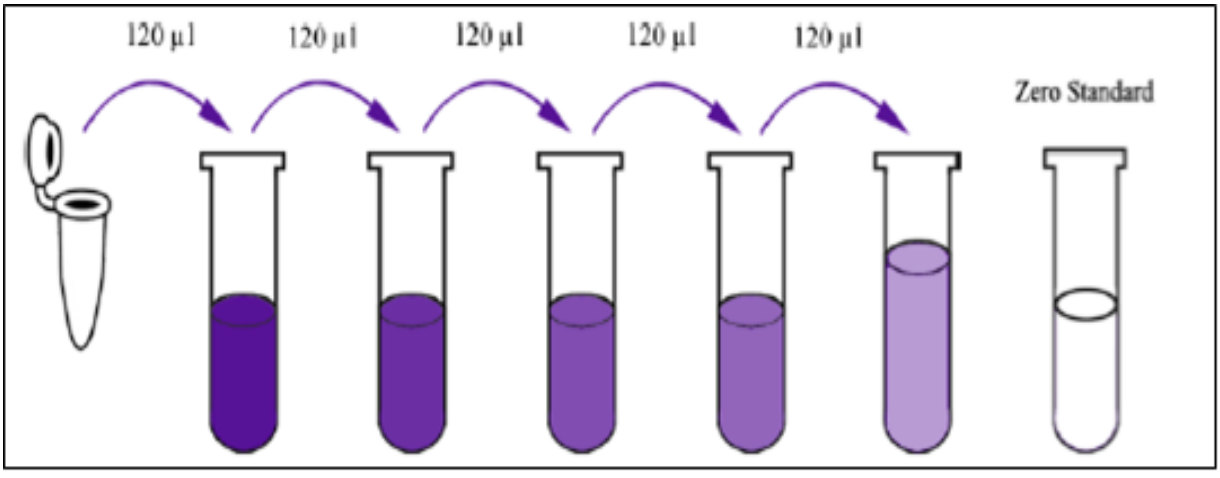


Figure (2-4): Preparation of standard concentration.

C-Assay Procedure

1. All reagents, Standard solutions and samples were prepared as instructed. All reagents were brought to room temperature before use.
2. A volume of 50ul Standard was added to Standard well**.**
3. A volume of 40ul sample was added to sample wells and then a 10ul human antibody was added to sample wells,
4. a volume of 50ul streptavidin-HRP was added to sample wells and Std. wells. Mixing well. Covering the plate with a sealer. Incubation 60 minutes at 37°c.
5. The sealer was removed and the plate washed 5 times with wash buffer. Wells soaked with 300ul wash buffer for 30 seconds to 1 minute for each wash.
6. A volume of 50ul substrate solution a was added to each well and then a volume 50ul substrate solution b was added to each well.
7. Plate covered with a new sealer and incubated for 10 minutes at 37°c in the dark.
8. A volume of 50ul stop solution was added to each well, the blue color will change into yellow immediately.
9. Absorbance in each well was read at 450 nm in a micro – plate reader.
10. The results should be read within 15 minutes of adding the stop solution. The standard curve is depicted in Figure 2-5

.

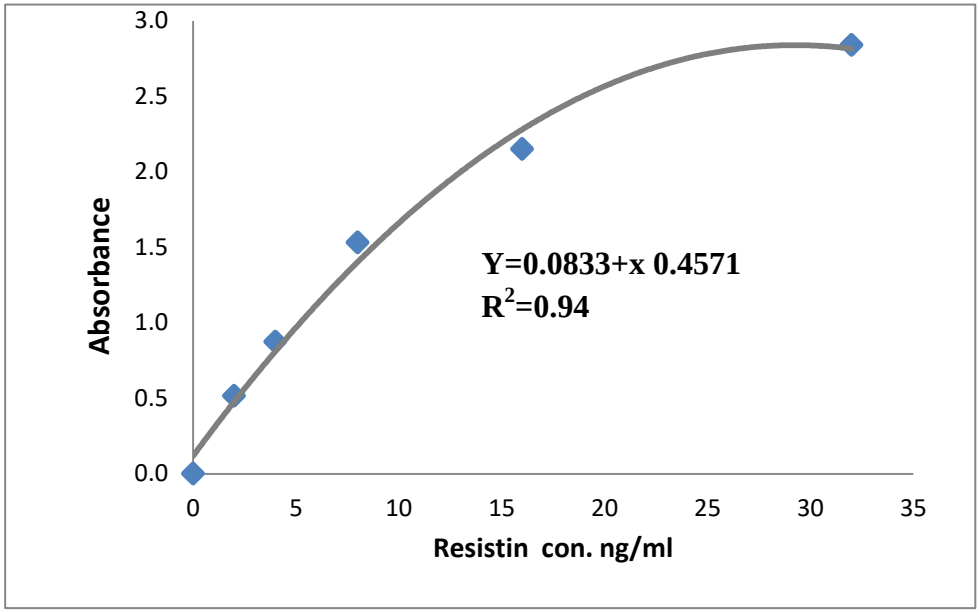


Figure (2-5): Standard curve for retn concentration by ELISA .

2.12 Determination of insulin resistance

HOMA-IR stands for homeostatic model assessment of insulin resistance. The meaningful part of the acronym is “insulin resistance”. It marks for both the presence and extent of any insulin resistance that you might currently express. It is a terrific way to reveal the dynamic between fasting blood sugar and the responsive hormone insulin.

The HOMA-IR (Mass Units) is an approximating equation for insulin resistance [169].

HOMA-IR was calculated using the formula:

HOMA-IR = fasting insulin µIU/ ml * fasting glucose mg/dl / 405

2.13 Determination of blood glucose concentration by enzymatic method

Principle

Ultra violet test / Enzymatic reference method with hexokinase.

Hexokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate by ATP. Glucose-6-phosphate dehydrogenase oxidizes glucose-6-phosphate in the presence of NADP to gluconate-6-phosphate. No other carbohydrate is oxidized. The rate of NADPH formation during the reaction is directly proportional to the glucose concentration and is measured photometrically[170].

2.14 Measurement of glycated hemoglobin (HbA_1c)

This test to determined glycated hemoglobin A1c (HbA1c) by COBAS INTEGRA400®plus by used COBAS HbA_1c kit, normal value of HbA_1c level less than 5.7%, a level between 5.7% to 6.4% shows pre-diabetes while level above 6.5% is indicative of diabetes mellitus .

Principle

Turbidity**:** Turbid metric measurement is made with spectrophotometer to determine the concentration of particulate matter in a sample. The amount of light blocked by suspension of particles depends not on concentration but also on size, because particles tend to aggregate and settle out of suspension sample handling becomes critical. Instrument operation is the same as for any spectrophotometer [171].

Calculation

Automatic calculation by using the COBAS INTEGRA® 400 plus.

2.15 Deoxyribonucleic Acid Extraction(Favogen) .

Principle

Genomic DNA was extracted from patients' peripheral blood in the laboratory of the Department of Biochemistry at College of Medicine, University of Babylon, the DNA was kept at (-20)°C.

Total DNA can be extracted from a variety of sources quickly and easily using a frozen DNA extraction kit (Whole Blood), peripheral blood monocytes can be used for DNA collecting, the Chaotropic salt causes cell lysis and protein degradation, allowing DNA fragments to adhere to the spindle fiberglass matrix. Elution buffer, low salt was used to elute purified DNA after DNA contaminants were removed with wash buffer solution containing ethanol [172] as Figure (2-6).

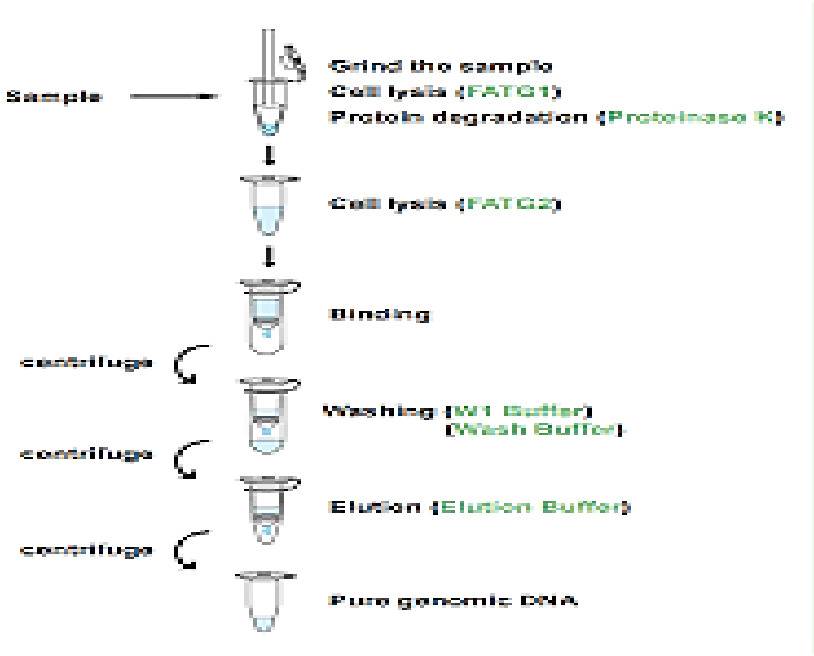


Figure (2-6): Principle of deoxyribonucleic acid extraction.

Preparation of Solutions

As recommended on the kit's leaflet, with a few changes DNA was extracted from venous blood by means of the preferred genomic DNA purification kit and another G-spin, total DNA Extraction buffer was prepared, briefly as follows:

1- Proteinase K was dissolved in 1.1 mL of deionized water.

2- Before the first used, a volume of 100 mL of absolute ethanol was placed in the washing solution.

3- The elution buffer (EL) solution was heated in a 70 °C water bath, before starting the extraction process.

Step 1: included the following steps

1- A volume of 200 µL from frozen blood in 1.5mL microcentrifuge tube was added.
2- The blood tube had been mixed thoroughly by pulse- vortex with 40µL of proteinase K.
3- The mixture was incubated at 60 ºC for 15 minutes to lysis the leucocytes, the tube was inverted 2-3 times during the incubation period.
4- A volume of 5 µL RNase (10mg/mL) was added to the mixture and it was shaken strongly then followed the DNA binding step.

Step 2: Cell lysis

1- A volume of 200 µL FABG buffer was added to the sample mixture and shaken by vortex then incubated the mixture at 25ºC for 10 min, during incubation period, the tube was inverted every 3 minutes.
2- A volume of 5 µL RNase (10mg/mL) was added to the mixture and shaken strongly.

Step 3: DNA binding

1- A volume of 200 µL absolute ethanol was added and was mixed by Shaking for 10 min, then it centrifuged at 18000 xg for 5 minutes.
2- A column of FABG was placed in a 2mL collection tube.
3- Supernatant including any precipitate was transferred to FABG column, and centrifuged at 18000 xg for 1 minute.

Step4: Wash

1- Collection tube containing the flow-through was discarded and 400 µL of W1 buffer was added, then centrifuged at 18000 xg for 1 minute.

2- Collection tube containing the flow-through was discarded and 600 µl of wash buffer was added, then centrifuged at 18000 xg for 1 minute.
3- The FABG column placed in a new 2mL collection tube, re-centrifuged after discarded flow rate for 3minutes at the same speed to dry matrix column.

Step 5: DNA elution

1- Eluted DNA in new eppendorf tube 50 µL of pre-heated elution buffer was added to column and left 10 minutes at 37ºC to absorb it.
2- The tube was centrifuged at 18000 xg for one minute to elute the purified DNA.
3- Additional DNA samples were obtained by placing the FABG column in a new eppendorf and added 50 µl of pre-heated elution buffer solution and left 10 minutes at 37ºC to absorb it.
4- Repeated centrifuged at 18000 xg for one minute to elute the purified DNA.

2.16 Ribonucleic acid extraction(GENEzol™ TriRNA Pure):

The GENEzol™ TriRNA Pure Kit is a phenol and guanidine isothiocyanate plus spin column system for convenient purification of high-quality total RNA from a variety of samples. Initially, samples are homogenized in GENEzol™ Reagent without chloroform phase separation or isopropanol RNA precipitation. Following sample homogenization, simply bind, wash and elute the high-quality, total RNA in RNase-free Water and use in a variety of sensitive downstream applications[173].

A. Sample homogenization and lysis:

1. A volume of 200 µl of blood was added to a 1.5 ml of microcentrifuge tube (RNase-free).

2. About 3 volumes of GENEzol™ Reagent were added to 1 volume of sample (3:1) then mixed well by vortex.

3. The sample mixture was incubated for 5 minutes at room temperature.

B. RNA binding

1. The sample was Centrifuged at 16000 rmp for 1minute to remove cell debris then transferred the clear supernatant to a new 1.5ml microcentrifuge tube (RNase-free).

2. One volume of absolute ethanol was directly added to 1 volume of sample mixture (1:1) in GENEzol™ reagent then mixed well by vortex.

3. The mixture was placed into a RB Column in a 2 ml collection tube.

4. A volume of 700 µl of the sample mixture was transferred to the RB Column then Centrifuged at 16000 xg for 1 minute then discarded the flow-through.

5. The RNA binding step was repeated by transferring the remaining sample mixture to the RB Column.

6. The mixture was centrifuged at 16000 xg for 1 minute.

7.The flow-through was discarded and the RB Column was placed into a new 2 ml collection tube.

C. RNA wash

1. A volume of 400 µl of pre-wash buffer containing ethanol was added to the RB Column then centrifuged at 16000 xg for 30 seconds.

2. The flow-through was discarded then the RB column was placed back in the 2 ml Collection Tube.

3. A volume of 600 µl of wash buffer containing ethanol was added to the RB column.

4. The mixture was centrifuged at 16000 xg for 30 seconds then the flow-through was discarded then the RB column was placed back in the 2 ml Collection Tube.

5. A volume of 600 µl of wash buffer containing ethanol was added to the RB column.

6.The mixture was centrifuged at 16000 xg for 30 seconds then the flow-through was discarded.

7. The RB column was placed back in the 2 ml collection tube then the RB column was washed with 600 µl of wash buffer.

8. The mixture was centrifuged at 16000 xg for 3 minutes to dry the column matrix.

D. RNA elution

1. The dry RB column was placed in a clean 1.5 ml microcentrifuge tube (RNase-free).

2. A volume of 25-50 µl of RNase-free water was added into the center of the column matrix then incubated for at least 3 minutes to ensure the RNase-free Water has been completely absorbed by the matrix.

3. The mixture was centrifuged at 16000 xg for 1 minute to elute the purified RNA.

2.17 Quantitative and purity determination for DNA and RNA

The concentrations of DNA and RNA were measured by a nanodrop device, figure (2-7 and 2-8).The concentration is proportional to the absorbance, proteins have a maximum absorbance capacity of 280nm and DNA has a maximum absorbance capacity of 260nm where the A260/A280 ratio was used to determine DNA purity. If it is approximately (1.8-2.2), the DNA considered pure. Similar to the absorbability at 230 nm, the absorbability at 230 nm was the result of other pollutants, and thus A260/A230 was also

evaluated, the DNA concentration assessed by the nanodrop was usually in the range of 1.8–2.2 for unstained DNA[174].

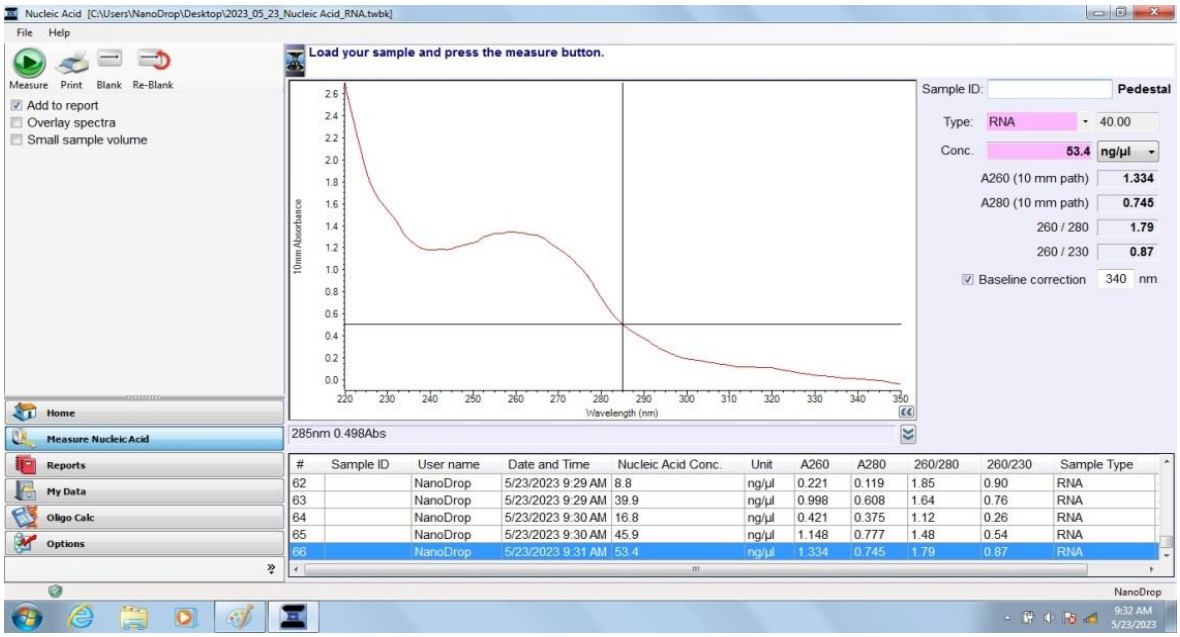


Figure (2-7): Screen shot of nanodrop 2000 program for quantitative determination of DNA.

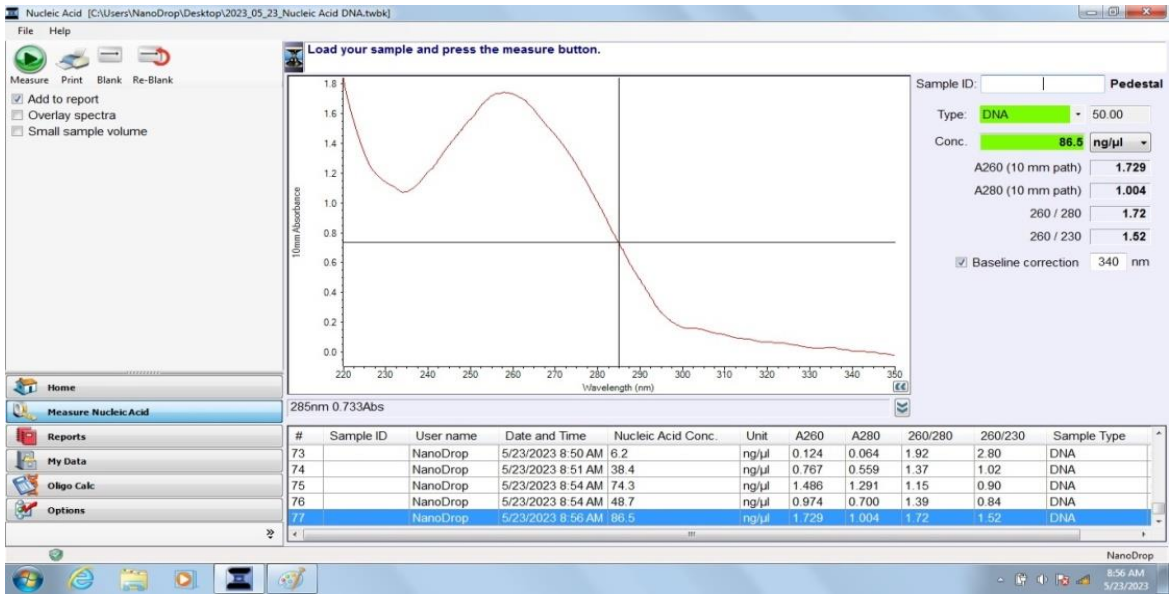


Figure (2-8): Screen shot of nanodrop 2000 program for quantitative determination of RNA.

2.18 Qualitative determination for DNA

The detected of DNA by using agarose gel electrophoresis technique.

A-Agarose Gel Electrophoresis

The standard method for separating, identify, and purify the DNA fragments is electrophoresis on agarose gel, bands containing less than 1-10ng of DNA can be directly determined by staining with low concentration of safe red fluorescent dye.

Electrophoresis is the process through which charged molecules in solution, primarily protein and nucleic acids, travel in response to an electric field, their rate of migration or mobility in the electric field, was high, and it was dependent on the field's strength, net charge, size, and shape of molecules as well as the ionic strength, viscosity, and temperature of the medium in which the molecules were moving. The following process was used to move of DNA in the gel-based on its molecular weight, conformation, and concentration of the agarose, the voltage applied, and strength of the electrophoresis buffer[175].

1-The TBE buffer (10X) PH 8.3 of the stock solution (100mL of stock solution 10X was dissolved in 900mL DDW to prepared 1X of TBE).

2- To make 100 mL of a 2% agarose solution, 2g of agarose was weighted and putted into conical flask, then 100mL of TBE 1X was added.

3-The agarose was completely dissolved and the solution becomes clear, the solution was allowed to cool to about 45-50°C and 4µL of safe red stain was added.

4-The gel was cast in the gel tray and allowed to harden at room temperature for about 20 minutes, with a thickness of less than 0.5 cm because a thick gel could reduce sensitivity.

5-The comb placed in the gel tray about 1 inch from one end of the tray and position the comb vertically, so that the teeth are about 1–2mm above the surface of the tray.

6-After carefully removing the comb, the tray was placed in the electrophoresis chamber and electrophoresis solution (1X-TBE) was applied to the wells until just covered (the same buffer used for agarose preparation).

7-At this step the DNA product (4µL) was mixed with (3µL) loading dye (bromophenol blue and glycerol) had been loaded on each well with extreme cautions to avoid damages of the wells and cross contamination of neighboring wells.

8-Later supplement to the power supply, run at 50V for 10min and then at 100V for 30 min.

9-Observed the DNA bands which will be visible under short wave UV light.

B-Photo Documentation

The agarose gel was positioned a top the UV trans illuminator device, the agarose gel was visualized in a UV supplied with the gel documentation device. The gel was exposed to UV light, and images were taken using the gel documentation of the computing device's digital camera, a tracking dye (bromophenol blue) was added to DNA in loading step of the electrophoresis method because DNA was colorless.

2.19 Conversion of RNA to cDNA

This product is an efficient, stable and fast reverse transcription system that can remove genomic DNA contamination. RTMaster Mix is a one-tube reverse transcription master mix, which contains a variety of reagents required for reverse transcription (H RTase, RNase Inhibitor , dNTP mixture, buffer), just add template RNA primers and water to carry out the reaction. The reverse

transcriptase used in the kit removes RNase H activity, and has stronger thermal stability, which can withstand 55 ℃ reaction and improve the reverse transcription of complex RNA templates. The heat-sensitive double-strand-specific nuclease is added to master mix, which can directly degrade and remove residual genomic DNA (gDNA) contamination in RNA samples during reverse transcription. Oligo dT & random primer is provided in a single tube, if you need to use a specific primer, you can directly replace it[176].

● **Instructions:**

1. The template RNA and reagents was thawed on ice, and each solution was mixed by vortexing gently before use .

2. The mixture was centrifuged to collect liquid remaining on the tube wall to the bottom of the tube.

3. The following reaction system was prepared on ice in an RNase free tube Table (2-4):Contents of the reaction.

Component	Volume
Total RNA/mRNA	2 µl (0.1-2 µg)
RT MasterMix	4 µl
Oligo dT & Random Primer or specific primer	1 µl
RNase free H_2O make up	13 µl
Total	20 µl

4. The mixture was gently mixed with a pipette, then putted into the PCR machine then the following program was ran:

- Standard procedure: 25˚C (10min.), 55 ˚C (30~60min), 85 ˚C (5min.).

5. The obtained cDNA product was used for qPCR reaction immediately

2.20 Primers

According to genome-wide association studies (GWAS), there are more than fifty genes linked to T2DM [177]. Association of T2DM with *retn* gene located in chromosome 19 that is the two SNPs (NG023447 C>G in exon 3 and 4 of *retn* gene and rs34861192 G >A in prompter of *retn* gene)as shown in appendix2 (a and b) were selected due to both SNPs were more polymorphic and more related with the target protein(retn). In addition the housekeeping gene selected in this study was *GAPDH*.

●Sequence of primers

Real Time- PCR- HRM , ARMs , gene expression primers and controls DNA was design as following

1- The sequence of the *retn* gene was taken from NCBI site according to the add gene site, and then the required SNPs were identified and through (flank) selection was taken the sequence.
2- Transferred the sequence to the primer site 3, then determined the length of the required primer around the SNPs, as well as selected the sequence of DNA fragment that contain the SNPs (wild and mutant) and sequence of DNA complementary the selected primers, and then obtained a sequence of the resulting primer.
3- Then the primers went to sms-primer state bioinformatics for certification or to the Optimase ProtocolWriter™ .
4- After going through all the steps, the prefix for the SNPs on *retn* gene (NG023447sequencing) was using NCBI to avoid false-positive amplification to determine the primer (forward and reverse) location and SNPs on the gene as in Table (2-5). [178].

Table (2-5**):** Primers used in present study.

Primers	Name of primer	Sequences of primer	L	Tm	GC %
HRM rs34861192 G>A	HRM F	5′-TGCTGTGATCATAAGTCACTGTAG-3′	24	57.4	42
	HRM R	5′-TGACGTGAGAGAATTGCTTGA-3′	21	58.1	43
ARM NG023447 C>G	ARMS 1 G	5′-CCCGGATGTGGGACG-3′	15	63.9	73
	ARMS 2 C	5′-CCCGGATGTGGGACC-3′	15	64.4	73
	ARM R	5′-GCCCCCAACCCTCCC-3′	15	69.5	80
retn – 420 C/G gene expression	*retn* F	5′-TGGTATGTCATTCTCACCCAGAG-3′	23	61.3	48
	retn R	5′-CAGCTAACCAAATCCGGCAC-3′	20	61.2	55
GAPDH	GAPDH F	5′-TTGGCTACAGCAACAGGGTG-3′	20	60.5	55
	GAPDH R	5′-GGGGAGATTCAGTGTGGTGG-3′	20	60.5	60

L(length),Tm(meltingtemperature),G(guanine),C(cytosine),A(adenine),GAPDH(glyceraldehyde3-phosphate dehydrogenase)

- **The Bioinformatics Software's Web Site Link Used to Design Primers and controls DNA are seen in appendix (3).**
- **Reconstituting and diluting primers**

The Macrogen primers were delivered as a lyophilized state. The units of the lyophilize primer were given as a mass in picomoles. To create the stock, one would reconstitute the primer in sterile nuclease-free water. The following steps were followed for reconstituting and diluting the primers:

1- The tube of primers were spined down before open up the cap.

2- According to the manufacturer's protocol, the required amount of sterile nuclease-free water was added to produce 100 pmoles/µl (Master Stock).

3- Vortex duly to evenly re-suspend primers.

4- Working Stock has been prepared by transferring 10µl of master stock to the Eppendrof tube, that contains 90µl of sterile nuclease-free water.

5- The master and working stock have been stored at -20°C after the additions were completed.

2.21 High resolution melt assay for genotyping method(HRM)

Principle

Analysis of HRM was performed on double-stranded DNA samples, typically using the real-time polymerase chain reaction prior to HRM analysis to amplify the DNA region in which their mutation, essentially the real-time

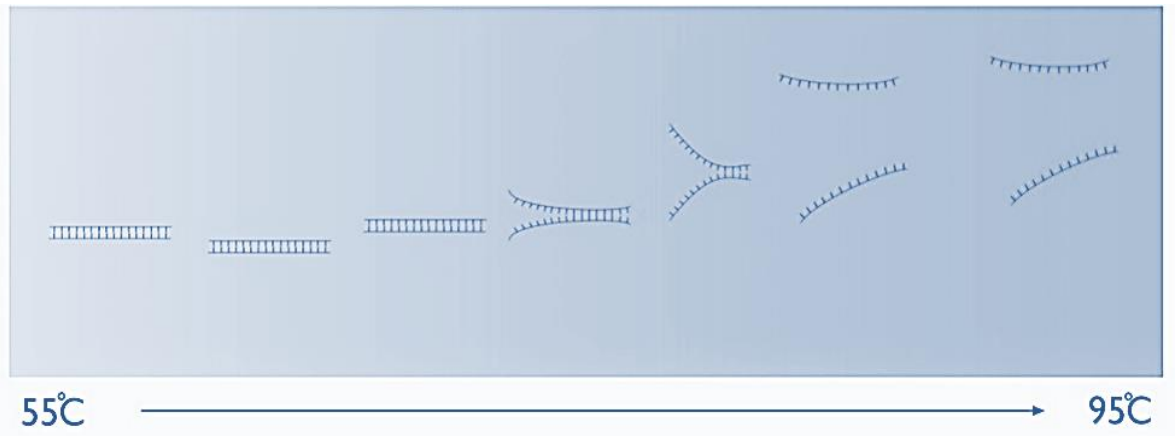


Figure (2-9): Temperature gradient for HRM analysis.

PCR process turns a tiny amount of region DNA into a large amount, the region is called amplicon, the HRM analysis is began after the PCR process. In this process , the amplicon DNA is simply precise warming from around 55°C up to around 95°C as in figure (2-9). During this process and at some point, the melting temperature of the amplicon was reached and the two strands of DNA “melt” apart[179].

The fluorescent dye that is used for HRM known as intercalating dyes. This dye binds specifically to double-stranded DNA and when they are bound they fluoresce brightly.

For this and at the beginning of the HRM analysis there is a high level of fluorescence in the sample due to the presence of billions of copies of the amplicon. But the two strand is separated when the sample is heated up and there is no longer any double stranded DNA present and thus fluorescence was reduced, the PCR has a camera to measure the fluorescence, the data is plotted as a graph called melting curve that shows the level of fluorescence vs. the temperature. HRM real time PCR was simple, fast, cost-effective and efficient genotyping technique, there was no need for costly probe synthesis and labelling, also time-saving (completed in about two hours) and had a low risk for DNA contamination, one current limitation of HRM is the possibility had designed amplicon that as short.

Wild, Heterozygote or homozygote type are the three genotypes that each one gives a melt curve and is slightly different. It is possible to distinguish between all three genotypes due to a high-quality HRM assay, as show in figure (2-10).

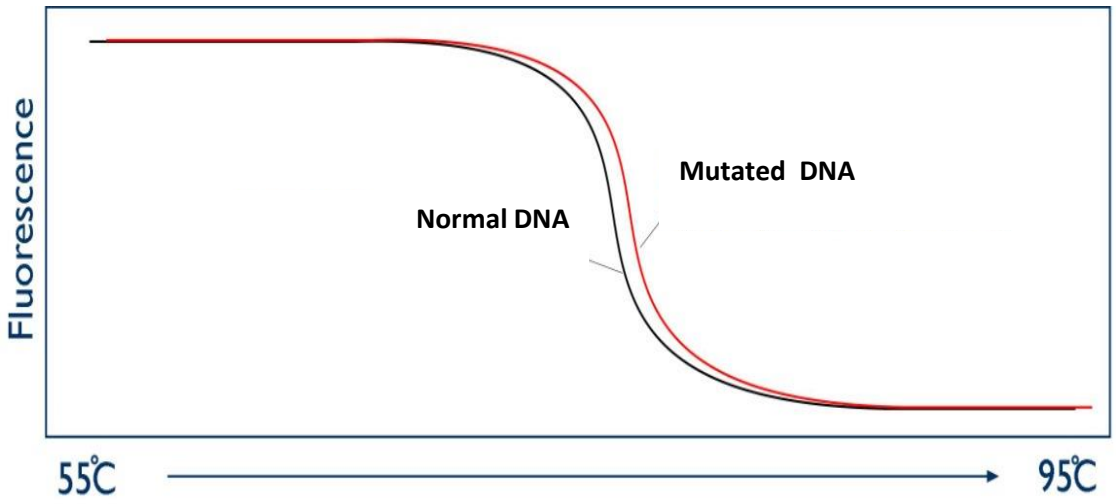


Figure (2-10): Genotyping curve by high resolution melt PCR.

2.22 Real time PCR -HRM

The reaction of real time-PCR was done by mixing PCR components with DNA solution and used the several different thermal attempts to PCR as in Tables (2-6) and table (2-7) respectively.

Table (2-6) **:** Mix reaction of polymerase chain reaction for genotyping of *retn* gene rs34861192 G>A

Component	Volume
SYBR Green qPCR mix	**25 µL**
Forward Primer	**1 µL**
Reverse Primer	**1 µL**
ROX Reference Dye	**1 µL**
Sample DNA	**2 µL**
nuclease-free H_2O	**20 µL**
Total	**50µL**

Table (2-7) : Optimization of polymerase chain reaction for genotyping of *retn* gene rs34861192 G>A.

NO. of Program	Tm	Time	No. of cycle	Product
Preheating	**95 °C**	**2 min**	**1**	**Success**
Denaturation	**95 °C**	**15 sec**	**40**	
Annealing rs34861192 G>A	**60 °C**	**15-30 sec**	**40**	
Extension HRM	**72 °C**	**30 sec**	**40**	

Tm (melting temperature).

2.23 Amplification refractory mutation system for genotyping method

Principle

This method is based on allele specific primers when DNA is amplified. The annealing and so that the amplification can be reduced when PCR mismach at the 3’ end of the primer is occurred. This is due to the absence of

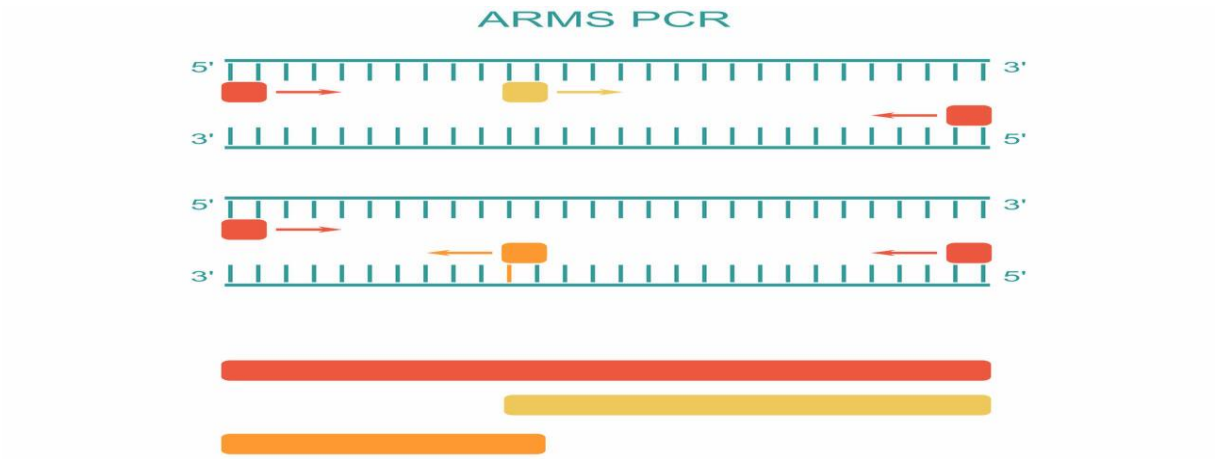


Figure (2-11): Principle of ARMs PCR.

3’ to 5’ exonuclease proofreading activity of Taq polymerase, that have High fidelity DNA polymerases, cannot be used in ARMS figure(2-11). This method is useful for identification of point mutations or polymorphisms. Moreover it is also important to identify whether the change in DNA is heterozygous or homozygous. A heterozygote or homozygote is differentiated by using ARMS primers for the mutant/polymorphic and the normal (wild type) alleles. The reactions for the mutant and the normal alleles are usually carried out in separate tubes. But these may be done in the same tube after labeling the two primers with different fluorescent dyes.[180]

2.24 Real time PCR - ARMs

The reaction real time-PCR- ARM was done by mixing PCR components with DNA solution and used the several different thermal attempts to PCR as in Tables (2-8) and Table (2-9) respectively.

Table (2-8) **:** Mix reaction of polymerase chain reaction for genotyping of *retn* gene NG023447 C>G.

Component	Volume
2* Taq PCR Smart mix 1	**25 µL**
Forward 1 Primer	**2 µL**
Forward 2 Primer	**2 µL**
Reverse Primer	**2 µL**
ROX Reference Dye	**1 µL**
Sample DNA	**2 µL**
nuclease-free H_2O	**18 µL**
Total	**52µL**

Table (2-9): Optimization of polymerase chain reaction for genotyping of *retn* gene NG023447 C>G .

NO. of Program	Tm	Time	No. of cycle	Product
Preheating	**95 °C**	**2 min**	**1**	**Success**
Denaturation	**95 °C**	**20 sec**	**40**	
Annealing NG023447	**72 °C**	**1 min**	**40**	
Extension ARM	**72 °C**	**5 min**	**40**	

Tm (melting temperature).

2.25 Expression of *retn* gene

Principle

Gene expression profiling is a fundamental feature of systems biology studies, which are ever-increasing in use to unravel the complexity of biological systems and their disease states. In particular, measurement of gene expression ('transcriptomics') in blood is an effective tool for the discovery of biomarkers of disease, and remains a key component of studies requiring large numbers of samples due to ease of collection and storage. Although the advent of single-cell RNA sequencing has made it possible to measure gene expression of specific blood cell types, there remain financial and technical barriers to its widespread use. Hence, the most common methods of blood RNA profiling either investigate RNA extracted from all blood cell types (i.e. whole blood) or only peripheral blood mononuclear cells (PBMCs)[181].

2.26 Housekeeping gene primers

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene primer was used as a control and design to measure and know the effect of gene expression under study as a gene from Housekeeping gene from the Origene[182]. This primer was supplied in its original lyophilized form, diluted in sterile dd H_2O to a final concentration of 100 µM, and stored in a deep freezer at -20 oC until use. When monoplex PCR was used, forward and reverse primers were designed specifically for these genes. All primers are created using the Primer3 software. A concentration of 10µM of the stock primers was created to be used as a work primer, Table (2-5).

2.27 The Polymerase Chain Reaction for gene expression:

The reaction of real time-PCR was done by mixing PCR components with DNA solution and used the several different thermal attempts to PCR as in Tables (2-10) and Table (2-11) respectively.

Table (2-10) : Mix reaction of polymerase chain reaction for genotyping of *retn* – 420 C/G

Component	Volume
SYBR Green qPCR mix	**25 µL**
Forward Primer	**1 µL**
Reverse Primer	**1 µL**
cDNA template	**1 µL**
ROX Reference Dye	**1 µL**
nuclease-free H_2O	**21 µL**
Total	**50µL**

Table (2-11) : Optimization of polymerase chain reaction for genotyping of *retn* gene *retn* – 420 C/G.

.

NO. of Program	Tm	Time	No. of cycle	Product
Preheating	**95 °C**	**2 min**	**1**	**Success**
Denaturation	**95 °C**	**15 sec**	**40**	
Annealing (*retn* – 420 C/G)	**60 °C**	**15-30 sec**	**40**	
Extension	**72 °C**	**30 sec**	**40**	

Tm (melting temperature).

2.28 Statistical Analysis

Data of the study participants were transferred into computerized database, revised for errors or inconsistency and then managed, processed and analyzed using the statistical package for social sciences (IBM-SPSS) version26.0. The normality of data distribution was tested by the Kolmogorov-Smirnov and Shapiro-Wilk tests. Present data were Non-normally distributed . Mann-Whitney U test was applied for comparison between two groups that expressed as median, while test statistic(mean rank difference) of Krusskal Wallis Post Hock test was used for multi-comparison among studied groups. Correlations between variables were performed using Spearman correlation. ROC analysis was used to determine the diagnostic markers for studied variables. The area under the curve (AUC) provides a useful tool to compare different biomarkers good and excellent range from (80-100)**.** ($P \leq 0.05$) consider as a significant value.

Test for Hardy-Weinberg equilibrium of allelic or genotypic association in cases versus control were evaluated by Chi – square (x^2) test, this analysis was performed for all genotypes in this study using Hardy-Weinberg equilibrium online calculator.

To assess the predictability of T2DM, logistic analysis of both SNPs was applied, to measured odds ratio (OR). Also the 95% confidence interval was calculated which is good estimator for the significance of the OR; when the value of "one" included within interval, this is an indicator that the OR is not significant [183], web sites were used for statistical analysis, as shown in appendix (4).

3. Results and discussion

3.1 Demographic Characterization of Studied Groups

Demographic data of the studied groups was summarized in Table (3-1) and (3-2) for age and BMI respectively, while distribution of sex demonstrated in Figure(3-1).

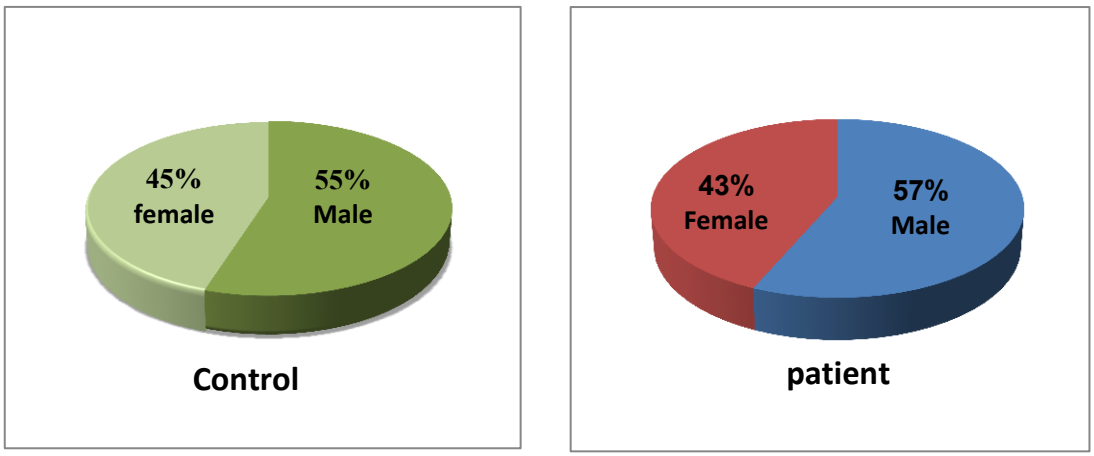


Figure (3-1): Sex distribution in studied groups

Results of (Table 1) have showed, median(50,49), minimum(41,42), maximum(59,58) of age among control and patient groups respectively; there was non significant (P>0.05)differences between control and patient groups.

Table (3-1): Comparison of age between patients and control using Mann-Whitney U test.

Variable	groups	No.	Median	Minimum	Maximum	P- value
Age (years)	Patients	60	49	42	58	0.127
	Control	60	50	41	59	

The age matching is required to remove the potential discrepancies inparameter findings which may occur due to the effects of these factors A. Al-Aaraji ,*etal* ,[184].

The differences of BMI between subgroups (obese and non obese) have determined to explain the principle line of the present study as in (Table 3-2). These results go with the same line with other findings in previous study Antuna-Puente B,*etal*, [185].

Table (3-2)**:** Krusskal Wallis Post Hock multi-Comparison for body mass index among studied groups.

Parameters	Groups	Study sub groups	Mean rank difference	SE	Std.	P values
BMI (Kg/m^2)	Non obese without T2DM	Non obese T2DM	-10.133	8.979	-1.129	.259
		Obese without T2DM	-60.133	8.979	-6.697	.000
		Obese with T2DM	-70.000	8.979	-7.796	.000
	Non obese T2DM	Obese without T2DM	50.000	8.979	5.568	.000
		Obese with T2DM	-59.867	8.979	-6.667	.000
	Obese without T2DM	Obese T2DM	-9.867	8.979	-1.099	.272

P value≤0.05 was significant, T2DM (Type 2 diabetes mellitus), SE (Standard error) Std. (standard deviation), BMI(body mass index).

3.2 Biochemical results

3.2.1 Insulin , retn and insulin resistance of studied groups

Data in Table (3-3) had elucidated the hormonal level changes and IR among studied subgroups. The results were indicated a significant differences between patient with T2DM and control (non-obese and obese) without T2DM, insulin, retn, IR were significantly (P<0.05) elevated in T2DM patient compared to control groups. While there was

non significant(P>0.05) difference in retn between obese without T2DM and obese with T2DM (mean rank difference = -1.4).

Table (3-3): Krusskal Wallis Post Hock_multi-Comparison of hormonal parameters and IR among the studied groups

Parameters	Groups	Study groups	Mean rank difference	SE	Std.	P values
Insulin (mIU/L)	Non obese without T2DM	Non obese T2DM	-19.600	8.971	-2.18	.029
		Obese without T2DM	-49.783	8.971	-5.54	.000
		Obese with T2DM	-74.683	8.971	-8.32	.000
	Non obese T2DM	Obese without T2DM	30.183	8.971	3.364	.001
		Obese with T2DM	-55.083	8.971	-6.14	.000
	Obese without T2DM	Obese with T2DM	-24.900	8.971	-2.77	.006
Resistin (ng/ml)	Non obese without T2DM	Non obese T2DM	-37.117	8.981	-4.13	.000
		Obese without T2DM	-69.167	8.981	-7.70	.000
		Obese with T2DM	-70.583	8.981	-7.85	.000
	Non obese T2DM	Obese without T2DM	32.050	8.981	3.569	.000
		Obese with T2DM	-33.467	8.981	-3.72	.000
	Obese without T2DM	Obese with T2DM	-1.417	8.981	-.158	.875
IR	Non obese without T2DM	Non obese T2DM	-33.600	8.981	-3.74	.000
		Obese without T2DM	-59.083	8.981	-6.57	.000
		Obese with T2DM	-86.517	8.981	-9.63	.000
	Non obese T2DM	Obese without T2DM	-25.483	8.981	-2.83	.005
		Obese with T2DM	-52.917	8.981	-5.89	.000
	Obese without T2DM	Obese with T2DM	-27.433	8.981	-3.05	.002

P value≤0.05 was significant, T2DM (Type 2 diabetes mellitus), SE (Standard error) Std. (standard deviation), IR(insulin resistance).

These results are in agreement with previous study by Chanchay S *etal*, [186] that recorded severe insulin resistance as results of increasing insulin and resistin levels in obese diabetic patient Majid Kadhum ,*etal*,[187].While other study Sinorita H *etal*, etal, [188] was disagreement with this present and shown that the obesity, retn and insulin resistance changes in different lines. The discrepancy in the results of this study and others may be resulting from the differences in the duration of T2DM disease, BMI, presence of complications from T2DM or not and the procedures used in measurement serum retn with different cutoff levels.

3.2.2 Levels of FBS and HbA_1c of studied groups:

Table (3-4) showed the mean rank differences, standard error and standard deviation of biochemical parameters among studied subgroups. FBS, HbA1c results were indicated significant (P<0.05) differences between patient with T2DM and subjects (non-obese and obese) without T2DM, were significantly elevated in T2DM patients compared to non-obese and obese without T2DM groups. While there was no significant(P>0.05) in both FBS and HbA1c between obese without T2DM and obese T2DM (mean rank difference =-8.46,-7.9 respectively).

Table (3-4**)** Krusskal Wallis Post Hock multi-Comparison of biochemical parameters among the studied groups

Parameters	Groups	Study groups	Mean rank difference	SE	Std.	P values
FBS (mg/dl)	Non obese without T2DM	Non obese T2DM	-22.333	8.973	-2.489	.013
		Obese without T2DM	-66.933	8.973	-7.459	.000
		Obese with T2DM	-75.400	8.973	-8.403	.000

	Non obese T2DM	Obese without T2DM	-44.600	8.973	-4.970	.000
		Obese with T2DM	-53.067	8.973	-5.914	.000
	Obese without T2DM	Obese with T2DM	-8.467	8.973	-.944	.345
HbA1c %	Non obese without T2DM	Non obese T2DM	-24.300	8.974	-2.708	.007
		Obese without T2DM	-68.200	8.974	-7.599	.000
		Obese with T2DM	-76.100	8.974	-8.480	.000
	Non obese T2DM	Obese without T2DM	-43.900	8.974	-4.892	.000
		Obese with T2DM	-51.800	8.974	-5.772	.000
	Obese without T2DM	Obese with T2DM	-7.900	8.974	-.880	.379

P value≤0.05 was significant,T2DM (Type 2 diabetes mellitus),SE (Standard error) Std. (standard deviation),FBS(fasting blood glucose),HbA_1c(hemoglobin A_1c).

In diabetic obese patients, the glycaemic control was significantly shooted up when compare with non obese without T2DM as shown by significant increase in FBS and HbA_1c levels in diabetic patients. These results are in the same line with other finding of previous study Mohammad zadeh G, *etal*,[189] .

3.2.3 The correlation between retn , IR,FBS and BMI:

Spearman Correlation was used to determine whether the relation among present parameters related with development of IR, present results were described in Table (3-5).

Table (3-5**):** Spearman correlation between the Levels of Parameters in patients with T2DM .

Variable		Insulin	Resistin	FBS	HbA1c	IR	BMI
Insulin mIU/ml	r		.947	.530	.246	.983	.988
	Sig.	.	.000	.000	.058	.000	.000
	N		60	60	60	60	60
Resistin ng/l	r	.947		.678	.247	.983	.924
	Sig.	.000		.000	.057	.000	.000
	N	60		60	60	60	60
FBS mg/dl	r	.530	.678		.147	.633	.456
	Sig.	.000	.000		.263	.000	.000
	N	60	60		60	60	60
HbA1c %	r	.246	.247	.147		.168	.166
	Sig.	.058	.057	.263		.061	.06
	N	60	60	60		60	60
IR	r	.983	.983	.633	.168		.963
	Sig.	.000	.000	.000	.061		.000
	N	60	60	60	60		60
BMI Kg/m^2	r	.988	.924	.456	.166	.963	
	Sig.	.000	.000	.000	.06	.000	
	N	60	60	60	60	60	

IR (insulin resistance), FBS (fasting blood sugar), BMI(body mass index),N(number of patients),Sig(significant), r(regression).

In diabetic individuals, serum retn levels was positively correlated with IR (r= 0.983, P=< 0.05). Previous research were reported also the same results between the retn levels and IR in the same line with this finding Gharibeh MY, *etal*, [190]. A positively significant correlation between serum retn levels ,insulin and FBS in diabetic patients was noted (r= 0.947, P<0.05) (r= 0.8, P<0.05) respectively and this is in agreement with other study manifested the same correlations [187]. Otherwise, this association was not determined in previous study [189]. The correlations between retn, IR and FBS may be referred to chemical

nature of retn , is a cysteine-rich peptide hormone which is directly connected obesity with diabetes. Retn is secreted by macrophages of adipose tissue. It binds to toll-like receptors (TLR) and promotes downstream inflammatory pathways like nuclear factor kappa-light-chain-enhancer of activated B cells (NFkβ), LRR, and pyrin domain-containing protein (NLRP) 3. These steps further induce TNF-α and IL-6, that stimulate serine-threonine kinase which causes down regulate of insulin receptor substrate (IRS) and, insulin receptor (IR) leading to insulin resistance in adipose tissue Al-Harithy RN, *etal*,[191].

The difference of BMI among studied groups was used to determine its effects on retn and determine the effects of later in insulin resistance. Such positive correlation was currently documented between serum retn levels and BMI in diabetic patients($r= 0.988$, $P< 0.05$). Previous research done by Komal Rawal, *etal* were recorded correlation between retn level and BMI in the same line with present finding,[192] Actually, retn plays a pivotal role in inflammation process because of its expression and secretion from human macrophages is promoted by various pro-inflammatory stimuli Yanran Li, *etal*, [193], a variant of decurin (Δ-DCN) is one of retn receptor that was mainly presented in white adipose tissue, lung and bone marrow, and its production is increased in obese individuals to regulate white adipose tissue expansion Al Hannan, F, *etal*,[194] Daquinag A.C., *etal*, [195].So that, adipose tissue is expanded resulting in adipocyte hypertrophy and the release of adipokines. The latter causes increased cytokine production, leading to adipose tissue dysfunction and impairment of glucose tolerance Kageyama, H., *etal*,[196].

All data have been added potential fact that obesity is related to high level of plasma retn and is directly associated with IR Khanna, D.

, *etal*,[197]. From these observations, retn is the factor that resist insulin function that supported by the data obtained from present study that demonstrated FBS, fasting insulin and IR were raised in T2DM. The idea of such observation is when insulin reaches a critical levels, the retn might cause initiation of resistance to insulin or vice versa. Many of studies have documented that adipokines, such as leptin, retn and adiponectin, is changed in T2DM and these may be contributed to the development of resistance to insulin José Luis , *etal*,[198] Rajala MW, *etal*, [199] Antuna-Puente B, *etal*,[200].

3.2.4 Receiver operating characteristic curve (ROC)of Retn:

Present data of ROC analysis confirmed that retn could potentially be used as biomarker for obese T2DM patients and differentiated them from non obese T2DM patients and obese patient without T2DM with high sensitivity and specificity of retn in this group figure (3-2),Table(3-6).

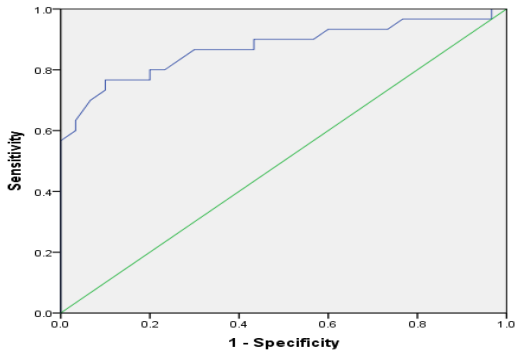


Figure (3-2): Roc curve of retn patient with T2DM.

Table (3-6) Receiver operating characteristic (ROC) curve of retn
Area under the curve (AUC).

AUC	specificity	sensitivity	SE	P-values	Confidence interval
0.87	90%	76%	0.048	0.000	0.78 - 0.96

AUC(area under curve), SE(standard error).

3.3 Molecular analysis

3.3.1 Detection of genomic deoxyribonucleic acid

The DNA that extracted from blood samples of studied groups was illustrated in figure (3-3) that indicated high quality of DNA bands , by using agarose gel electrophoresis staining with safe red fluorescent dye.

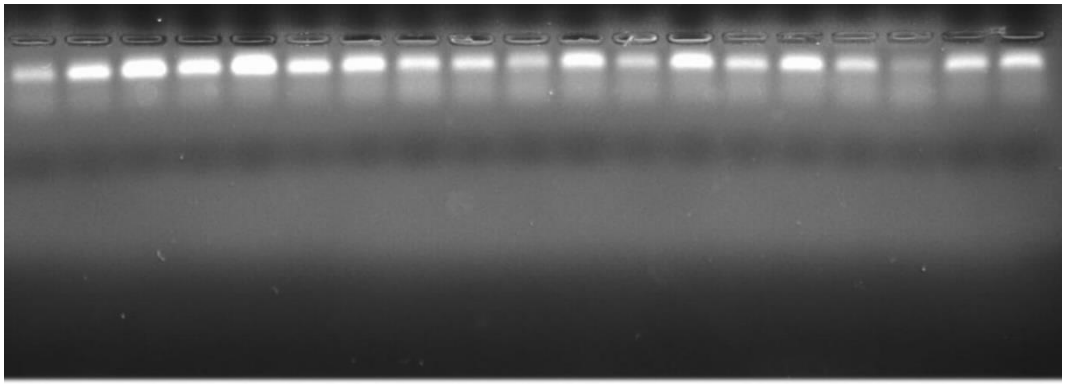


Figure (3-3): Detection the presence of genomic DNA by using agarose gel electrophoresis staining with safe red fluorescent dye.

3.3.2 Measurement of concentration and purity of nucleic acid :

A ratio of absorbance at 260/280 nm and 260/230 nm were used to measure the DNA and RNA purity that expressed as range (1.82-1.98) and (1.88-2.1) for DNA, (2.03-2.1) and (2.09- 2.14) for RNA respectively, the range of DNA and RNA concentrations were (78 - 93.9) and (70.1- 89.2) respectively. Data was demonstrated in Table(3-4).

Table (3-7): The range of concentration and purity of extracted nucleic acid

DNA,RNA variables	No.of samples	Range
DNA concentration(ng/µl)	120	78 - 93.9
DNA purity(260/280)	120	1.82-1.98
DNA purity(260/230)	120	1.88-2.1

RNA concentration(ng/µl)	120	70.1- 89.2
RNA purity(260/280)	120	2.03-2.1
RNA purity(260/230)	120	2.09- 2.14

At 260/ 280 ratio ,the acceptable range of absorbance is optimal ~1.8 that recorded in the present samples. A ratio of < 1.7 suggests protein or acidic phenol contamination, while a value > 2.0 suggests remnants of the basic solution used to lyse the cells**.** In contrast, the 260/230 ratio is used to indicate the presence of unwanted organic compounds such as Trizol, phenol, Guanidine HCL, and guanidine thiocyanate. Generally, acceptable 260/230 ratios are in the range of 2.0 – 2.2 Wei Liu, *etal*,[201].

3.3.3 Gene polymorphism of retn in studied subgroups

The gene polymorphism of retn was studied in patients with T2DM(obese and non obese) and control groups(obese and non obese). There are two SNPs were included in the present study; rs34861192 G>A in promoter region and NG023447 C>G exon 3and 4 within the *retn* gene, the genotypes were detected by HRM and ARM -real time PCR respectively; from data based on NCBI, allele C for NG023447 C>G designated as new SNPs studied in T2DM patients M. Cohen, *etal*,[202].

3.3.3.1 Analysis of rs 34861192 G>A and NG -023447 C>G Polymorphism

a. Analysis results of amplification reaction and genotype of rs 34861192 G>A

The fluorescent signal of amplification curve for (G>A) illustrated in figure (3-4). Genotyping analysis by HRM was performed from 65˚C to 90˚C with a temperature increase of 0.2˚C/s.

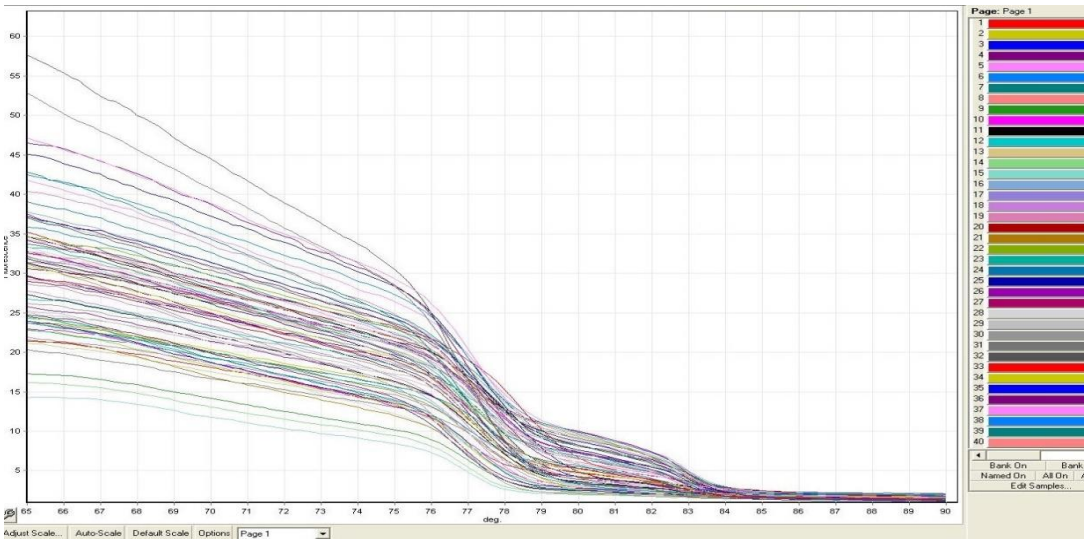


Figure (3-4): Amplification curve of rs 34861192 G>A.

The HRM assay melt curve results were normalized to identify the genotype as in figure (3-5 and 3-6) The top, middle and bottom lines represent GG,GA and AA genotypes for control and patients respectively.

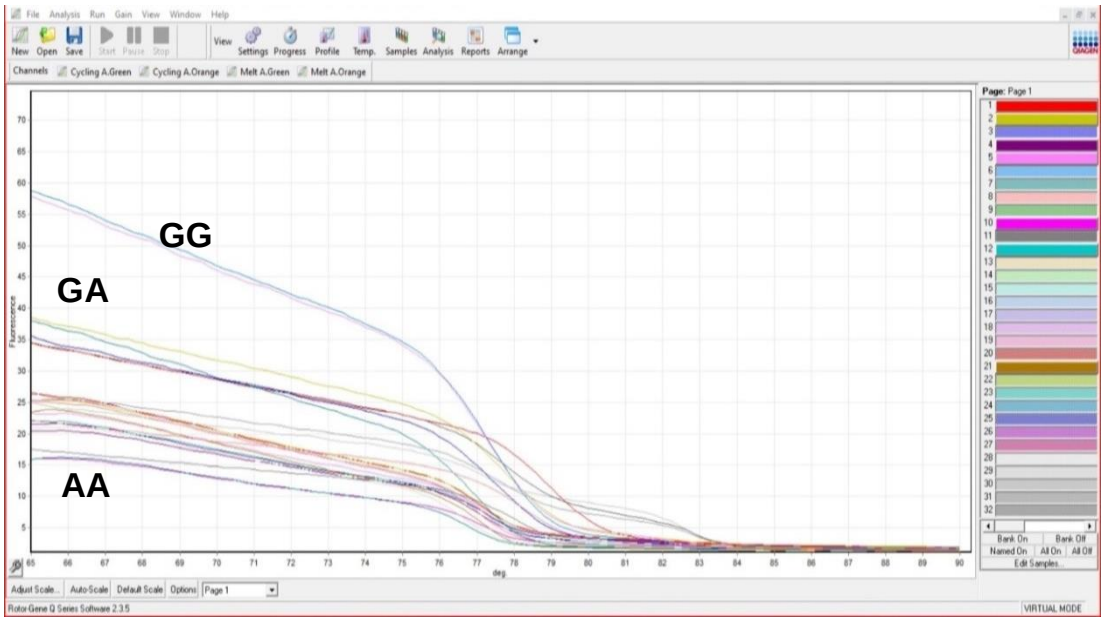


Figure (3-5): Genotype steps for control of G>A , GG wild, GA (hetero mutant), AA (homo mutant) for 1 run of PCR .

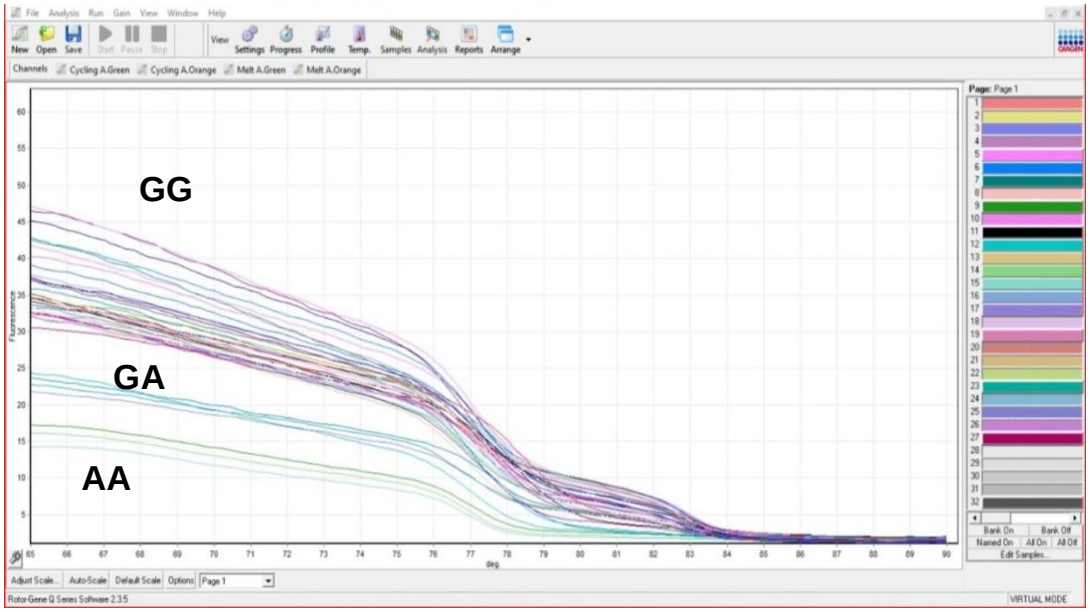


Figure (3-6): Genotype step for patients of rs34861192 G>A , GG wild, GA (hetero mutant), AA (homo mutant)for 1 run of PCR.

b. Analysis results of amplification reaction and Genotype of NG023447 C>G

The amplification curve for NG023447 (C>G) shown in figure (3-7). ARMs assay melting curve results were normalized to identify the genotype in figure (3-7) for control and patients of NG023447C>G.

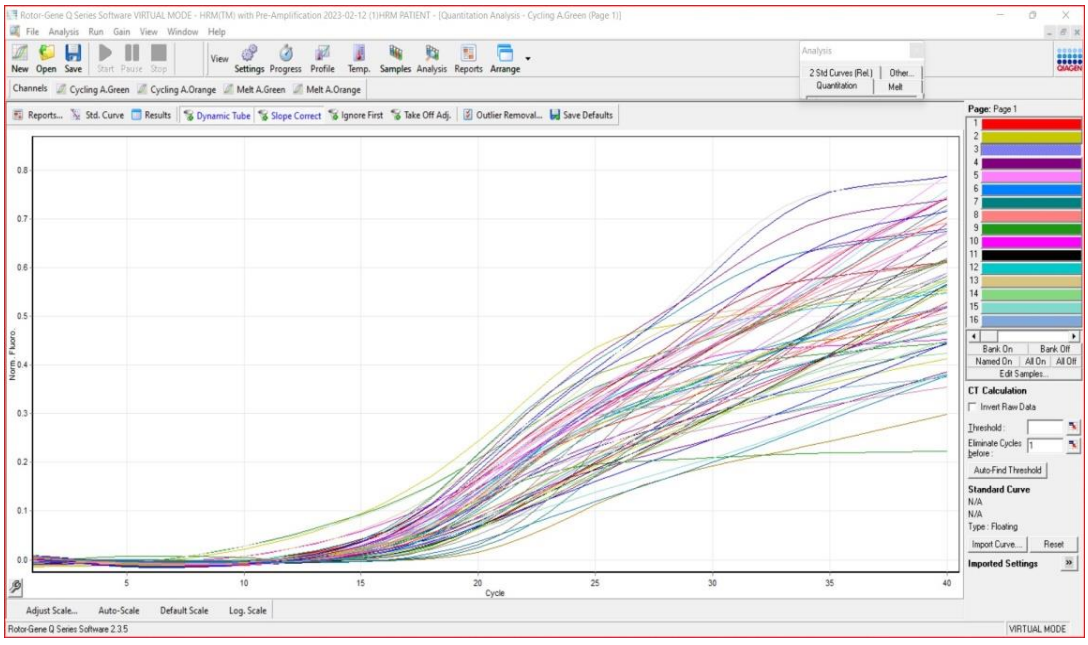


Figure (3-7): Amplification curve of NG023447 C>G.

3.3.4. Alleles frequency for rs34861192 G>A and NG023447 C > G

Allele frequency of *retn* gene variants rs34861192(G>A) and NG023447C>G in T2DM patients were represented (20.8 and 24) % of G and C allele, (79 and 76)% of A and G allele respectively, while in control groups were represented (68.3 and 75)% of G and C allele, (31.6 and 25) % of A and G allele respectively.

All data were conducted to assess the effects of of *retn* gene variants [GG (wild type), GA (heterozygous type), and AA (homo mutated type)] on T2DM development, the results were summarized in Table (3-8) that indicated a higher risk model was recorded in genotype AA [P<0.0001, OR (95% CI) = 8.2(4.5 to 14.7)], while in NG023447 C>G the higher risk was recorded in GG genotype[P<0.0001;OR (95% CI) = 9.8(5.4 to 17.8)].

Table (3-8) :Alleles frequency and allelic association of rs34861192 G>A and NG023447 C>G of *retn* gene polymorphism between Patients and control groups.

retn SNPs	Allele	Frequencies (%)		Odd ratio (95% CI)	P value
		Control (n=60)	Patient (n=60)		
rs34861192 G>A	G	68.3% (n=82)	20.8% (n=25)	0.1 (0.06 to 0.21)	< 0.0001
	A	31.6% (n=38)	79% (n=95)	8.2 (4.5 to14.7)	
NG023447 C>G	C	75% (n=90)	24% (n=28)	0.1 (0.05 to 0.183)	< 0.0001
	G	25% (n=30)	76% (n=92)	9.8 (5.4 to17.8)	

G- major allele, A-minor allele for rs 34861192, CI: confidence interval, SNP(single nucleotide polymorphism), P value ≤0.05 was significant.

The *retn* gene polymorphisms results showed that A allele of (rs34861192 G>A) was potential risk for T2DM development, inversely, the G allele was protective with higher frequent in control groups as shown in Table (3-8).

Asano, H. *etal*, [203], have directed that there is a significant association between polymorphism with T2DM the G allele was protective, and A allele was a risk model because of higher frequent was saw in patients group.

This study tries to reveal whether NG023447 in *retn* gene played a potent role in development of T2DM. Allele frequency results indicates that C allele cannot be considered a risk factor in patients with (OR 0.1 CI 95% 0.05-0.183) ,while allele G cannot be considered a protective factor in healthy subjects with (OR9.8 CI 95% 4.5-17.8).

Alleles frequency and allelic association of NG023447 C>G in *retn* gene have revealed that there is a highly significant difference between (control and patients). From the results that have been appeared , it can be said the SNP that revealed in the study has heterogeneous effects on the community and on the groups of this study.

3.3.5. Hardy–Weinberg equilibrium of studied SNP_S

Genotype frequencies of of *retn* gene polymorphism were not agreement with Hardy Weinberg Equilibrium ($P<0.05$) in T2DM patient and control groups as in Table (3-9). Whereas genotype frequencies of NG023447 of *retn* gene polymorphism were in agreement with Hardy Weinberg Equilibrium ($P>0.05$) in T2DM patient and control groups as in Table (3-10).

Table(3-9):Hardy-Weinberg equilibrium law of *retn* gene polymorphism rs34861192 G>A observed and expected genotype frequency for control and patient

rs34861192 G>A	GG	GA	AA	P value
Control	63% (n=38)	10% (n=6)	26% (n=16)	<0.05
Patient	16% (n=10)	8% (n=5)	75% (n=45)	<0.05

G(guanine) (major allele), A(adenine)(minor allele), P value ≤0.05 was significant.

Table(3-10):Hardy-Weinberg equilibrium law of *retn* gene polymorphism NG023447 C>G observed and expected genotype frequency for control and patient

NG023447 C >G	CC	CG	CC	P value
Control	52% (n=31)	46% (n=28)	2% (n=1)	0.058
Patient	5% (n=3)	36% (n=22)	59% (n=35)	0.56

C(cytocine)(major allele), G(guanine)(minor allele), P value ≤0.05 was significant.

3.3.6 Genotyping of rs34861192 G>A and NG023447 C>G

All data have conducted to assess the effects of polymorphism (rs34861192) of *retn* gene variants [GG (wild type), GA (heterozygous type), and AA (mutated type)] on T2DM development, the results have summarized in Table (3-11) which indicated a higher risk model was recorded in genotypes AA co-dominant model which was [P<0.05, OR (95% CI) = 10.68(4.34 to 26.29)], followed by AA recessive model which was [P<0.05; OR (95% CI) = 8.2(3.6 to 18.7)], also GA-AA dominant model which was [P<0.05; OR (95% CI) = 8.6(3.6 to 20.3)]. While there was no significant(p>0.05) association found in GA over all models.

Table (3-11): Association of rs34861192 G>A genotypes with T2DM under different models of inheritance. .

Model	Genotype	Control (No.60)	Patient (No.60)	Odd ratio (95% CI)	P. Value
Co.dominant	GG	70% (n=38)	16% (n=10)	References(OR=1)	
	GA	8% (n=6)	8% (n=5)	3.1 (0.79 to 12.5)	0.1
	AA	21% (n=16)	75% (n=45)	10.68 (4.34 to 26.29)	<0.0001
dominant	GG	70% (n=38)	16% (n=10)	References (OR=1)	
	GA-AA	30% (n=22)	83% (n=50)	8.6 (3.6 to 20.3)	<0.0001
Recessive	GG-GA	78% (n=44)	25% (n=15)	References (OR=1)	
	AA	21% (n=16)	75% (n=45)	8.2 (3.6 to 18.7)	<0.0001
Over dominant	GG-AA	91% (n=54)	91% (n=55)	References (OR=1)	
	GA	8% (n=6)	8% (n=5)	0.8 (0.23 to 2.8)	0.75

G(guanine), A(adenine).

The comparison was also conducted in (obese and non obese) T2DM and control to determine the effect of the obesity under three basic genotype,Table (3-12) showed the highly significant difference (P<0.001) of AA genotype in patients(non obese and obese) compared to control(obese and non obese), while non-significant differences recorded among studied groups underline GA genotype.

Table (3-12): Genotype frequency of rs34861192 G >A of *retn* gene in patient and control subgroups (normal weight and obese).

Genotypes	Groups	Control	Patient	P. Value	
GG	Non Obese	24	9	References	
	Obese	14	1		
GA	Non Obese	1	3	0.08**	0.08*
	Obese	5	2	0.19***	
AA	Non Obese	5	18	0.0004**	0.01*
	Obese	11	27	0.001***	
Total	All subject	60	60		

*** between obese (patients and control), **between normal weight (patients and control) ,* between normal weight and obese in patients only, P value ≤0.05 was significant.

The results of the current study are similar to those of Asano, H. et al(2010) [203] which reported higher frequency of the mutant AA genotypes of SNP, and exhibited a significant association with the risk of T2DM mainly in obese patients. The present results also have documented that mutant AA has highly frequent in obese patients.

On the other hand, NG023447 C>G SNP that has described in Table (3-13) showed the highly significant difference (P<0.001) of GG genotype in non obese patients and obese in a comparison with the control group. Also there was highly significant difference (P<0.01) of CG genotype normal-weight patients as compared with control group.

Table(3-13): Genotype frequency of NG023447 C>G of *retn* gene polymorphism between Patient and control group within subgroup.

Genotypes	Groups	Control	Patient	P. Value	
CC	Non Obese	22	2	References	
	Obese	9	1		
CG	Non Obese	8	13	0.009**	0.8*
	Obese	20	9	0.2***	
GG	Non Obese	0	15	0.0004**	0.4*
	Obese	1	20	0.0004***	
Total	All subject	60	60		

*** between patient and control was obese , ** between normal weight (patient and control) , * between normal weight and obese in patient only.

3.3.7 Influence of *RETN* gene polymorphisms (G>A)and (NG023447) on RETN levels in T2DM patients

Table(3-14) described the probable influence of promoter *retn* gene SNP rs34861192G>A in serum retn level of T2DM patients. Higher concentration of retn was observed in mutant genotype(AA) that was significantly (p<0.05) associated with retn level when compared with(GG)genotype the mean rank difference was (-12.909).

Table (3-14**):** Krusskal Wallis Post Hock multi-Comparison of RETN level according to G>A genotypes in T2DM patients.

Parameters	Genotype	Mean rank difference	SE	Std.	P values
Resistin (ng/ml)	GA-GG	0.145	9.417	0.015	0.988
	GA-AA	-13.055	8.240	-1.584	0.113
	GG-AA	-12.909	5.885	-2.193	0.028

G(guanine) , A(adenine), P value ≤0.05 was significant. SE (Standard error) Std. (standard deviation)

The findings in Table (3-14) of present work have synergistically with previous studies Asano, H. *etal,* [203] Osawa H, *etal*,[204].These authors have explained many genetic lines contribute to the changes in circulating concentration of retn, especially SNPs of the *retn* gene that is located in the promoter region such as , rs3745368 and rs1862513 .

Table(3-15) has described the probable influence of *retn* gene SNP NG023447 C > G in circulating retn concentration of T2DM patients. Present results didn't show any significant (p>0.05) association between retn level and all genotypes model.

Table (3-15**):** Krusskal Wallis Post Hock multi-Comparison of retn with genotypes of NG023447 C>G among the studied subgroups.

Parameters	Genotype	Mean rank difference	SE	Std.	P values
Resistin (ng/ml)	GC-CC	0.417	6.981	-.188	0.775
	CG-GG	-5.055	4.240	-1.584	0.34
	CC-GG	-10.44	3.885	-3.0	0.16

C(cytosine), G(guanine),SE(Standard error)Std. (standard deviation).

3.3.8 Analysis results of amplification reaction of gene expression for *retn* gene -420 C>G in control and T2DM patients.

The amplification curve of *retn* and *GAPDH* gene expression -420 C>G in control and T2DM patients was shown in figure (3-8)and (3-9) respectively.

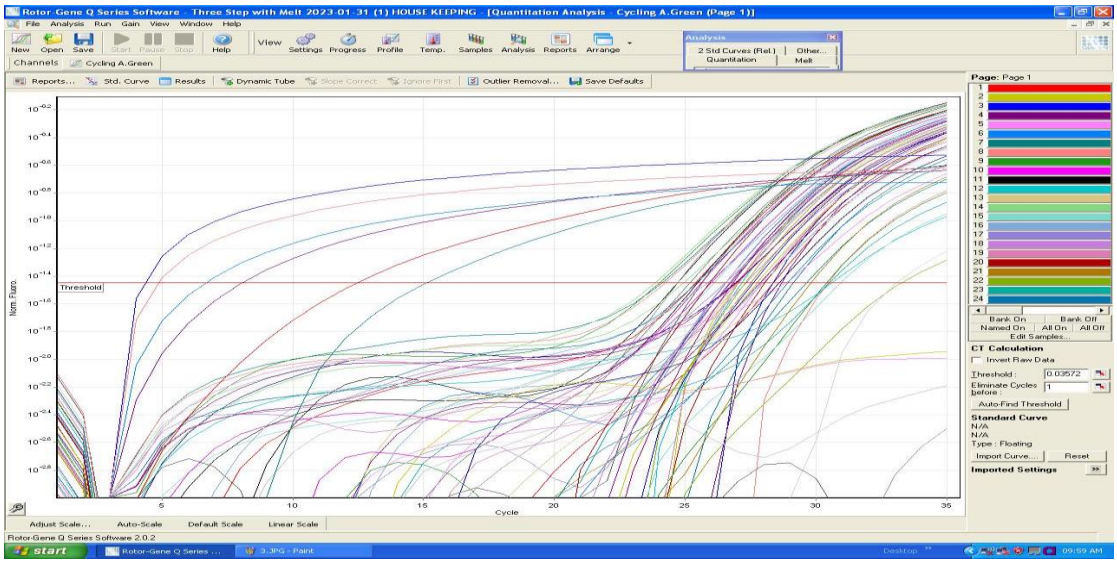


Figure (3-8): Amplification reaction curve in control and T2DM groups for *GAPDH* gene.

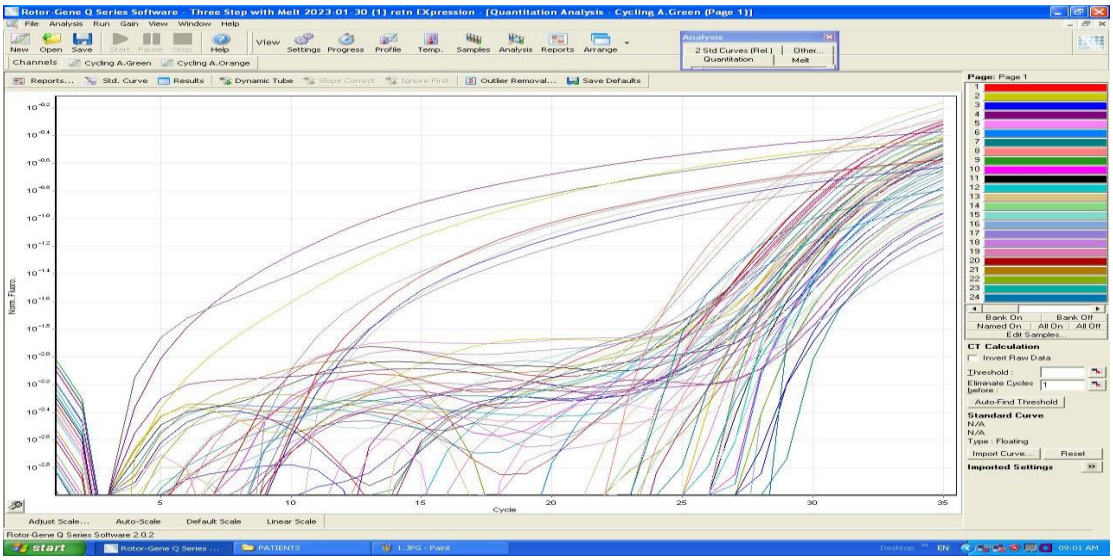


Figure (3-9): Amplification reaction curve of *retn* gene expression in control and T2DM groups.

3.3.9 R*etn* – 420 C/G gene expression and its relationship with retn levels in control and T2DM patients:

The probable influence of promoter *retn* gene SNP rs34861192G>A on the folding change of *retn* gene expression(gene of interest (GOI)) in T2DM patients was described Table (3-16). The results was expressed as mean±SD for folding change and revealed that there was high folding change (156.8±17.66**)** for patients compared to control(19.69±6.56).

Table (3-16**) :**Krusskal Wallis Post Hock multi-Comparison of folding change with genotypes among the studied subgroups.

	House Keeping Gene	*retn* "GOI"	ΔCT	ΔΔCT	Folding change
Average mean±SD CT Of **Control**	23.06± 7.84	25.19± 6.10	2.128	10±2.32	19.69±6.56
Average mean±SD CT Of **Patients**	17.35±10.04	17.61± 9.22	0.257	1.87±3.98	156.80±17.66

GOI, gene of interest ,CT (cycle threshold) ,ΔCT = CT (GOI)−CT (GAPDH gene), ΔΔCT=ΔCT(GOI)−ΔCT (GAPDH gene).

Table(3-17) described the probable influence of promotor *retn* gene SNP rs34861192G>A on the folding change of *retn* gene expression. Higher level of folding change was observed in mutant genotype(AA) that was observed significant ($p<0.05$) changes when compared with(GG)genotype the mean rank difference (-23.456).

Table (3-17) **:**Krusskal Wallis Post Hock multi-Comparison of folding change with genotypes of rs34861192 G>A between control and patient.

Parameters	Genotype	Mean rank difference	SE	Std.	P values
Folding change	GA-GG	-8.350	9.561	-.873	0.382
	GA-AA	-6.806	5.1	-1.32	0.41
	GG-AA	-23.456	8.228	-2.851	0.004

G(guanine) , A(adenine), P value ≤0.05 was significant.SE (Standard error) Std. (standard deviation)

The expression of *retn* gene was measured in this study to explain weather the effect of rs43861192 on retn level, the disturbance of *retn* rs34861192 in promoter region and the variation in this region considers as underlying causes for increasing *retn* expression that may be a risk of T2DM development Osawa H**,** *etal*, [205].

The present study has indicated strong link between rs34861192 G>A genotype and *retn* gene expression specially of AA genotype. This SNP present in promoter region of *retn* gene dependent on NCBI data base, previous researcher Dilip Kumar 1, *etal*, [206] focusing on promoter region to indicate the SNPs that related with the *retn* expression, actually, many of them indicate strong effect of these SNP on circulating retn levels. Authors of Japanese recorded that there were genetic association between rs1862513 and retn levels that represent for Japanese cohort but this association was completely loss when conducted with influence of rs34861192. This effect was particularly striking with methylation at CPG SNP within the regularity region of the gene One of the strong evidence was recorded in previous study that refer the effect of on DNA methylation.

DNA methylation which is one of an epigenetic factor has important role in the regulation of gene expression. Approximately 23% of DNA methylation in blood cells has also been found to be heriTable ,and SNPs have been related to many differences in DNA methylation level. Indeed, one of the most convincing explanation of many studied SNPs have been shown to influence mRNA levels through effects on DNA methylation. The SNPs in the *retn* promoter region might thus affect DNA methylation around *retn gene* in monocytes and macrophages and thereby regulate retn levels Masahiro Nakatochi, *etal*,[207]. To confirm those evidence

further studies were needed to reach the cause that interfere with retn expreression in T2DM Iraqi population.

Conclusions

1-Retn might be considered as a strong link between obesity, and IR through derangement of the signaling pathway of insulin, eventually this may lead to development of T2DM.

2-Polymorphisms of *retn* gene (rs34861192 G >A) may be play an important role in development of T2DM.

3-Mutant AA genotypes of rs34861192 G>A play an important role on the elevated levels of retn in the circulation that consider as a enhancement factor for T2DM.

4-The direct correlation between serum retn concentration and the genotypes rs34861192 G>A revealed a strong association of GG to AA, while NG 023447 genotype has not such effect.

5-*Retn* expression is a good indicator for T2DM development in obese individuals that have AA genotype of *retn* gene rs34861192 G>A .

Recommendations

1. The future study should be dependent on the adequate sample size for the reliable genetic power to determine exact effect of genetic variants on T2DM.

2. Study of DNA methylation and other epigenetic factors to determine their effect on *retn* gene expression.

3. Real time HRM PCR is a simple, fast, cost-effective and efficient genotyping technique, with low risk for DNA contamination , so it is advised to use in gene analysis.

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Appendix

1. Questionnaire

أسم المراجعة : العمر: الجنس:

التاريخ و اليوم : رقم العينة:

السكن الحالي :

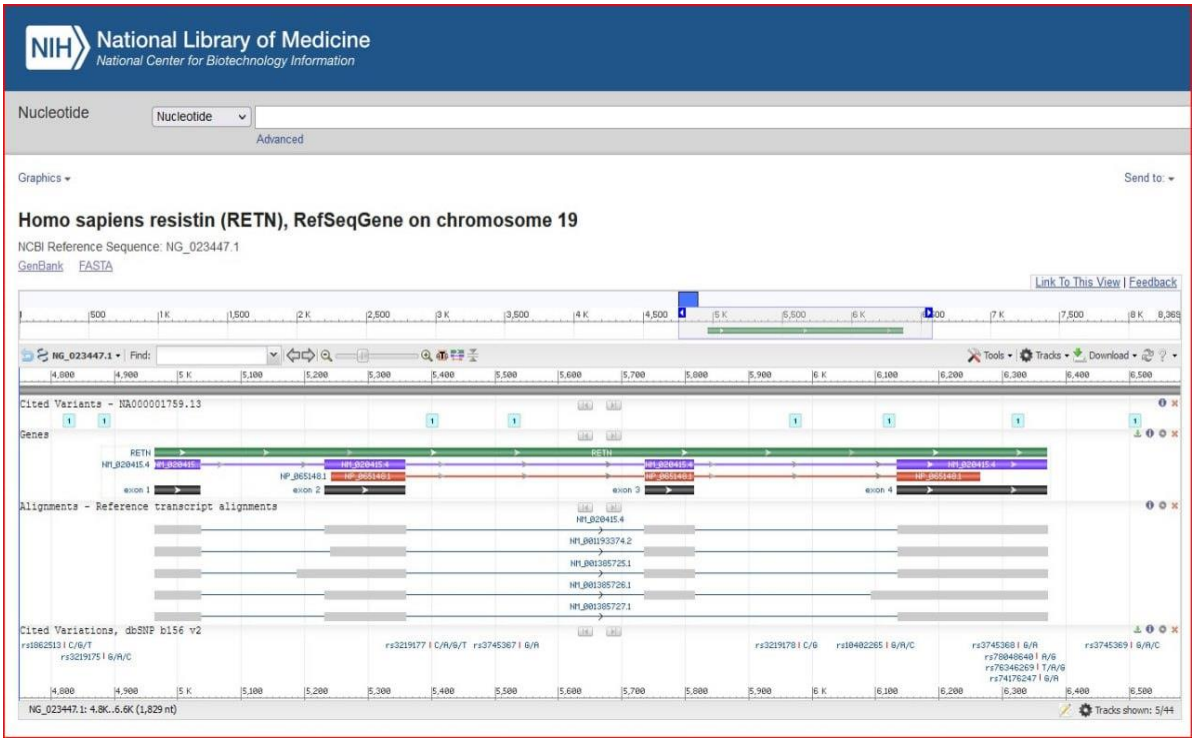
رقم الهاتف : فترة الاصابة بمرض السكري:

أمراض مزمنه : نعم , كلا الوزن : الطول:

أمراض مناعية: نعم , كلا

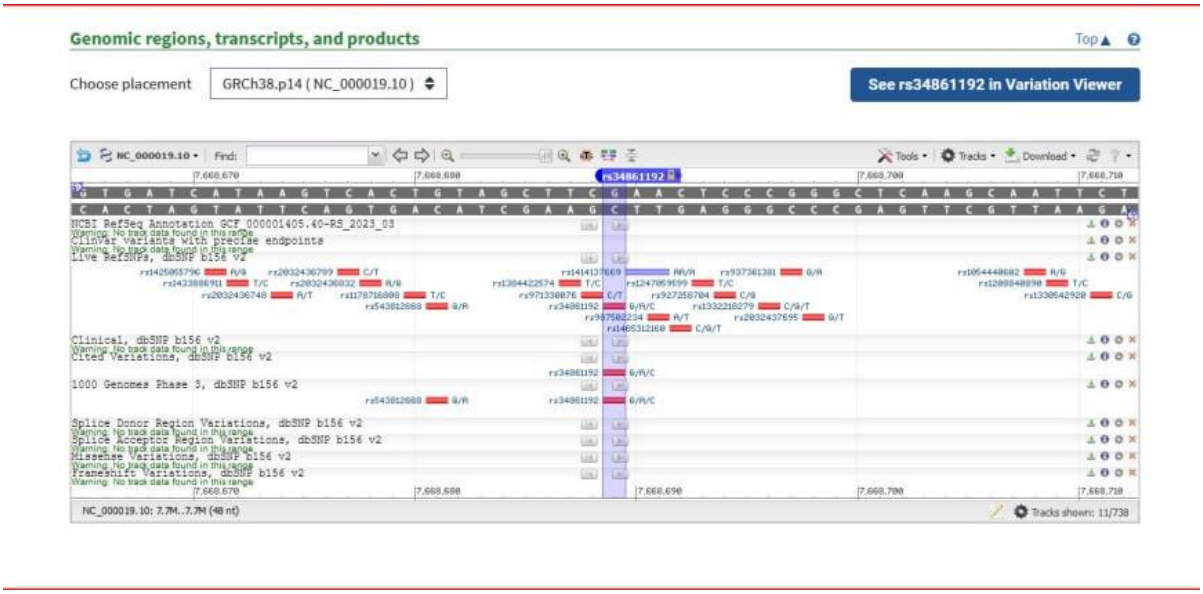
هل يوجد في العائلة او الاقارب شخص مصاب بمرض السكري : نعم , كلا

2- a-The information of NG 023447 from NCBI website.



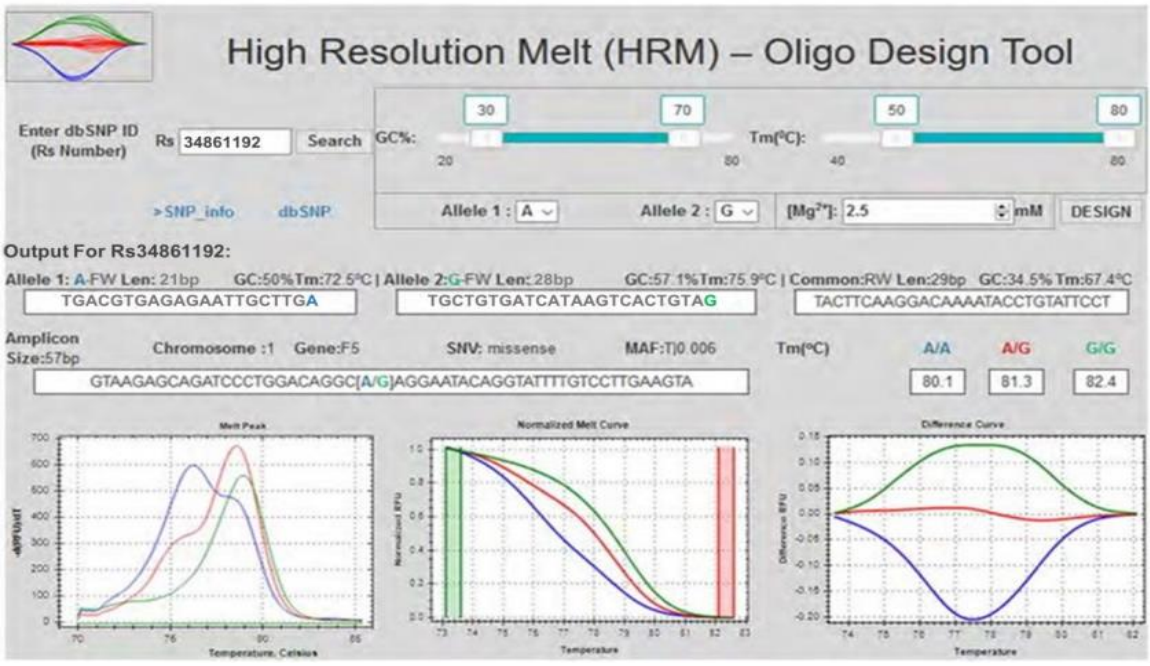
Appendix

2-b- The information of rs 34861192 from NCBI website.



3- A-NCBI /gene / SNPs rs34861192 G>A

1-https://www.ncbi.nlm.nih.gov/snp/rs34861192 G>A
https://www.ncbi.nlm.nih.gov/projects/sviewer/?id=NC_000019.10&v=7668689:7668689&mk=7668689|rs34861192 G>A|008000.
2-Programming design method of "HRM- Oligo design tools"



.

B-Programming design method of "HRM- Oligo design tools" NCBI /gene / SNPs NG023447 C>G
https://www.ncbi.nlm.nih.gov/gene/?term=-420+C%3EG+NG_023447
https://www.ncbi.nlm.nih.gov/genome/gdv/browser/gene/?id=56729

C- Retn – resistin

https://www.ncbi.nlm.nih.gov/search/all/?term=*retn*
Homo sapiens resistin (retn), RefSeqGene on chromosome 19
https://www.ncbi.nlm.nih.gov/nuccore/302058260

D-primer3 in put
https://www.rosaceae.org/primer3/cgi-bin/primer3web_results.cgi

E-Optimase ProtocolWriter™
http://mutationdiscovery.com/md/MD.com/screens/optimase/OptimaseProtocol.jsp?action=create

4-

A-odds ratio was measured by an online software program

(https://www.medcalc.org/calc/odds_ratio.php)

B-Hardy Weinberg equilibrium was measured by

(https://scienceprimer.com/hardy-weinberg-equilibrium-calculator)