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A comparative Study of Some Biomarkers in Blood and Saliva of Type II Diabetes Mellitus

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بسم الله الرحمن الرحيم

(يَا بَنِي آدَمَ خُذُوا زِينَتَكُمْ عِندَ كُلِّ مَسْجِدٍ وَكُلُوا وَاشْرَبُوا وَلَا تُسْرِفُوا ۚ إِنَّهُ لَا يُحِبُّ الْمُسْرِفِينَ)

صدق الله العظيم

سورة الأعراف

الآية (31)

Dedication

I dedicate this work to my great country, Iraq.

Beloved parents.

Beloved family and my husband.

Dear brothers and sisters.

Wonderful friends.

Belqis Hamed

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Summary

Type II Diabetes is a disease with a variety of causes and complex complications. Many people suffer from it in both developed and developing countries. It has been closely linked to the development of individuals' lifestyle and unhealthy diet. It often appears in the elderly, but recently it has begun to appear in young people. Its environmental and genetic causes vary among people and races in the world.

The current study was conducted with the participation of 54 patients with type II diabetes (T2DM) group (27 male and 27 female) and 34 healthy non-diabetics-(ND) group (17 male and 17 female).

The age ranged for both groups from 40 to 55 year. The subjects attended the laboratory of the consulting clinic at Baghdad Teaching Hospital in Baghdad – Iraq, from December 2021 to February 2022.

Oral consent was taken from the groups of participants (T2DM and ND) to collect saliva and blood samples, and to obtain information on age, weight and height for the calculation of body mass index (BMI) and measurement of waist circumference (WC) and neck circumference (NC).

The oxidase-peroxidase method was used using the assay kit from Randox Company –UK to determine the concentration of fasting glucose (FG) in saliva, and the results of the serum (FG) test and (HbA1c) for both groups were obtained from the consulting clinic laboratory records, in addition, Enzyme Linked Immuno Sorbent Assay(ELISA) technique was used to determine the level of type II diabetes millets biomarkers (insulin, resistin, visfatin, interleukin -18 and vitamin D3) in the blood and saliva for both groups.

Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) technique was used to identify alleles and genotypes of single nucleotide polymorphisms (rs1862513- *resistin gene*, rs11977021- *visfatin gene*, rs3842752 - *insulin gene* and rs360717- *interleukin-18 gene*) and polymerase chain reaction - single strand conformation polymorphism (PCR-SSCP) technique was used to identify genotypes of single nucleotide polymorphisms (rs 12255372-*TCF7L2*

gene and rs13266634-*SLC30A8 gene*). Sequence analysis of nucleotides was perfo-rmed by Macrogene, South Korea.

The results of the current study showed a significant increase ($p \leq 0.05$) in (BMI), (WC), and (NC) in the T2DM group compared to the ND group.

Moreover the results showed a significant increase ($p \leq 0.05$) in the percentage of (HbA1c) and the levels of fasting biomarkers in serum and saliva (glucose, insulin, resistin, visfatin, interleukin-18 and vitamin D3) in the T2DM group compared to ND group.

The current results, showed a significant medium positive correlation ($r = 0.695$, $p<0.05$) between (BMI) and (WC), and a weak correlation between (WC) and (NC) ($r = 0.310$) in the T2DM group.

In addition to the existence of a strong positive correlation ($r = 0.844$) between (HbA1c) and (FG) in the serum, the presence of a strong positive correlation ($r = 0.741$) for insulin levels, the presence of a medium positive correlation ($r = 0.528$) for visfatin levels, and the presence of a positive medium correlation ($r = 0.544$) for vitamin D3 levels, between serum and saliva for T2DM group.

The distribution of alleles and genotypes showed that the allele (C) of the single nucleotide polymorphism (rs1862513) in the *resistin gene* represents a risk factor for developing type II diabetes among Iraqis. While there was no significant difference in the level of resistin among carriers of the genotypes (G/G, G/C, C/C).

However, no significant differences($P>0.05$) were found in the distribution of alleles and genotypes, the levels of biomarkers among carriers of genotypes, between the T2DM group and the ND group for SNPs (rs3842752-*insulin gene*, rs12255372-*TCF7L2gene*, rs13266634- *SLC30A8gene*, rs 11977021-*visfatin gene*, rs360717-*interleukin 18gene*).

Sequencing analysis of the rs12255372-*TCF7L2 gene* revealed the presence of the (A) allele, an alternative to (T), amongT2DM group.

Based on the results of the current study, It was concluded that saliva could be used as a substitute for serum in evaluating the levels of some biomarkers related to type II diabetes. In addition, measuring waist circumference is a good indicator

of central obesity, which increases susceptibility to type II diabetes, the biomarkers that are measured in the fasting state (Glucose, insulin, resistin, visfatin and interleukin- 18) are good indicators for predicting or diagnosing type II diabetes in serum and saliva.

The appearance of correlations between serum and saliva of biomarkers (Insulin, Visfatin) strongly indicates the efficiency of saliva as a biological fluid substitute for serum, and the absence of correlations with other biomarkers indicates the need for more studies to clarify the factors that cause the change in the level of secretion.

It was also concluded that the genetic variants of single nucleotide polymorphisms (SNPs) may differ in different races and societies, and their functional role may also differ. It was also concluded that the allele (C) present in (rs1862513) in the *resistin gene* is a risk factor for developing type II diabetes among Iraqis.

Therefore, one of the recommendations was to measure other biomarkers related to type II diabetes in saliva and to investigate more single nucleotide polymerphisms that are expected to have a functional role in the occurrence of type II diabetes among Iraqis..

List of contents

List of Tables

List of figures

List of Abbreviation	
ATP	Adenosine Tri –Phosphate
BMI	Body Mass Index
CYP2R1	Cytochrome P450 family 2 subfamily R member 1
CYP2B1	Cytochrome P450 family 2 subfamily B member 1
DMT2	Diabetes Mellitus Type II
FPG	Fasting Plasma Glucose
GDM	Gestational Diabetes Mellitus
HbA1C	Glyco-Hemoglobin
HDL	High-Density Lipoprotein
HMG	High-Mobility Group Domain
IgE IgG	Immunoglobulin Epsilon Immunoglobulin Gama
IGT	Impaired Glucose Tolerance
IFG	Impaired Fasting Glycaemia
IL-1β	Interleukin 1 Beta
iNAMPT	Intracellular form Nicotinamide Phosphoribosyl Transferase
IDF	International Diabetes Federation
eNAMPT	Extracellular form Nicotinamide Phosphoribosyl Transferase
GWAS	Genome-Wide Association Studies
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
IL-6	Interleukin -6
LADA	Latent Autoimmune Diabetes in Adults
LPS	Lipopolysaccharide
LDL	low dense lipd
NC	Neck circumference
NIH	National Institute of Health
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NAD	Nicotinamide Adenine Dinucleotide
OGTT	Oral Glucose Tolerance Test
PBEF	Pre-Beta Cell Colony-Enhancing Factor
PRBC	Packed Red Blood Cells
RCTs	Randomized Controlled Trials
SLC30A8	Solute Carriers of family 30 member A8
2-h PG test	2-hour plasma glucose test
TCF7L2	Transcription factor 7-like 2
TCF/LEF	T-Cell Factor and Lymphoid Enhancer binding factor Family
TNF-α	Tumor Necrosis Factor Alpha
VDR	Vitamin D Receptor
WC	Waist Circumference
WHO	World Health Organization
ZnT	Zinc Transporter

Chapter One
Introduction

1.1 Introduction

Type II diabetes millitus-T2DM has gained its clinical importance from being a complex metabolic disease worldwide (Guariguata *et al*., 2014). It is characterized by the presence of insulin resistance or a decrease in insulin production or both (Deshmukh *et al*., 2015).

According to the World Health Organization, the rate of T2DM among Iraqis is estimated 8.5% to 13.9%, meaning that there are 1.4 million patients with T2DM (World Health Organization, 2019**).** The development of T2DM depends on both heredity and life style (Tuomi *et al.*, 2014**).** Decreased physical activity associated with an increase in body mass index are risk indicators for the occurrence of T2DM (Weinstein *et al.*, 2004**).** Acute and chronic high blood glucose levels cause many damages to the structure and function of tissues and organs in the body (Nawale *et al*., 2006 and Gurung & Jialal, 2021).

The secretion of insulin from the pancreas occurs in response to high blood glucose, allowing glucose to enter the cells, and at the same time inhibiting the process of glycogenolysis in the liver and muscles (Burhans *et al*.,2018).

Resistin is a cytokine that is involved in many inflammations. It binds to the insulin receptor, causing insulin resistance. Its secretion increases in the T2DM patients (Li *et al*., 2021).

Visfatin is a hormone secreted by adipose tissue that mimics the action of insulin, but does not connect to the insulin receptor. Its secretion increases in patients with T2DM (Wang *et al*., 2006).

Interleukin-18 is a cytokine whose levels are increased in inflammatory conditions, as well as complications of T2DM associated with cardiovascular disease (Cavalcante *et al*., 2020).

Vitamin D3 is a hormone that has an important role in all vital processes in the body. Its level decreases greatly in patients with T2DM. It plays an important role in increasing insulin sensitivity and glucose homeostasis (Holick, 2007 and Martínez Pizarro , 2020).

Blood is the approved vital fluid for diagnosis and monitoring of diseases and ailments (May *et al*., 2013)**.** Saliva is one of the vital fluids in the body that contains a lot of components that cross to the salivary glands from the blood (Chi- appin *et al*., 2007). Therefore, it can serve as a substitute for serum and can be used for diagnostic purposes (Ladgotra *et al* ., 2016)**.**

Genetic variations of single nucleotides polymorphisms-SNPs, according to their locations in genes, affect the process of gene expression and thus the level of protein production (Brett *et al*., 2005). SNP (rs3842752G>A) is located in the *Insulin gene* at position chr11:2159843 (GRCh38.p14), (Sherry *et al*., 2001). There is an association of rs3842752 with type I diabetes and circulation glucose level (Reddy *et al*., 2011 and Gootjes *et al*., 2022).

Promoter's SNP (rs1862513 C>G -*Resistin gene*) is one of many SNPs that have been studied for the *Resisten gene* because its effect on gene expression and concentration of the circulating resistin (Ukkola *et al*., 2008 and Hivert *et al*., 2009).

Numerous studies in different populations indicated that genetic variants of the *TCF7L2 gene* are associated with the risk of developing T2DM and its complications, rs12255372 (G/T) is an intronic single nucleotide polymorphism (SNP) in the *TCF7L2* gene that is closely associated with the risk of developing T2DM (Florez *et al*., 2006 and Li *et al*., 2018).

In many populations in the world, there is an association between the single nucleotide wild-type C allele of SNP rs13266634 (C>T)in the *SLC30A8gene*, and susceptibility to T2DM (Huang *et al*., 2019).

The SNP rs360717, is one of the SNPs present in the promoter of the *Interlukin-18(IL-18) gene*, has been tested in several studies to determine its relationship with IL-18 levels and its association with disease, as in the immune response to H. pylori (Myung *et al*., 2015), myocardial infarction susceptibility (Koch *et al*., 2011).

The SNP (rs11977021) is located in the promoter region of the *Visfatin gene*, and may affect the gene transcription process (Ooi *et al*., 2016). It is associated with serum visfatin levels, obesity, hypertension, and hyper-triglyceridemia in obese children (Ooi *et al*., 2014).

1.2 The aim of the study

The current study aimed to find the correlation of some biomarkers between serum and saliva of type II diabetic patients. This is to encourage the adoption of saliva as a vital fluid characterized by ease of collection and low cost as an alternative to serum in measuring the parameters of type II diabetes. Also to determine the genetic variations of some of these biomarkers and their relationship to susceptibility to type II diabetes, and this achieved by the following objectives:

1- Calculating the body mass index value based on height, weight, waist circumference, and neck circumference for the two study groups and finding the relationship with type II diabetes.

2- Estimating the levels of fasting biomarkers (glucose, insulin, resistin, visfatin, interleukin 18 and vitamin D3) in serum and saliva for both groups and finding the relationship with type II diabetes.

3- Studying the possibility of adopting saliva as an alternative to serum by studying the correlations of biomarkers between saliva and serum in aT2DM group.

4- Studying some genes polymorphism by using Polymerase Chain Reaction (PCR) technique.

5-Detection gene polymorphisms, using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) technique.

6. Analyzing the genetic results by sequencing method to identify the mutated site within the gene and identify genotypes.

7-Comparison the levels of each biomarker among genotype carriers to determine which allele affects the expression of the biomarker.

Chapter Two
Literature Review

2. Review of literatures

2.1 Diabetes mellitus (DM)

Diabetes includes several types, all characterized by high blood glucose (Das *et al*., 2018)**.** Diabetes is a non-communicable disease that affects all human societies, with differences in its prevalence rates between societies and races. This disease constitutes a huge health problem, as for individual or community, and it is still raising up and exacerbating, it is defined as a chronic disorder with multiple causes (genetic and environmental). It is characterized by an excessive or high level of blood glucose resulting either from a defect in insulin secretion or a defect in the action of insulin or both, in addition to suffering from the symptoms of diabetes, the chronic complications lead to various conditions that are life-threatening. Diabetes causes about 6% of all deaths worldwide (Alashbal , 2019)**.**

Hyperglycaemia is a common characteristic of all types of diabetes mellitus (Egan & Dinneen, 2019)**.** Moreover, Long-term damage and failure of a variety of organs, especially the eyes, heart, kidneys, veins, and nerves, are consequences of the hyperglycemia (Sneha & Gangil, 2019)**.** Coronary heart disease, heart failure and peripheral arterial diseases, are the most common illnesses developed by diabetes(Glovaci *et al.*, 2019)**.**

There are three common types of diabetes. Type I diabetes accounts for about 5% of total diabetes (WHO, 2019). Type I diabetes usually develops when the pancreatic beta cells are destroyed by autoimmune antibody, resulting in a low or complete absence of insulin production that is most prevalence in children, and may arise latent in adults, as latent autoimmune diabetes in adults (LADA) (Petersmann *et al*., 2018). Pancreatic Beta-cell damage is predominantly T-cell mediated. Subsequently, B-cells produce auto antibodies against islet antigens that serve as important diagnostic markers for the disease. Non-immune-mediated type I diabetes may occur as a result of damage or destruction of pancreatic Beta cells (Yang *et al*., 2014).

Weight loss, polydipsia, and urination are short-term symptoms of type I diabetes, in addition to a family history of autoimmune diseases for instance Hashimoto's thyroiditis or Graves' disease (Di Meglio *et al*., 2018). Treatment is primarily insulin-dependent, essential for survival, as the absolute absence of

insulin leads to ketoacidosis as a consequence of providing fuel to insulin-dependent tissues (Perilli *et al*., 2013)**.** Ketoacidosis is essential during starvation to supply fuel to tissues when the nutrient supply is insufficient (White &Venkatesh, 2011)**.** But long-term ketoacidosis is fatal due to low blood pH, which is why type I diabetes is the most deadly form of diabetes, although a subset of type II diabetics can also develop ketoacidosis (Perilli *et al*., 2013 and Gosm-anov *et al* ., 2014)**.**

The second major type of diabetes is type II diabetes, which accounts for 90-95% of all cases of diabetes worldwide (WHO, 2019)**.** It is Characterized by insulin resistance with a relative decrease in insulin production**.** Type II diabetes often accompanies other diseases such as metabolic syndrome (Petersmann *et al*., 2018)**.**

The third major type of diabetes is Gestational diabetes (GDM) that is characterized by high blood glucose level (impaired fasting glycaemia –IFG and impaired glucose tolerance-IGT) during pregnancy in women who have no previous history of diabetes**.** Later it may develop into type II diabetes (WHO , 2013 and Deshmukh *et al* ., 2015)**.** The proportion of women who develop gestational diabetes is estimated between 3% and 9% of all pregnancies (Genuth *et al*., 2015)**.**

The diabetic environment can be defined as a group of related substances (nutrients, hormones, cytokines) with variable concentrations**.** In a normal pregnancy, the mother's abnormal metabolic environment may generate stimuli within adipose tissue cells and placental cells resulting in increased production of inflammatory cytokines but with reduced expression. One of the main hypotheses for the promotion of maternal insulin resistance and gestational diabetes is an association between inflammation and metabolic changes caused by changes in the circulating concentrations of TNF-α, adiponectin, leptin and Resistin , of which high levels have been documented (Desoye & Hauguel deMouzon, 2007)**.**

According to Tummala *et al*.(2019) the incidence of gestational diabetes may be induced by increased age, pre-pregnancy weight gain, family history of diabetes, previous pregnancy complications, lack of exercise**.**

2.2 Type II diabetes mellitus (T2DM)

2.2.1 Definition of type II diabetes mellitus (T2DM)

Type II diabetes mellitus -T2DM is the most common metabolic disorder of clinical importance and in recent decades has become a global epidemic and constitutes a major burden on health care worldwide (Guariguata *et al*., 2014)**.**

Type II diabetes mellitus **,**also known as non-insulin dependent diabetes mellitus (NIDDM), or previously as "adult –onset diabetes"**.** It occurs as the development of insulin resistance, a condition in which cells cannot use insulin properly whether there is an increase or decrease in insulin production (Deshmukh *et al* ., 2015)**.**

World Health Organization (WHO) has defined diabetes as **"**a metabolic disorder of multiple pathogens characterized by chronic hyperglycemia with disruption of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both**"(**Thompson & Kanamarlapudi , 2013)**.**

2.2.2 Prevalence of type II diabetes

Diabetes mellitus (DM) is the fastest growing and most challenging health issue of the century (Meo *et al*., 2017). It is expected that the prevalence of diabetes will reach from 2017 to 2045 in 221 countries and territories, grouped into seven regions of the international diabetes federation (IDF) which includes : Europe (EUR), Africa (AFR), Middle East and North Africa (MENA), South and Central America (SACA), North America and Oceania Caribbean (NAC), Western Pacific (WP), and Southeast Asia (SEA). As follows: in countries with high incomes, it is estimated that approximately 87% to 91% of the total number of people with diabetes have type II diabetes, and it is estimated that around 7% to 12% have type I diabetes (Federation, I. D. , 2017).

The incidence of other types of diabetes is estimated from 1% to 3%**.**It is estimated that there are about 425 million people living with diabetes worldwide, 8.8% of adults aged 20-79 have diabetes. It is also estimated that the number of people with diabetes will rise to 451 million with the expansion of age from 18-99 years, it has been observed that not only adults develop type II diabetes, but it is also increasingly beginning to appear in children (WH O, 2019). It is estimated that

by 2045 there will be 693 million people from 18 to 99 years old, or 629 million people from 20 to 79 years old who will have diabetes (Federation, I. D. , 2017)**.**

In 2019, the Middle East and North Africa (MENA) region had the highest prevalence of diabetes in the world at 12.2% (El-Kebbi *et al.*, 2021)**.** On the other hand, it is estimated that the prevalence of diabetes will increase to 110% between 2017 and 2045 in the MENA region and the number of diabetes patients will reach 629 million worldwide in 2045 (Federation, I. D. , 2017)**.**

The highest prevalence of T2DM in the Arab countries was 31.6% in Saudi Arabia, 29% in Oman, 25.4% in Kuwait, 25.0% in Bahrain, and 25.0% in the United Arab Emirates. The lowest prevalence rate was (4.7%) in Mauritania (3.9%) in Somalia (Meo *et al*., 2017)**.**

In Iraq, it is still difficult to fully understand the prevalence of diabetes due to the insufficient number of randomized controlled trials (RCTs) and epidemiological studies related to diabetes (Abusaib *et al.*, 2020)**.** But WHO, (2019) mentioned that, in Iraq the prevalence of T2DM ranges from 8.5% to 13.9% , where it is estimated that there are about 1.4 million diabetics**.**

2.2.3 Causes of type II diabetes(T2DM)

Type II diabetes (T2DM) is a global epidemic that increases spending on health care and lowers an individual's quality of life**.** Several non-genetic risk factors have been elucidated, but their epidemiological validity has not been comprehensively assessed (Bellou *et al*., 2018)**.** Both genetic and environmental factors contribute to the development of type II diabetes (Tuomi *et al.*, 2014)**.** They are determinants of both insulin resistance and impaired beta-cell function. Beta cells produce insulin depending on the feedback loop with insulin-sensitive tissues to maintain normal blood glucose metabolism**.** In the case of insulin resistance, beta cells increase insulin production in order to tolerate normal glucose, but with continued resistance to the action of insulin by the body's cells, insulin production will decrease due to deterioration in beta cell functions, and thus blood glucose levels will rise (Kahn *et al*., 2014)**.**

Any defects in the molecular mechanisms involved in the processes of insulin release and activity could lead to impaired glucose metabolism that is responsible for the development of T2DM (Galicia-Garcia *et al*., 2020)**.**

Although an individual's genetic predisposition determines susceptibility to diabetes, rapid changes in environmental factors (such as lifestyle) are the most likely explanation for the increasing number of people with both forms of diabetes (Tuomi *et al.*, 2014)**.** The increase in the prevalence and incidence of type II diabetes during the past and present centuries is generally believed to be due to the increase in the percentage of the world's population who are overweight, accompanied by the adoption of more sedentary lifestyles (FB, 2011)**.**

Several risk factors for developing type II diabetes have been identified (Thompson & Kanamarlapudi, 2013 and Ardisson Korat *et al*., 2014)**.** Obesity is the main risk factor for developing type II diabetes, as the risk increases by 90 times for people who suffer from obesity, and the majority of people with type II diabetes are overweight or obese (Golay &Ybarra, 2005 and Kyrou &Weickert, 2010).

The risk of developing type II diabetes is positively correlated with an increase in Body mass index -BMI, and an increase in (BMI) above 30 increases the risk significantly (Kyrou & Weickert, 2010)**.** In Western countries, statistics indicate that about 50% of patients with type II diabetes have a BMI greater than 30 and 30-40% with a BMI of 25-30 (Nguyen *et al*., 2011)**.** Body mass index (BMI) and physical inactivity are considered as independent risk factors for type II diabetes (Weinstein *et al.*, 2004)**.** Moreover, increased fat deposition in visceral areas increases the risk of developing type II diabetes by more than double (Gastaldelli, 2008 and Thompson & Kanamarlapudi, 2013)**.**

Over the past few decades, it has also been shown that environmental changes can play a role as a cause of type II diabetes, such as the use of medicines, food additives used in food processing and packaging and pesticides (Corkey, 2012)**.** It has been shown that some environmental pollutants can alter and weaken the function of beta cells in animals, such as Bisphenol-A, which is used in the manufacture of plastic food containers (Alonso-Magdalena *et al.*, 2011)**.**

2.2.4 Pathogenesis of Type II diabetes(T2DM)

Type II diabetes mellitus (T2DM) is mainly caused by the involvement of two main factors: first, a defect in insulin secretion by pancreatic beta cells, and secondly, the inability to respond to insulin by insulin-sensitive tissues (Roden & Shulman, 2019)**.**

Metabolic demand precisely corresponds to insulin release and action; So there is tight regulation, between the molecular mechanisms involved in the synthesis and release of insulin, as well as the tissue response to insulin. The presence of defects in any of the mechanisms involved causes an imbalance in the metabolic balance and the occurrence of type II diabetes (Galicia-Garcia *et al*., 2020)**.** In addition to the defect in pancreatic beta cells, it is also characterized by increased function of pancreatic alpha cells, and resistance of peripheral tissues to insulin**.** These alterations result in hyperglycaemia due to impaired peripheral glucose uptake, dyslipidaemia (hypertriglyceridemia and low high-density lipoprotein [HDL]-cholesterol) due to impaired peripheral fat uptake, impaired amino acid uptake and ATP production into peripheral tissues, such as skeletal muscle, due to the impaired nutrient uptake, and increased glucagon production, which further amplifies hyperglycaemia and hyperlipidaemia (D'alessio, 2011 and Voet D. & Voet J. , 2011)**.**

Usually, T2DM does not appear acutely in individuals but is preceded by the presence of an insidious stage of pre-diabetes (Bansal, 2015). During pre-diabetes, the number of pancreatic beta cells decreases and their function slowly deteriorates, and symptoms of type II diabetes usually appear after a few years (Tabák *et al*., 2012)**.** Over time, beta cells are unable to compensate for insulin resistance and their ability to secrete insulin is impaired, and many of beta cells undergo programmed cell death (apoptosis), which is believed to occur as a result of a variety of stressors, such as oxidative stress, increased insulin demand, endo-plasmicreticulum, dyslipidemia, inflammatory stress, and amyloidal (Cerf, 2013 and Boland *et al*., 2017)**.**

The pre-diabetes stage and the onset of type II diabetes are characterized by hyperinsulinemia. Most of the insulin produced is proinsulin, which indicates weak

beta cell function in processing insulin into mature insulin (Breuer *et al*., 2010 and Pfützner *et al*., 2015)**.**

Pathology of beta-cell during type II diabetes begins with impairment of the first phase of insulin secretion, followed by ongoing insufficient secretion of insulin to promote normoglycemia and normolipidemia, increase in the proportion of proinsulin secretion and dysfunction of glucose sensing (Boland *et al*., 2017)**.**

2.2.5 Treatment of type II diabetes

Differences in diagnosis, severity of insulin deficiency and treatment requirements make type II diabetes a heterogeneous disease (Faerch *et al*., 2016 and Karalliedde & Gnudi, 2016)**.** For the good control of the metabolism in patients with diabetes and its long-term maintenance, a combination of lifestyle modifications and appropriate pharmacological treatment is required**.** Moreover maintaining a near-normal level of glycated hemoglobin greatly reduces the risk of complications in the macro-vascular and micro-vascular vessels (Marín-Peñalver *et al*., 2016)**.**

Energy balance is determined by dietary intake and physical exercise (National Diabetes Education Program , 2016)**.** This balance is the basic rule that must be followed in the treatment of diabetic patients. In addition, energy levels and well-being must be maintained by sleeping at least 7 hours each night (Garber *et al*., 2016)**.** Evidence supports the association of reduced cardio-metabolic risk factors with 6 to 9 hours of sleep each night (Cappuccio *et al*., 2011)**.** While the reduced hours of sleep leads to increased insulin resistance, hyperglycaemia, hypertension, and dyslipidemia (McNeil *et al*., 2013)**.**

The total caloric intake of the diet depends on several factors, such as determining obesity or weight gain based on (BMI)**.** Most patients with type II diabetes suffer from overweight that leads to insulin resistance and insulin secretion defects (Shoelson *et al*., 2006 and Nguyen *et al*., 2011)**.** Weight reduction is considered a positive factor in improving insulin sensitivity, improving parameters of glycemic control (Escalante-Pulido *et al*., 2003)**.**

Physical activity and exercise are key strategies in treating diabetes**.** Boosting exercise, within a defined plan, provides multiple benefits**:** increased tissue

sensitivity to insulin, and improved glycemic control(Phielix *et al*., 2010) and significantly lowers HbA1c (Chudyk & Petrella, 2011)**.**

Although pharmacological options offer more therapeutic possibilities, especially in patients with type II diabetes, lifestyle interventions are essential in the approach of these patients to achieve therapeutic goals (Nathan *et al*., 2009)**.** Choosing the most effective and most tolerable drugs for patients among the different types of diabetes medications must be made by Physicians, and treatment options should be based on the characteristics of each patient**.** Metformin remains a first choice as a treatment for most type II diabetes patients (Marín Peñalver *et al*., 2016)**.**

2.3 Blood as a diagnostic fluid

Blood is classified as a connective tissue with a complex formation that performs many functions such as transporting gases (oxygen and carbon dioxide), various nutrients, water, waste products resulting from metabolism, hormones, all immune factors, heat and many other substances (Patton & Thibodeau, 2013)**.** Diagnosis and monitoring of pathological conditions often depend on blood samples that can be collected relatively easily**.** In addition, plasma or serum can be extracted to perform certain tests when whole blood contains many interfering substances that may prevent obtaining accurate results (May *et al*., 2013)**.**

Whole blood consists of a mixture of cellular, colloidal, and crystalline components. Due to the different relative densities of hematopoietic materials and the different sediment rate and size, it was easy to separate them using centrifugal force **(**Hardwick, 2008)**.** In addition to that, the whole blood can be separated into many different blood components such as packed cell volume (PCV), fresh frozen plasma, cryoprecipitate and platelet concentration. Each of these components can be used to investigate a different blood indication; Thus, the process of separating these components has increased the benefit obtained from one unit of whole blood. Taking into consideration that these different ingredients need different storage and preservation conditions and temperature requirements to maintain their therapeutic efficacy (Basu & Kulkarni, 2014)**.**

On the other hand, blood culture can be relied upon as a method of reference at the present time to detect any microorganisms causes of diseases in the circulatory system, such as bacteria or fungi (Pilecky *et al*., 2019)**.** As well as ,whole blood point-of-care (POC) biomarkers are useful in establishing an accurate diagnosis, good treatment guidance, and accurate assessment of disease risk and predict-ability. These biomarkers are tests used to monitor the normal or pathological processes of a patient, especially in the fields of human and veterinary medicine, and they are of increasing importance in disease management, especially serious diseases**.** These biomarkers including lactate, triglycerides, glucose, cardiac troponin I, creatinine, serum amyloid, and many other tests (Radcliffe *et al*., 2015)**.**

2.4 Saliva as a diagnostic fluid

Saliva is a vital body fluid that is secreted from different groups of salivary glands include,three major large glands: the parotid glands with serous secretions, the sub-mandibular and sub-linguall glands with mixed secretions (mucosal serous), and from many minor salivary glands**.** Water makes up about 99% of its composition, and the rest contains various substances such as electrolytes and proteins that include enzymes, immuneglobulins, as well as mucous glycolproteins that have an important role in maintaining oral health. Saliva also contains different amounts of glucose, ammonia and urea (Carranza *et al.*, 2005 and de Almeida *et al*., 2008) **.**

Saliva, also contains ingredients that are derived from the oropharyngeal mucosa (oral mucosal transudate, fungi, bacteria, viruses, and gastrointestinal reflux liquid) (Aps & Martens, 2005 and Dame *et al*., 2015)**.** There is also a plasma transudate that is produced from crevicular fluid in the order of 2-3 µl /h per tooth and food debris as well as blood-derivated components for example, erythrocytes, plasmatic proteins and leucocytes as in the case of infections (Chiappin *et al*., 2007)**.**

Anatomically, the salivary glands are distributed in and around the mouth inside the head **.** The secreted saliva is transmitted through channels spread in the mouth**.** Saliva plays an important role in maintaining oral health by protecting and moisturizing the mucous membranes (Contreras-Aguilar and Gómez-García 2020)**.**

Salivary glands are classified according to their location, size, quantity and quality of saliva they secrete**.** The pathophysiology of salivary glands has been given attention in cases such as diabetes and metabolic disorders**.** Diabetes is a global health problem characterized by the occurrence of micro-vascular , macro-vascular complications and directly associated with periodontal and oral health lesions. There is evidence that saliva secreted contents are linked to the progression of diabetes (Fouani *et al*., 2021)**.**

Biological processes and the health of all body organs can be identified through the various bodily fluids of the human body, which include blood, urine and saliva through their high content of proteins that may arise with many systemic and oral diseases, which give great benefit as biomarkers in diagnosing and controlling diseases to maintain human health (Malathi *et al*., 2014)**.**

Biomarkers are used as indicators of normal or pathological processes. Many molecules found in vital fluids such as proteins, metabolites, lipids and microbes can act as biomarkers**.** Saliva is one of the biological fluids that plays an important role as a diagnostic tool because it reflects the pathological condition of the individual since it contains a small amount of plasma which represents plasma-derived biomarkers that facilitates continuous monitoring of the general health status and oral health as well as allows rapid, easily collect and inexpensive detection of biomarkers (Helmerhorst *et al*., 2018 and Buzalaf *et al*., 2020)**.**

Saliva is considered to be the most ethically suitable biological liquid for scientific investigations. The advantages of saliva testing include disease identi-fication, prediction and monitoring, in addition to other features such as low cost and ease of collection, which allows it to be collected several times a day, that making it easier to repeat the tests (Chojnowska *et al*., 2018 and Gug *et al.,* 2019)**.**

Due to its multiple advantages over other body fluids, saliva as a vital fluid has generated great interest among researchers (Roi *et al*., 2019)**.** The hormones that are diagnosed in the plasma can be found in saliva as well**.** Although some correlations are found between them, it is necessary to do more research to prove the correlation between the level of salivary hormone and the level of plasma hormone, so that this correlation is highly reliable in pathological and physio-logical conditions (Chiappin *et al*., 2007)**.**

In fact, blood is considered the gold standard biological fluid used in the diagnosis of diabetes mellitus (DM), but because saliva contains components of blood serum, it can be a substitute for serum as a biological fluid used for diagnostic purposes(Ladgotra *et al* ., 2016)**.**

2.5 Anthropometric measurements

The term anthropometric consists of two words "anthropo" means human and "metric" means measure. Anthropometrics is mainly concerned with determining the measurements of individuals in societies in order to find the physical differences between them**.** It is concerned with the study of the size and shape of the biological forms components and their variations within populations**.** Anthropometrics has an important role in medical sciences, biometrics, forensics, sports, criminology, etc**.** (Utkualp & Ercan, 2015 and Thamizhselvi & Geetha, 2019**).**

2.5.1 Body mass index (BMI)

Body mass index (BMI) is define as statistical indicator for individual's fatness based on characteristics of height and weight to classify them into categories. It is used widely as a risk factor to determine the likelihood of occurrence and prevalence of various health issues. However, BMI is considered as poor index for measuring the total percent of body fat . It does not provide any information of the fat mass in different sites of the human body (Nuttall , 2015**).**

BMI is calculated by the equation "(BMI = weight (in kg)/ height^2 (in m^2)". The resulted number represent the BMI number of each individual. The National Institute of Health (NIH) categorized people to groups of underweight, normal weight, overweight, or obese, **(**Table 2.1) (WHO, 2000 and Consultation, 2004**).**

Table (2.1) :Classification of obesity based on BMI(WHO, 2000).

	Groups	BMI
1	severely underweight	BMI <16.5kg/m^2
2	underweight	BMI <18.5 kg/ m^2
3	normal weight	BMI ≥ 18.5 to 24.9 kg/ m^2
4	Overweight	BMI ≥ 25 to 29.9 kg/ m^2
5	Obesity	BMI ≥30 kg/ m^2
6	class I obesity	BMI from 30 to 34.9 kg/ m^2
7	class II obesity.	BMI from35 to 39.9 kg/ m^2
8	class III obesity	BMI ≥40 kg/ m^2

Regarding clinical practice, increased BMI numbers are associated with great number of overweight patients (Sisodia & Chouhan , 2019)**.** BMI is a significant risk factor and it is strongly related to the risk of developing type II diabetes (Sanada *et al*., 2012 and Ganz *et al*., 2014)**.**

Overweight or obese patient with insufficient physical activity is related with an increased risk of developing diabetes (Cloostermans *et al*., 2015)**.** Apositive correlation has been found between high BMI and poor control of blood glucose level in patients with type II diabetes (Sisodia & Chouhan , 2019)**.**

In patients with new diagnosis of type II diabetes, increased body mass index is linked to increased risk of cardiovascular diseases and mortality (Bodegard *et al*., 2013)**.** Lastly, type II diabetic patients have higher mortality risk if their BMI is above 40 kg/m2 (Edqvist *et al*., 2018)**.**

2.5.2 Neck circumference (NC)

Neck circumference (NC) is a new and valuable tool for defining obesity that is simple and inexpensive, showing the distribution of fat in the upper body (Saka *et al*., 2014 and Silva *et al*., 2014)**.** In addition It is a good tool for assessing overweight and obesity when it is not possible to measure waist circumference and body mass index, especially in pregnant women and those who suffer from abdominal masses or a scites (Pei *et al*., 2018)**.**

It is the simplest tool for assessing central obesity in individuals with type II diabetes (Papaza firopoulou *et al*., 2016)**.** To measure all the circumferences of the

body a non-elastic tape with an accuracy of 1 mm is used (Zen *et al.*, 2012)**.** Neck circumference is measured in the middle of the neck, standing upright, looking straight ahead and shoulders down**.** In men with Adam's apple the measurement is taken just below the protrusion (Aswathappa *et al*., 2013) **.**

The normal value for measuring neck circumference in women > 34 cm and NC > 37 cm for men are the best cut-off points for determining the incidence of central obesity in individuals. (Yang *et al.*, 2010). Insulin resistance can be predicted by measuring neck circumference, which is an important risk marker for cardiovascular disease (Wang *et al*., 2015 and Saad *et al*., 2017)**.**

2.5.3 Waist circumference (WC)

The measurements widely used to measure obesity are waist circumference and body mass index, despite their limitations. Overweight and central obesity can lead to many diseases such as diabetes, metabolic syndrome, and cardiovascular disease (Pei *et al*., 2018)**.** A non-elastic measuring tape is used to measure the waist circumference within 1 mm horizontally at the level of the navel (Aswathappa *et al.*, 2013 and West *et al*., 2016)**.**

Increased waist circumference leads to increases the risk of developing type II diabetes **(**Tatsumi *et al*., 2015)**.** Central obesity is a better predictor than general obesity for predicting type II diabetes (Venkatrao *et al*., 2020)**.** Furthermore, abdominal obesity and waist circumference and its change were closely associated with the risk of developing type II diabetes, compared to indicators of general obesity ,as in Chinese adults (Fan *et al*., 2020) **.**

2.6 Physio-biochemical markers

2.6.1 Blood glucose

Glucose is the source of energy for all living organisms in the world**.** It is the main substance in the aerobic and anaerobic cellular respiration process**.** The structure of glucose is composed of 6 carbon atoms described by the chemical formula ($C_6H_{12}O_6$)**.**

Glycogen is the stored form of glucose in the body that is released during fasting times**.** Glucose is derived from many types of sugars that enter the body such as mono-saccharides (galactose and fructose), disaccharides (lactose and

sucrose) and polysaccharides (starch)**.** It is also derived from the breakdown products of proteins and fats through the process of creating glucose**.** When glucose enters the body, it is transported through the blood to tissues that require energy, which is obtained by breaking down glucose in a series of biochemical reactions that release energy in form of adenosine triphosphate -ATP (Hantzidiamantis & Lappin, 2019)**.**

Blood glucose level is mainly controlled by pancreas ,in Beta cells of pancreas, the glucose transporter (GLUT-2)facilitative glucose transporter protein is localized to the plasma membrane and functions as part of the glucose sensing mechanism for the stimulation of insulin secretion**.** The pancreas also secretes glucagon from alpha cells, which raises blood glucose when glucose level are low and also secretes somatostatin by delta cells, which has a net effect in lowering blood glucose levels (Vargas *et al*., 2021; El Sayed & Mukherjee, 2022 and Venugopal *et al.*, 2022)**.**

Blood glucose level Homeostasis (4 to 6 mmol or about 72 to 108 mg/dl) is affected by both the functional capacity of beta cells and the sensitivity of skeletal muscle cells, adipose tissue and liver to insulin**.** In cases of diabetes, a defect in (insulin production or absorption) can cause an increase in blood glucose levels (Fujii *et al*., 2019)**.**

Certain organs that do not have insulin receptors (do not require insulin to absorb glucose), such as the brain and red blood cells, are significantly affected by acute, chronic or recurrent decreases in blood glucose levels that are associated with significant morbidity (Iqbal & Heller, 2018 and Rehni & Dave, 2018)**.**

Acute or chronic hyperglycemia can cause diseases, as in both types of diabetes (I &II)**.** Poor control of chronically elevated blood glucose levels over time associated with significant morbidity occurs by several mechanisms **"** (Osmotic damage), glucose is osmotically active and can cause damage to peripheral nerves, (Oxidative stress), glucose participates in several reactions that produce oxidative byproducts, (Non-enzymatic glycation), glucose can complex with lysine residues on proteins causing structural and functional disruption**"** (Nawale *et al*., 2006 and Gurung & Jialal , 2021)**.** These mechanisms lead through macro and micro-

vascular complications to many clinical manifestations including chronic wounds and poor wound healing, peripheral neuropathy, retinopathy, cerebrovascular disease, coronary artery disease, and chronic kidney disease (Goyal & Jialal, 2022 and Goyal *et al*., 2022)**.**

Because the degree and duration of hyperglycemia is associated with micro-vascular and macro-vascular complications of diabetes, so normal blood glucose levels should be targeted by monitoring blood glucose levels, which is accomplished through various tests to determine the blood glucose level (Valeri *et al.,* 2004)**.**

Plasma glucose criteria enable the diagnosis of diabetes through several tests such as determining the fasting plasma glucose value(FPG) or the two-hour plasma glucose value (2-h PG) via the (75-g)oral glucose tolerance test(OGTT) or the A1C test (Committee , 2009)**.**

Criteria for diagnosing diabetes are based on the values of the following tests: fasting plasma glucose test (FPG) ≤126mg/dl or (7.0 mmol /L)**.** Fasting means not eating for at least 8 hours before the test is done to achieve a calorie fast; 2-h plasma glucose test(2-h PG) ≤ 200mg/dl (11.1 m mol /l) throughout of oral glucose tolerance test (OGTT); Glyco-hemoglobin test (HbA1c) ≤ 6.5% or (48 m mol/ mol)**.** Besides, the random plasma glucose test for hyperglycemic patients or hyperglycemic crisis patients is ≤ 200 mg/dl or (11.1 mmol/L)(American Diabetes Association , 2020)**.**

2.6.2 Insulin

Insulin is an important endocrine hormone in metabolism regulation of carbohydrate, protein and fat**.** It binds to receptors associated with the plasma membrane in target cells such as adipocyte, hepatocyte, and skeletal muscle cells, to facilitate the uptake of glucose available in the blood**.** Insulin classified as a polypeptide hormone synthesized and secreted by pancreatic beta cells (Jin Chan & Steiner, 2000 ; Shen *et al*., 2019 and Dağaşan & Erbaş , 2020)**.**

The molecular weight of Insulin is (6000) Daltons, with (51) amino acid in humans (Steiner *et al*., 1985)**.** These amino acids are arranged in two chains, A chain (21 amino acids) and B chain (30 amino acids), and these two chains are linked together by disulfide bonds (Steiner *et al*., 1985; Derewenda *et al*., 1989)**.**

Insulin production occurs in response to elevated levels of glucose in the blood **,** in addition to the main function of insulin, which is to transport glucose from the blood to target cells, insulin also performs other functions, such as inhibiting gluconeogenesis in the liver and facilitating the storage of excess glucose in the form of glycogen by glycogenogenesis and in the form of fat by adipogenesis. Glycogen and fat are the body's short- and long-term energy stores, respectively (Burhans *et al*., 2018)**.**

After eating meals, insulin is secreted at its highest peak and for a short duration (Huang *et al*., 2020)**.** Insulin exhibits a pulsatile release and blood insulin concentrations oscillate at a rate of 5-15 minutes per pulse, resulting in significant changes in plasma insulin levels within a short period of pulsatile time (Porksen *et al*., 2002 and Crofts *et al*., 2015)**.** Insulin concentrations during its presence in the peripheral plasma are affected by the kinetics of insulin distribution and its degradation. Where the volume of insulin distribution is greater than the volume of insulin present in the plasma several times (De Leon & Stanley, 2013).

Insulin levels are estimated by the balance between insulin release into the blood stream and its disappearance. It is estimated that 20 - 80% of the secreted insulin does not enter the blood circulation , but it is removed by the liver (Polidori *et al*., 2016).

In healthy subjects, the normal range for fasting insulin varies greatly between laboratories, it ranges from 3 to 30 μU/ml or 18 to 189 pmol/l (Crofts *et al*., 2015). Although measuring insulin levels is essential for research and clinical purposes, there is currently no reference method for insulin hormone assays. Different assays for measuring the concentration of insulin in the blood showed a maximum variation of 1.8-fold, and therefore care must be taken when making comparisons of insulin levels produced by different laboratories and research studies (Tohidi *et al.,* 2017).

2.6.3 Resistin

Resistin (*RETN*) a cysteine-rich protein (Li *et al*., 2021), was first discovered 20 years ago in mice and has been identified as a secretory factor specific to adipose tissue (Kim *et al*., 2001)**.** Later on, it was renamed resistin due to its resistance to insulin action (Steppan *et al*., 2001)**.**

In humans, resistin is secreted by white blood cells that infiltrate adipose tissue as a result of the low-grade inflammatory response associated with obesity, rather than by the adipocytes themselves (Hannan & Culligan, 2015)**.** The molecular weight of resistin is 12.5 kDa and consists of 108 amino acids (Pine *et al*., 2018)**.** According to Codoner-Franch & Alonso-Iglesias (2015) the main source of resistin in the blood is peripheral mononuclear white blood cells, bone marrow cells and macrophages**.** Resistin is regarded as a pro-inflammatory molecule that is expressed by immune cells(Li *et al*., 2021)**.**

The regulation of the synthesis and secretion of resistin is dependent on the cell type and the type of micro-environmental stimuli**.** In human, the expression of resistin is up-regulated at the stage of monocyte differentiation into macrophages (Savage *et al*., 2001 and Patel *et al*., 2003)**.** It was clearly indicates the role of resistin in the functions of monocytes and macrophages**.** It has been shown that some pro-inflammatory mediators such as Lipopolysaccharide (LPS), interleukin 1β (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin6 (IL-6) can strongly induce the expression of resistin in monocytes and macrophages (Lu *et al*., 2002 ; Patel *et al*., 2003 and Lehrke *et al*., 2004)**.**

Human resistin has a significant role in inflammation and in stress biology due to several extraordinary properties that enable it to act as chaperone-like molecule in the cellular stress biology. In addition, it represents a diagnostic biomarker used to evaluate disease status and outcome of treatment (Tripathi *et al*., 2020)**.**

High levels of resistin stimulate insulin resistance and have a significant effect on the occurrence of inflammation. Moreover, resistin plays an essential role in many diseases, such as metabolic diseases, autoimmune diseases, and inflammatory (Acquarone *et al*., 2019)**.**

2.6.4 Visfatin

Adipose tissue acts as an endocrine gland where it produces several substances known as adipocytokines, which have various effects on metabolic syndrome, lipid metabolism, and cardiovascular risk**.** Visfatin or nicotinamide phosphoribosyl transferase (NAMPT) is one among the adipokines. It is mostly expressed by visceral adipose tissue **(**Saddi Rosa *et al*., 2010 and Dakroub *et al*., 2021**).**

Visfatin is also known as PBEF which refers to its role as pre-B cell colony-enhancing factor (Adeghate, 2008). It is classified as a homodimeric protein (Wang *et al*., 2006**).** Each of the two monomers has 491 residues, that consists of 19 beta-strands and 13 alpha-helicals arranged together in two structural domains (Kim *et al*., 2006**).** Visfatin exists in two forms: an intracellular form (iNAMPT) in which it plays a regulatory function in nicotinamide adenine dinucleotide (NAD+) biosynthesis, and an extracellular form (eNAMPT) involved in several pathways such as intracellular signaling cascades and hormone-like signaling pathways (Revollo *et al*., 2007 and Verdin, 2015**).** In addition it has a very important role in organizing a wide range of physiological and pathological processes, such as obesity, cardiovascular disease and diabetes (Zheng *et al*., 2019**).**

Visfatin has a hypoglycaemic action by exerting insulin mimicking effects such as stimulating glucose transport into muscle and adipocytes and inhibiting glucose production by hepatocytes, through binding to insulin receptors at other sites that insulin does not bind to (Fukuhara *et al*., 2005and Abd Rabo *et al*., 2013**).**

The plasma level of visfatin increases in cases of chronic inflammatory diseases such as arthritis, psoriasis and obesity, as visfatin stimulates the production of many cytokines such as tumor necrosis factor in human leukocytes and interleukin 6 (IL-6) (Adeghate, 2008). In addition in abdominal obesity and type II diabetes mellitus (Fukuhara *et al*., 2005**).**

2.6.5 Interleukin-18 (IL-18)

Interleukin-18(IL-18) is an pro-inflammatory cytokine (Cavalcante *et al*., 2020). It has been identified as an interferon-gamma (IFNγ) stimulating factor by Gu *et al.* (1997). IL-18 belongs to the super family of interleukin-1 (IL-1) cytokines, which is characterized by the absence of a signal peptide**.** IL-18 is produced in an

inactive precursor form (24 k Da) which then converts to the active form (18 kDa) by caspase-1**.** It has a major role in inducing T helper-1 and natural killer cells to produce interferon-gamma (IFNγ)(Gu *et al*., 1997; Dinarello *et al*., 1998-a and Dinarello *et al*., 2013-b).

Interleukin-18 is encoded by the *interleukin-18 gene* into a protein with 193 amino acids (Okamura *et al*., 1995)**.** There is a natural balance of IL-18 activity in the presence of a high affinity for IL-18 binding protein, where the increase in the severity of the disease in humans is associated with the loss of this balance (Dinarello *et al*., 2013-b)**.**

Many cells produce IL-18 such as monocytes /macrophages, smooth muscle cells and endothelial cells, etc., and it stimulates the expression of chemokine receptors, adhesion molecules, granulocyte macrophage- colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNFα), interferon gama (IFNγ), and inter-leukin- 1β (IL-1β) activates both types of immune response (type 1 and type 2) to helper T cells (Nakanishi *et al*., 2001 and Dinarello, 2002)**.**

IL-18 stimulates many types of cells and performs pleiotropic functions (Yasuda *et al*., 2019), such as facilitating type I immune responses, and inducing cell-mediated immunity in association with interleukin 12 after infection with microbial products such as (lipo-polysaccharides), and inhibition of interleukin-4 production, which is dependent on the production of IgE and IgG1 and stimulation of plasma cell production of IgG2 (Jia *et al*., 2018)**.**

Interleukin-18 levels were significantly higher in patients with newly diagnosed type II diabetes than in non-diabetic subjects (Aso *et al*., 2003 and Esposito *et al*., 2003). Elevated IL-18 levels are considered as part of chronic low-grade inflammatory processes, which are associated with obesity, type II diabetes, and metabolic syndrome (Zaharieva *et al*., 2018)**.** This elevated level has been associated with a variety of risk factors used to assess the incidence of metabolic syndrome, such as: (WC, BMI, cholesterol, HDL , triglycerides, basal insulin, blood pressure control, fasting plasma glucose) (Aso *et al*., 2003 ; Hung *et al*., 2005 and Evans *et al*., 2007), and index of insulin resistance (Fischer *et al*., 2005)**.** In addition, the high levels of IL-18 that associated with metabolic syndrome can predict the risk of developing cardiovascular diseases (Troseid *et al*., 2009).

2.6.6 Vitamin D3

Vitamin (D3) is one of the fat-soluble vitamins that plays an important role in bone health (Kulie *et al*., 2009). Vitamin (D) was described upon its discovery as a substance capable of treating rickets and named "D" for its sequence as the fourth discovered vitamin (De Luca , 2014).

In the early twentieth century, the discovery of vitamin (D3) represents a revolution in the treatment and prevention of epidemic rickets. This was achieved by public health that increased the supply of vitamin (D3) to the general population, fortifying food with vitamin (D3), ultraviolet radiation, taking cod liver oil, taking vitamin (D3) supplements (Rajakumar *et al*., 2007).

There are two main isoforms of vitamin (D), vitamin (D3: cholecalciferol) and vitamin (D2 :ergocalciferol), which share a similar metabolism. It is estimated that vitamin (D) production in the skin resulting from ultraviolet-B (UV-B) induction is about 80% of the vitamin (D) supply, while vitamin (D) from dietary intake (such as fish, eggs, foods fortified with vitamin D) about 30% (Macdonald *et al*., 2011).

Vitamin (D) is produced in the skin by converting 7-dehydrocholesterol (7-DHC) to 25-hydroxy vitamin D [25-(OH)D] and then to 1,25-dihydroxy vitamin D3 [1,25 (OH)2D3], the hormonally active form of vitamin D, 25-hydroxylation is carried out by a number of enzymes, the most important of which is CYP2R1. On the other hand, it is possible that renal CYP27B1 is responsible for the production of most of the 1,25(OH)2D in the circulation (Bikle, 2014 and Christakos *et al*., 2016).

The vitamin D hormone 1,25-dihydroxy vitamin D3 [1,25(OH) 2D3] acts by binding to a vitamin D receptor (VDR) on the genome in order to activate or sometimes inhibit transcription (Pike & Meyer , 2010).

Recommended concentrations of serum 25-hydroxyvitamin D [25 (OH)D] are 50 nmol/l (20 ng/ml), and serum 25(OH)D concentrations lower than 25 or 30 nmol/l (10- 12 ng/ml) should be prevented and treated (Pilz *et al*., 2019).

Vitamin (D) has received great attention in research studies in recent years due to evidence supporting the presence of many biological responses to the active form of vitamin (D) such as inhibiting the progression of colon, prostate and breast

cancer cells, providing protection against autoimmune diseases such as inflammatory bowel disease and multiple sclerosis. In addition to its effect on the skeleton, it has also been found that vitamin (D) improves the control of blood glucose in patients with type II diabetes (Holick, 2007and Martínez-Pizarro, 2020).

Currently there is a great deal of interest in the role of vitamin (D) in type II diabetes mellitus and insulin resistance in addition to its essential role in bone / calcium metabolism (Sacerdote *et al*., 2019). So it turns out that vitamin (D) deficiency is a common condition in patients with type II diabetes. Moreover, inverse associations have been found between vitamin (D) levels, metabolic control and insulin resistance (Calvo-Romero & Ramiro-Lozano, 2015).

2.7 Molecular study

2.7.1 Single nucleotide polymorphism (rs3842752G>A) of *Insulin gene*

Insulin is a hormone essential for the health of all vertebrate species (Irwin, 2021). The gene of insulin is located on chromosome 11 on the short arm, it is a small gene, consisting of 1425 base pairs divided into three exons and two introns. The first exon consists entirely of 5' un-translated region (5'UTR) sequences, but the second and third exons contain the sequences encoding the proinsulin precursor protein, which are translated into 110 amino acids (Bell *et al*., 1980 and Owerbach *et al*., 1981).

The N-terminal part of the proinsulin protein sequence, which is a signal peptide to allow insulin secretion from pancreatic beta cells, is removed by the enzyme peptidase to produce proinsulin. Further processing of the pro-hormone by convertase enzymes leads to the separation of the internal C-peptide to produce the double-chain insulin hormone, consisting of A and B chains linked together by disulfide bridges (Steiner *et al*., 1985 and Steiner, 2011).

Single nucleotide polymorphisms (SNPs) in the *insulin gene* and *insulin receptor gene* may influence the interaction between the two molecules. In addition, it may be associated with a susceptibility to diabetes (Reddy *et al*., 2011and Massarenti *et al*., 2022).

According to the information available to the public through, data base (dbSNP) in national center for biotechnology information (NCBI) web site, (rs3842752

G>A) SNP is located in the *insulin gene* at position of chromosome (chr11: 2159843) and then located in the mRNA 3′un-translated region (3′ UTR) (Sherry *et al*., 2001).

There is an association of (rs3842752) SNP with type1diabetes and circulation glucose level (Reddy *et al*., 2011and Gootjes *et al*., 2022). The presence of this SNP in the 3'untranslated region (3' UTR) of the insulin mRNA, probably causes mRNA instability (Julier *et al*., 1994).

2.7.2 Single nucleotide polymorphism (rs1862513) in *Resistin Gene*

Resistin gene, which encodes the protein resistin, is located on chromosome 19p13.2 (Menzaghi *et al*., 2006)**.** It consists of four exons separated by three intr-ons. The *resistin gene* is polymorphic containing some single nucleotide polymorph-isms SNPs in its regions like promoter, introns and 3′untranslated region (3′UTR) (Hivert *et al*., 2009)**.**

Promoter's SNP (rs1862513 C>G) is one of many SNPs that have been studied for the *resistin gene* because of its reported effect on *resistin gene* expression and concentration of the circulating resistin (Ukkola *et al*., 2008 and Hivert *et al*., 2009)**.** It is considered as one of the strong determinants for resistin levels in circulation (Kawamura *et al*., 2016). In addition, this polymorphism of *resistin gene* appears to be an important risk factor that increases susceptibility to type II diabetes (Rathwa *et al*., 2019). And gestational diabetes (Takhshid & Zare, 2015).

The (rs1862513 C>G) SNP is associated with other diseases, such as: nonalcoh-olic fatty liver disease (Zhang *et al*., 2015). Coronary artery disease-CAD (Tang *et al*., 2008). Hypertrophic cardiomyopathy (Hussain *et al*.,2010). Polycystic ovary syndrome (Urbanek *et al*., 2003). Chronic kidney disease (Axelsson *et al*., 2006).

2.7.3 Single nucleotide polymorphism(rs12255372 G>T) of *TCF7L2 gene*

Transcription factor 7-like 2 (TCF7L2) acts as a gene repressor and activator, many of its variants being associated with the presence of diabetes (Yüzbaşıo-ğulları *et al*., 2020). The strong genetic association with type II diabetes has generated a great deal of interest in the TCF7L2 gene, which is known as a transcr-

iption factor 7-like 2 (TCF7L2) a member of the T-cell factor and lymphoid enhancer binding factor family (TCF/LEF), a group of transcription factors that bind to DNA by a high-mobility group domain (HMG) (Grant *et al*.,2006 and Sladek *et al*., 2007).

The (TCF/LEF) is considered an essential part of the (Wnt) signaling pathway. This pathway is a complex network of interacting proteins that perform regulated cellular communications at multiple levels and that generate many effects that are initially associated with the biology of development.

The proteins of (Wnt) pathway, as with TCF7L2, are associated with various common diseases, in addition to different models of cancers, which indicates the importance of this pathway in human diseases (van Amerongen & Nusse, 2009 ; Lucero *et al*., 2010 and Wenzel, *et al*., 2020).

The human *TCF7L2 gene* is located on chromosome (10q25.3), and it contains 17 exons (Duval *et al*., 2000-a and Duval *et al*., 2000-b). This gene regulates biosynthesis and insulin secretion at the level of beta cells in the pancreas (Basile *et al*., 2014).

Several studies have shown that polymorphisms in the *TCF7L2 gene* are associated with a higher risk of developing type II diabetes. Numerous other studies in different populations also indicated that genetic variants of the *TCF7L2 gene* are associated with the risk of developing type II diabetes and its complications.

However, (rs12255372 -G/T) is an intronic single nucleotide polymorphism in the *TCF7L2 gene* that is closely associated with the risk of developing type II diabetes (Florez *et al*., 2006 and Li *et al*., 2018-a).

2.7.4 Single nucleotide polymorphism(rs13266634) of *SLC30A8 gene*

Solute Carriers of family 30 member 8 (SLC30A8) a member of the zinc transporter (ZnT) family, is encoded by the *SLC30A8 gene* located on chromosome (8q24.11), has a length of 37 kb, with eight exons (Chimienti *et al*., 2004). Expression of zinc transporter-8 (ZnT8) is unique to islets, as it regulates the concentration of zinc in cells by transporting zinc from the cytoplasm to secretory vesicles and also acts as a zinc sensor (Chimienti *et al*., 2004 and Cotsapas *et al*., 2010). Zinc plays an important role as a second cellular messenger in insulin

signaling pathways for the process of insulin synthesis, storage in secretory vesicles, secretion when needed and thus blood glucose homeostasis (Huang *et al*., 2019).

In type I diabetes, (ZnT8) represents an antigen target, and the presence of auto antibodies to (ZnT8) is an important diagnostic tool. In type II diabetes, it plays a mediator for transporting zinc to insulin-secreting granules. Moreover, genome-wide association studies (GWAS) reported that the presence of the mutation in the *SLC30A8 gene* is associated with type II diabetes (Sladek *et al*., 2007 and Kambe , 2012).

In many populations in the world, there is an association between the single-nucleotide wild-type (C- allele) of (rs13266634 -C>T) SNP and susceptibility to type II diabetes (Huang *et al*., 2019), also in Arab countries such as Tunisian (Kifagi *et al*., 2011). Saudi Arabian (Al-Daghri *et al*., 2014 and Bazzi *et al*., 2014). Whereas, the (T-allele) frequencies were higher in Asian papules. Furthermore, the meta-analysis also indicated that the (rs13266634) polymorphism is among the genetic markers that most confirm susceptibility to type II diabetes (Cauchi *et al*., 2010 and Li *et al*., 2018-b).

2.7.5 Single nucleotide polymorphism(rs360717)in *Interleukin -18 gene*

Human *interleukin-18 gene* is located on chromosome (11) at the position (11q 22.2-q22.3), where it contains 6 exons (Kaplanski, 2018). Several binding sites for transcription factors in the promoter site of the *IL-18 gene* are affected by the presence of a single nucleotide polymorphism (Wawrocki *et al*., 2020). Which appeared in many studies its effect on the synthesis of cytokines (Barbaux *et al*., 2007 and Yang *et al*., 2013).

The (rs360717) SNP, is one of the SNPs present in the promoter of the *IL-18 gene*, has been tested in several studies to determine its relationship with IL-18 levels and its association with disease, as in the immune response to (*H. pylori*) (Myung *et al*., 2015). Myocardial infarction susceptibility (Koch *et al*., 2011). Some immune diseases, such as types of cancer and rheumatoid arthritis (Stassen *et al*., 2003 and Pawlik *et al*., 2009).

2.7.6 Single nucleotide polymorphism (rs11977021) in *visfatin gene*

Visfatin is produced by many tissues, but it is produced mainly by adipose tissue (Wang *et al*., 2021). In human, the gene that encodes visfatin is located on chromosome (7), and consists of eleven exons spanning (34.7 kb) (Arya *et al*., 2002).

Single nucleotide polymorphism (rs11977021) SNP is located in the promoter region of the *visfatin gene*, and may affect the gene transcription process (Ooi *et al*., 2016). It is associated with plasma visfatin levels, obesity, hypertension, and hypertriglyceridemia in obese children (Ooi *et al*., 2014). Furthermore, Single nucleotide polymorphism (rs11977021)SNP has been associated with low dense lipd (LDL) cholesterol and may have a role in susceptibility to type II diabetes (Bottcher *et al*., 2006).

Genetic variants of visfatin have been associated with obesity and obesity-related complications and cardiovascular diseases, which indicates that visfatin has a role that cannot be ignored in the occurrence of diseases caused by obesity and heart disease (Ooi *et al*., 2014).

Chapter Three Materials and Methods

3. Materials and methods

3.1 Materials

All materials used to conduct laboratory tests in this study were listed in table (3.1). The devices used to perform laboratory tests were listed in table (3.2).

Table (3.1) :Materials used in the study

No.	Material	Company – Origin
1	Acrylamide	Himedid - India
2	AcuI restriction enzyme kit	Sib Enzyme - Russia
3	Agarose	Pronadisa - Spain
4	Ammonium persulphate APS	Sigma - German
5	Bis acrylamide	Scr - Chain
6	Boric acid	Thomas baker – India
7	BspACI restriction enzyme kit	Sib Enzyme - Russia
8	BstV2I restriction enzyme kit	Sib Enzyme - Russia
9	DNA ladder(100-1000 bp)	Cyntol - Russian
10	DNA loding dye	Promiga - USA
11	Double distilled water	-
12	EDTA	Thomas baker – India
13	Ethanol Alcohol	Cristalce - France
14	Ethedium bromide	Intron - Korea
15	Gel and clot activator tube – 5ml	China
16	Glucose (GLUC-PAP)	Randox - UK
17	Human IL-18 (Interleukin)ELISA Kit	Elabscience -USA
18	Human INS(Insulin) ELISA Kit	Elabscience - USA
19	Human RETN(Resistin)ELISA Kit	Elabscience - USA
20	Human VF(Visfatin)ELISA Kit	Elabscience - USA
21	Isopropanol Alcohol	Sharlo - Spain
22	K3 EDTA tube -2ml	China
23	Mgcl2	Cyntol - Russian
24	Nacl	SCR - China
25	Nuclease free water	Cyntol - Russian
26	PCR Master mix	Cyntol - Russia
27	Plain tube with screw cup -10ml	China
28	Primers	Macrogen - Korea
29	Proteinase K	Bioneer - India
30	Pst I restriction enzyme kit	Sib Enzyme - Russia
31	ReliaPrep TM Blood(g DNA Miniprep System)	Promega - USA
32	SDS	Thomas baker - India

33	Specimen container- 60ml	China
34	Sucrose	SCR - China
35	TEMED	Sigma - German
36	Tris –base	Thomas baker - India

Table (3.2) :The equipment used in the study

No.	Device and tools	Company – Origin
1	Analytikjena	Flex cycler - Germany
2	Autoclave	Hirayama -Japan
3	Centrifuge	Hettich - Germany
4	Cooling incubator	IL-11 - USA
5	Different size Eppendorf tube	Biobaseic - Canada
6	Different size tips	Biobaseic - Canada
7	Different size tube	Biobaseic - Canada
8	Digital camera	Canon - China
9	Digital timer	China
10	Distillatory	Shinsaeng - Korea
11	Flask different size	Chemical - Lab - China
12	Hoot plate	Medico - USA
13	Horizontal gel electrophoresis	Biorad - Japan
14	Multichanal Micro pipette(12tips)	Slamed - Germany
15	Microcentrifuge	Hettich - Germany
16	Micro pipette (100-1000)µl	Slamed - Germany
17	Microplate Reader-Epoch	Biotek -USA
18	Microwave	Argos -Germany
19	Nano- drop	Bio-drop - England
20	Refrigerator	Hisense -24 C - China
21	Refrigerator	SIEMENS 8C - Japan
22	Shaker-centrifuge	Bioneer - Koria
23	Sensitive Electron Balance	Entric - Germany
24	Spectro Photometer	Apel - Japan
25	Thermo-cycler	BIOMETRA - Germany
26	UV Transilluminator	Quantum - France
27	Vertical gel electrophoresis	Cleaver - England
28	Vortex	Griffen and George Ltd - UK
29	Water bath	Mermert -Germany

3.2 Methods

3.2.1 Study population

The current study was conducted in the consulting clinic lab of Baghdad Teaching Hospital in Baghdad city and the laboratories of the College of Science, University of Babylon. Volunteers attended from December 2021 to February 2022 in the consulting clinic laboratory in Baghdad Teaching Hospital.

The study included 88 subjects:54 patients with type II diabetics-T2DM group and 34 non diabetics -ND(control) group. Their ages ranged from 40 to 55 for both genders. BMI, NC and WC and were measured for each subject as shown in Table (3.4).

3.2.2 Inclusion criteria

Fifty four (54) subjects (27 females and 27 males) with pre-existing type II diabetes diagnosed depending on HbA1c and fasting glucose test, dependent on treatment with oral hypoglycemic medications only, and 34 subjects (17 females and 17 males) who were apparently healthy and non diabetic, diagnosed by HbA1c and fasting glucose test .

3.2.3Exclusion criteria

Type II diabetes-T2DM group and non-diabetics -ND group. Subjects with systemic diseases, oral diseases, taking vitamin D supplements were excluded ,in addition patients with type II diabetes who took insulin with oral hypoglycemic therapy were excluded, patients had body mass index (BMI) more than 27 were excluded for control group.

3.3 Anthropometric measurements

Aging combined with being overweight are the most risk factors for developing type II diabetes (Care, 2020). Body mass index (BMI) is a measurement of a subject's obesity. which is used to determine human characteristics, using height and weight in adults and categorize them into groups according to the value of the body mass index and is considered as a risk factor for the development and occurrence of many diseases (Nuttall , 2015). BMI was calculated by dividing

weight (in kilograms) by height (m) squared .According to the WHO protocol in 2011, the waist and neck circumference was measured using a flexible tape .where the waist circumference was measured from the area of the lower rib and the navel, and the neck circumference in the upper prominence of the larynx, where the head is straight and the eyes are looking forward (Zhong *et al.*, 2017; World Health Organization, 2011). The anthropometric measurements of the two study groups (T2DM and ND groups) are represented in Table(3.4) .

Table (3.4) :Anthropometric Measurements

Groups	Gender	BMI ($kg/m^{2)}$	NC (cm)	WC (cm)	Age (year)	Total	
T2DM	Female	27.34 - 44.44	38- 44	80- 114	40 - 55	27	54
	Male	27.1 - 33.57	39- 45	83-106		27	
ND	Female	21.48 -26.85	36 - 39	70- 91		17	34
	Male	23.99 - 26.99	38 - 44	83- 108		17	
Total : 88						88	88

Abbreviations: BMI: body mass index, NC: neck circumference, WC: waist circumference, T2DM: Type II diabetes millets, ND: non-diabetics.

3.4 Collecting of blood samples

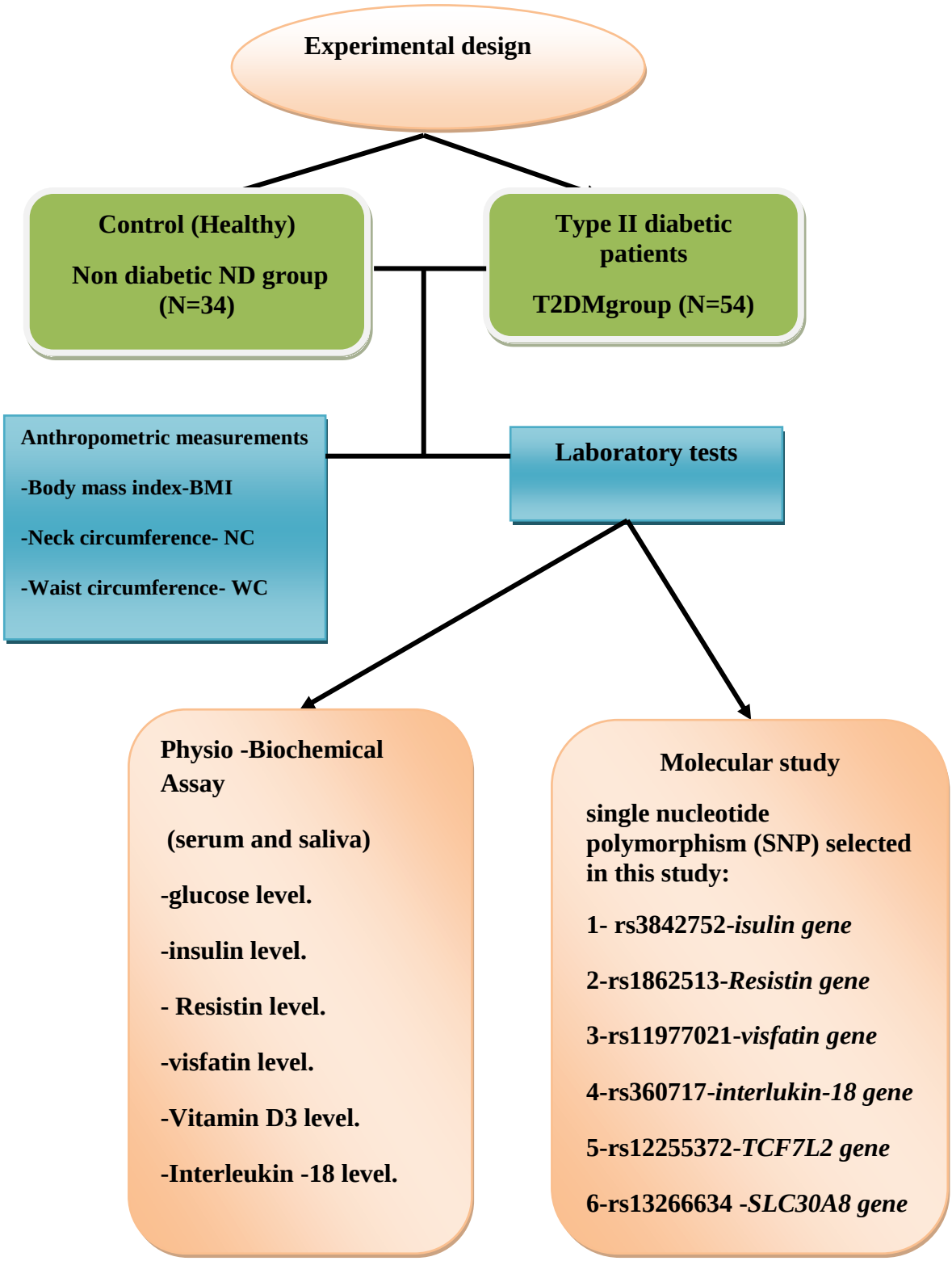
Blood samples for both groups were taken in the early morning from (8 am) until (11 am) after fasting for at least (8)hours, (3 ml)of venous blood was collected by gel tubes (Gel and clot activator tube - 5ml) to extract serum for both the diabetic and the non-diabetics (control group), and 2 ml of venous blood was also collected in (K3 EDTA) tubes for DNA extraction. Serum was obtained from whole blood samples by allowing the samples to coagulate for one hour at room temperature before centrifuging for (20) minutes at 1000xg. The isolated serum was divided into (5) portions, which were kept in eppendorf tubes frozen at (-20 ° C) until the tests were carried out.

3.5 Collection of saliva

According to previous studies, the draining method was used to collect the whole un stimulated saliva. The person is asked to sit silently and tilt his head down with his mouth open to allow saliva to drip into a sterile collection tube (Yamuna

Priya & Muthu Prathibha, 2017). Before collecting saliva, the person should be asked to rinse his mouth with (10 ml) of distilled water for (30) seconds to get rid of any remnants of cells and to moisten his mouth, and after resting for (10) minutes, (4 ml) of saliva was collected in sterile tubes kept in ice. Saliva was collected in the early morning from (8) am to (11) am. The subjects were fasting at least (8) hrs (Rantonen, 2003; Nunes *et al*., 2015). After the collection was completed, saliva is immediately centrifuged at (3000) rpm for (15) minutes. The supernatant was transferred and divided into (6) portions. They are placed in eppendorf tubes. All tubes are kept frozen at (-20°C) until the required tests are performed (The storage period should not exceed one month).

3.6 Study design



3.6.1 Physio -biochemical assay

3.6.1.1 Blood glucose level

An estimate of the serum fasting blood glucose concentration for the two groups T2DM and ND (control) groups was obtained from the records of the consulting clinic lab in Baghdad Teaching Hospital - Iraq in conjunction with taking serum and saliva from both groups.

3.6.1.2 Salivary glucose level

Salivary glucose level for T2DM and ND groups was estimated according to (Jouda *et al.*, 2016), which adopted the principle of oxidase-peroxidase method by using Randox assay kit. This method is used to determine the amount of glucose in body fluids such as serum, plasma and urine (Barham and Trinder, 1972).

Procedure :

1- The solutions were mixed and prepared according to the instructions attached to the test kit consisting of:
   - R1a. Buffer(Phosphate Buffer, Phenol) ,
   - R1b. GOD-PAP Reagent (4-aminophenazone , Glucose oxidase , Peroxidase)
   - CAL. Standard **(** Glucose)

2- Fresh distilled water was used to perform a fresh calibration in the cuvette .

3- GLU was selected in the Run Test screen and a water vacuum was performed as instructed.
   - Sample/Standard (5 µl - Standard S1or ample) ,(0 µl - Reagent Blank S0)
   - Reagent R1 (500 µl - Standard S1or ample) , (500 µl - Reagent Blank S0)

4-(1 ml) of the previously prepared solution was added to a tube with 10 µm of serum, thoroughly mixed, incubated for 10 min at 37 °C and the reading was carried out at a wavelength of 500 nm.

3.6.1.3 Measurement of glycosylated hemoglobin (HbA1c)

The percentage of glycosylated hemoglobin (HbA1c) was obtained from the records of the consulting clinic laboratory - Baghdad Teaching Hospital for both

groups T2DM and ND (control) groups in conjunction with obtaining serum and saliva samples.

3.6.2 ELISA tests

All the Enzymatic Linked Immune Sorbent Assay(ELISA) tests that were conducted to determine the levels of biomarkers in the current study had the same assay procedure and according to the sandwich (ELISA) principle. There is only some difference in the steps 1 and 2 of the assay procedure to determine the level of vitamin (D3).

3.6.2.1 Measurements of insulin, visfatin, resistin and interleukin-18 levels

Insulin, visfatin, resistin , and interlukin-18 levels were measured in serum and saliva samples for both groups of (T2DM and ND) according to procedure of (ELISA) kit by Elabscience-USA as mentioned in the following sequence of steps:

1. Hundred (100) µL were added from each dilution of the standard, blank, and sample into appropriate pre-selected wells. The plate was covered with a sealer provided in the kit. The incubation was carried out for 90 minutes at 37°C.
2. Liquid was poured out from each well, and the washing step was not performed. 100 µl of Biotinylated Detection Ab working solution was immediately added to each well. Then the plate was covered with a new sealer. and incubated for 1 hour at 37°C.
3. The solution was poured out from each well, and 350 µl of wash bufferwas added to each well. The soak was done for 1 min and the solution was withdrawn or poured from each well and left to dry on clean absorbent paper. The washing step was repeated 3 times.
4. Hundred (100)µL of HRP-conjugated working solution was added to each well. The plate was covered with a new sealer. The incubation was carried out for 30 minutes at 37°C.
5. The solution was poured out from each well, and the washing process was repeated for 5 times as was done in step 3.
6. Ninety (90) µL of Substrate Reagent was added to each well. The plate was covered with a new sealer. The plate was protected from light and incubated for

15 min at 37 °C. The micro plate reader was heated for ~15 min before the OD was measured.

7.Fifty (50) µl of the stop solution was added to each well.

8.A micro plate reader set at 450 nm was used to determine the optical density (OD value) of each well once.

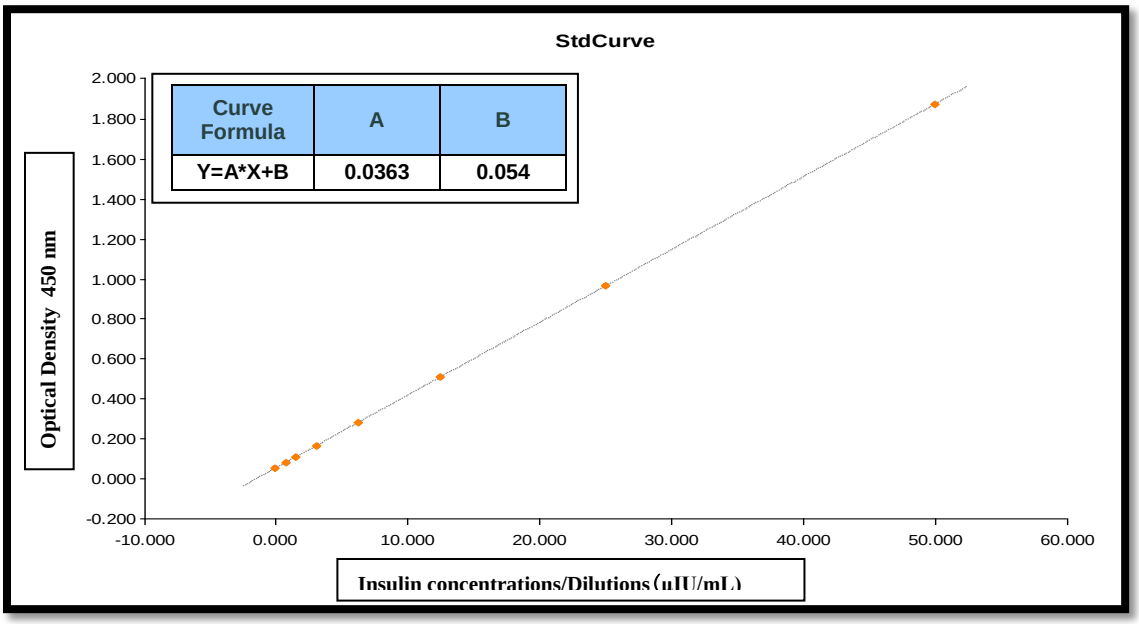


Figure (3.1): Standard curve of insulin levels.

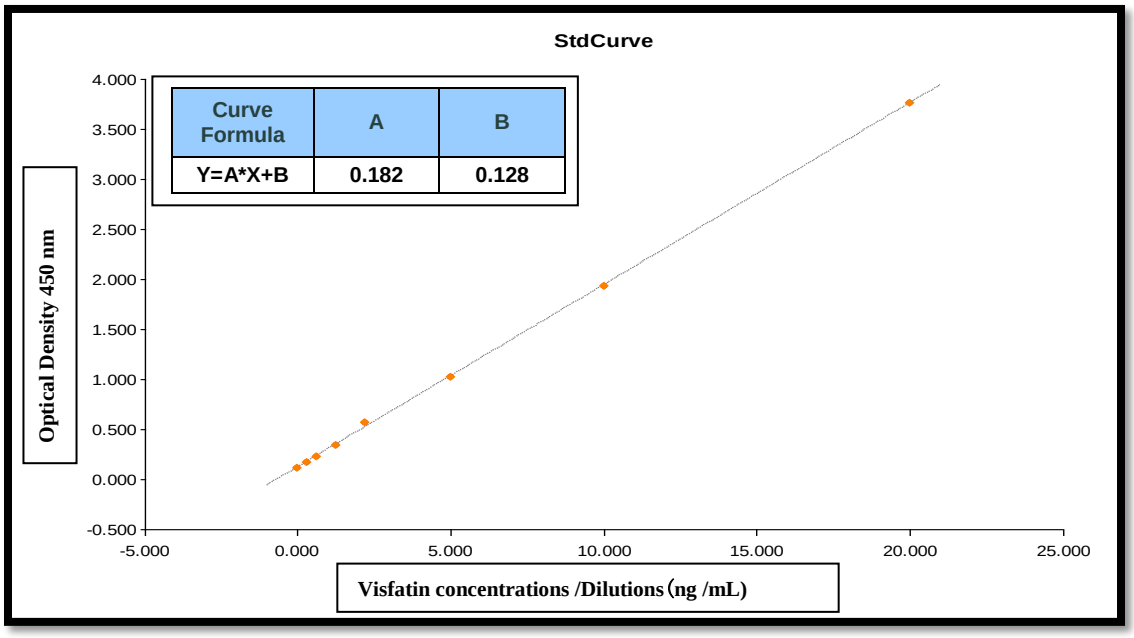


Figure (3.2) :Standard curve of visfatin levels.

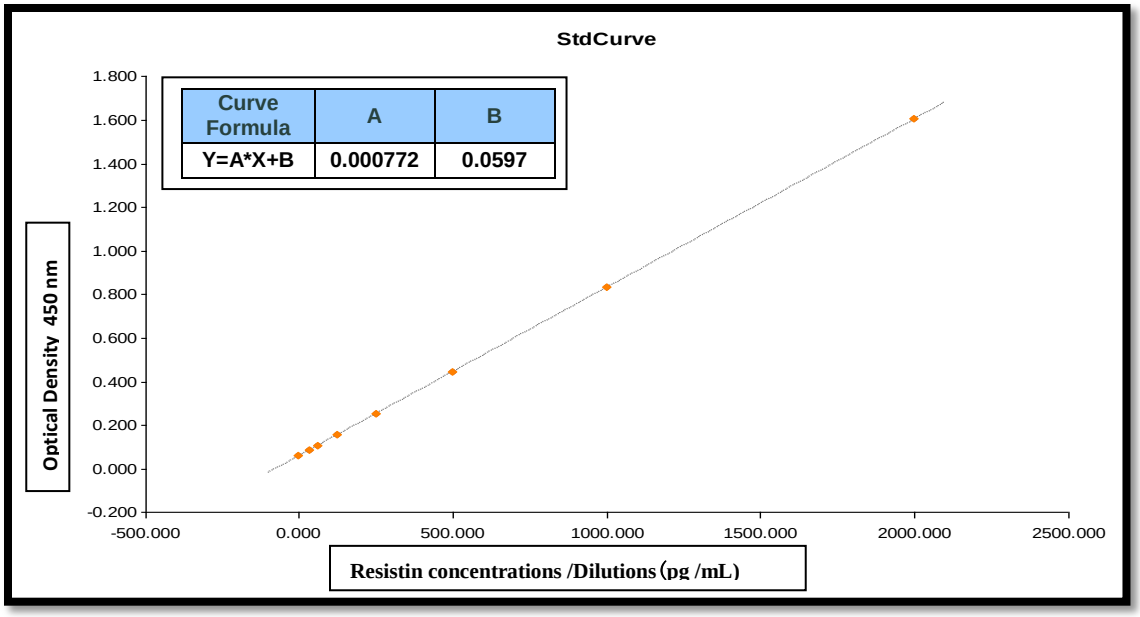


Figure (3.3): Standard curve of resistin levels.

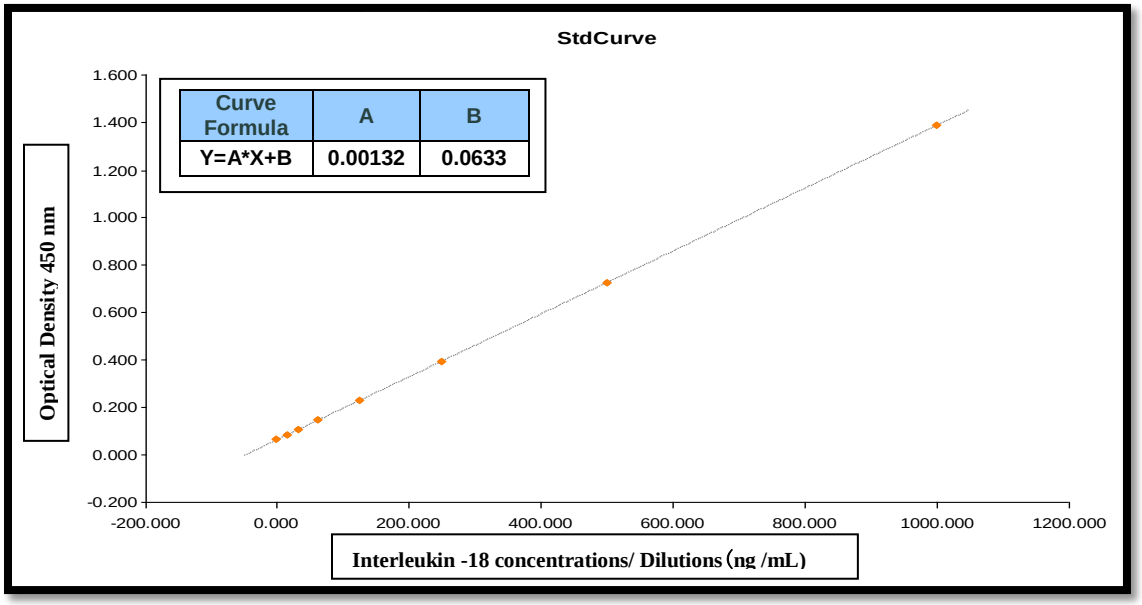


Figure (3.4) :Standard curve of interleukin-18 levels.

3.6.2.2 Vitamin D3(VD3) level

Vitamin D3 concentration was measured in serum and saliva samples for both groups of patients T2DM and ND groups according to procedure ELISA kit Elabscience-USA as mentioned in the following sequence of steps:

1.Fifty (50) µL of each diluted standard, blank and sample was added into the appropriate pre-selected wells. 50 µL of Biotinylated Detection Ab working solution was immediately added to each well. The plate was covered with a sealer provided in the kit. Then it was incubated for 45 minutes at 37℃.

2.The solution was poured out from each well, and 350 µl of wash buffer was added to each well. The soaking was done for 1 min and the solution was poured out from each well and left to dry on clean absorbent paper. The washing step was repeated 3 times.

3.Hundred 100 µL of HRP-Conjugate working solution was added to each well. The plate was covered with a new sealer. Then, it was incubated for 30 minutes at 37°C.

4.The solution was poured out from each well, the washing process was repeated for 5 times in the same way as in step 2.

5.Ninety (90) µL of Substrate Reagent was added to each well. The plate was covered with a new sealer. The incubation was carried out for 15 minutes at (37 °C). Before OD measurement the Microplate reader was heated for ~15 min.

6.Fifty (50) µL of the stop solution was added to each well.

7.A micro-plate reader set at 450 nm was used to determine the optical density (OD value) of each well once.

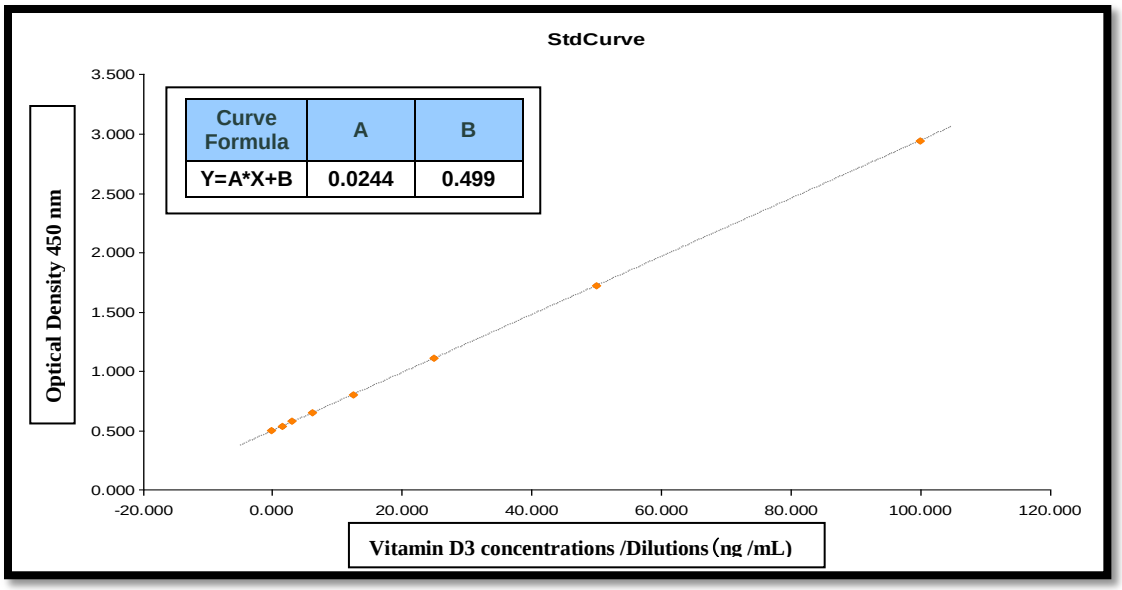


Figure (3.5) :Standard curve of vitamin D3 levels.

3.7 Molecular assay

3.7.1 Primers design of PCR– RFLP and PCR– SSCP techniques

The selection of genotyping technique, Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR– RFLP) and Polymerase Chain Reaction - Single Strand Conformation Polymorphism (PCR- SSCP) according to (Hashim, & Al-Shuhaib, 2019). The design of PCR primers according to the study of (Hashim , *et al*., 2015) briefly as follow:

The primers were designed by the aid of NCBI-primer BLAST online software (http://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?LINK_LOC=BlastHome) , at the same time the produced primers was checked for specificity for their target sequences by performing the BLAST against the human genome , then the primers pair was selected according to the demand criteria such as : product length , the similarity of melting temperature , primers length , specificity , etc. Then the mutations was interred according to the design demands .

The primer ability to form secondary structure was checked by the aid of Oligo Calc online software (http://www. basic.northwestern. edu/biotools/oligocalc .Html), the primer would be rejected if it had 5 bases or more able to form self-dimerization and/or it had 4 bases able to form hairpin. Each primers pair was checked for dimer formation by the aid of "Multiple Primer Analyzer" online software from Thermo Fisher Scientific Inc.© , the sensitivity of the software was adjusted to the value 2 , the primer pair would be rejected if it made any dimers in this degree of sensitivity .

3.7.2 Restriction enzyme selection for RFLP-PCR technique

The selection of the suitable restriction enzyme was performed by the aid of Snap Gene viewer software (V6.0.5), we selected the restriction enzyme according to several criteria such as: the lesser primer mutations needed , the distance of mutation from the variant , compatibility of the produced primers , cost and avail-ability .

3.7.3 Genotyping design of (rs1862513C>G) SNPs in *resistin gene*

The primer pair used to amplify the rs1862513 region (high frequency, promoter variant, C>G) is shown in Table (3.5). Figure (3.6) showed the amplified ampl- icon that was used for genotype rs1862513. Figure (3.7) showed the PCR-RFLP genotyping design for rs1862513 in *resistin gene.*

Table (3.5) :Designed primers sets for rs1862513(C>G) in *resistin gene*

Primer gene name	Sequence (5'->3')	Length bp	Amplicon Length bp
Resistin gene Forward	TGTCATGTTTGCATCAGCCAC	21	245
reverse complement	TTGGCTAATAAGTCCCTGGGC	21	

Abbreviations: bp: base pair

TGTCATGTTTGCATCAGCCACCCTTGCATGCAAAACTCTGCTTGTCTACCTGTTCCTCAGACCACAGCCCCTG

GCATTATCCCTGGGGCACCACCTCCTGACCAGTCTCTGGACATGAAGACGGAGGCCCTGTTGGAAGTGGGAA

GGCTCCCTTCCTCCTCCAGCCCTTACTGTCTGCTCAGGGGCTTCCTCTTGGCCCCGGATGTGGGACCGGAGGG

TTGGGGGCCCAGGGACTTATTAGCCAA

Restriction enzyme = BstV2I
C=124+121
G=245

GAAGAC(N)$_2$↑
CTTCTG(N)$_6$↓

Figure (3.6) :The amplified amplicon that was used for rs1862513 genotype.
The sequence with blue shade represents the forward and reverse primer. The(C) colored in red, represents (C) allele of rs1862513Polymorphisms. The green shaded sequence represents the restriction site of the (BstV2I) enzyme in which the enzyme will cut the amplicon with the C allele into two pieces: 142 base pairs and 121 base pairs, while in the presence of the G allele the enzyme will not cut the amplicon and the piece produced is 245 base pairs.

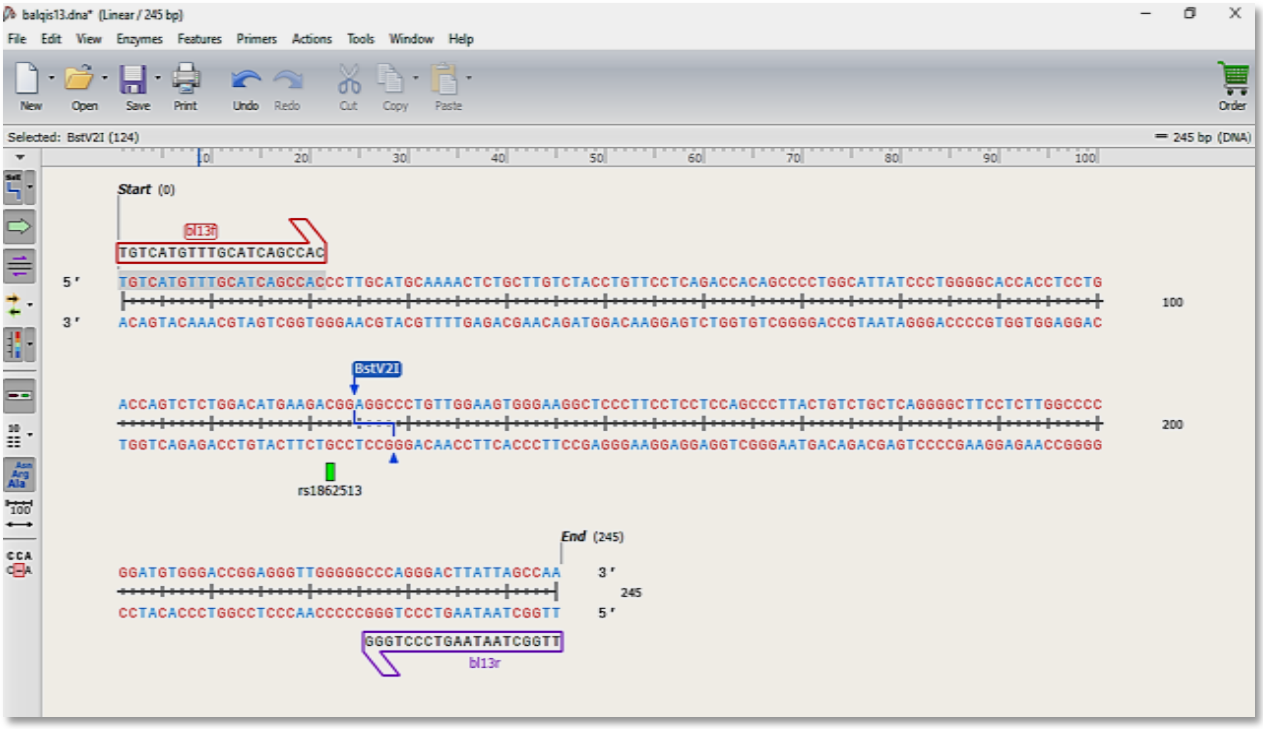


Figure (3.7) :PCR-RFLP genotyping design of rs1862513 in *resistin gene*

3.7.4 The design of PCR-RFLP genotyping technique for rs11977021 a polymorphism of *visfatin gene*

The primer pairs used to amplify the rs11977021 region are shown in table (3.6). The amplified amplicon that was used for rs11977021 genotype is shown in Figure (3.8). PCR-RFLPgenotyping design of rs11977021in *visfatin gene* is shown in Figure (3.9).

Table (3.6): Designed primers sets for rs11977021(C>T) in *visfatin gene*

Primer gene name	Sequence (5'-3')	Length(bp)	Amplicon Length bp
visfatin gene Forward	ATGGGCCCCCTCACTTGATT	20	307
reverse complement	GTTGCAGGTAGGTGAGTTCCA	21	

Abbreviations: bp: base pair

ATGGGCCCCCTCACTTGATTCAAAGCCCTGGATTATTATACCATCCTACCAAATAGCAATCAGGCACTGCCA
ATTGGGCATGCAGCTCCTTGTCTCCCTCCCTCAGAGTGCTATGAAAGACCTAGGGCTCATATATTTGTTCAG
GCAAAGTGAGTGTATCTGTGCCACTCCTGTTGCCACACACCCTTGCAGACAGCCTCGGGTGCACCTTTATAT
TTCAAAGTTGCCTCAGATACTCCAAT/CTGACCTGATTTGACtTCAGTAAAAAACACTGGTGAAGTAGGTA
TGGAACTCACCTACCTGCAAC

Restriction enzyme = **Acu I**

CTGAAG(N)$_{16}$↑
GACTTC(N)$_{14}$↓

C=307
T=242+65

Figure (3.8) :The amplified amplicon that was used for rs11977021 genotype.
The sequence with blue shade represents the forward and reverse primer. The (T) colored in red, represents (T) allele of rs11977021Polymorphisms. The yellow shaded sequence represents the restriction site of the (Acu I) enzyme in which the enzyme will cut the amplicon with the(t) allele into two pieces 242 base pairs and 65 base pairs, while in the presence of (C) allele the enzyme will not cut the amplicon and the piece produced is 307 base pairs.

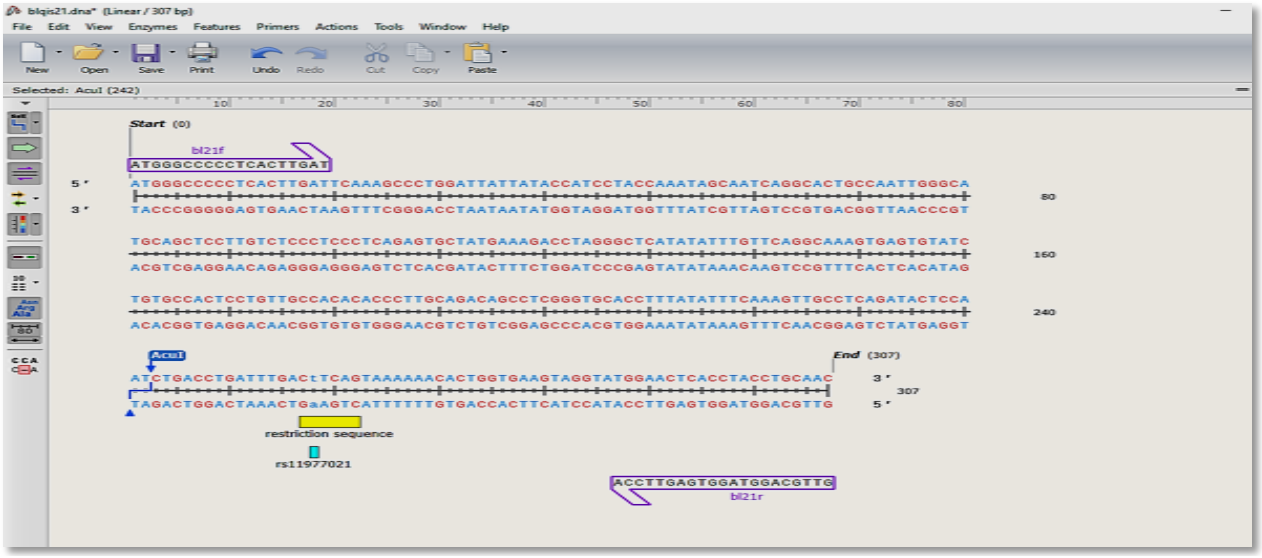


Figure (3.9): PCR-RFLP genotyping design of rs11977021 in *visfatin gene*

3.7.5 The design of PCR-RFLP genotyping technique for (rs3842752-G/A) SNP of *insulin gene*

The primer pairs that are used to amplify the SNP rs3842752 region of insulin gene are shown in the Table (3.7). The amplified amplicon that was used for rs3842752 genotype is shown in the Figure (3.10). Figure (3.11) represent the PCR-RFLP genotyping design of rs3842752 in Insulin Gene.

Table (3.7): Designed primers sets for rs3842752(G/A) in *insulin gene*

Primer gene name	Sequence (5'->3')	Length(bp)	Amplicon Length(bp)
Insulin gene Forward	CCCCCAAGACACACAGACG	19	329
reverse complement	GTGTCTCCCTGACTGTGTCC	20	

Abbreviations: bp: base pair

CCCCCAAGACACACAGACGGCACAGCAGGGCTGGTTCAAGGGCTTTATTCCATCTCTCTCGGTGCAGGAGGC
GGCGGGTGTGGGGCTGCCTGCa/GGCTGCGTCTAGTTGCAGTAGTTCTCCAGCTGGTAGAGGGAGCAGATG
CTGGTACAGCATTGTTCCACAATGCCACGCTTCTGCA/GGGACCCCTCCAGGGCCAAGGGCTGCA/GGCTGC
CTGCACCAGGGCCCCCGCCCAGCTCCACCTGCCCCACTGCCAGGACGTGCCGCGCAGAGCAGGTTCCGGAAC
AGCGGCGAGGCAGAGGGACACAGGAGGACACAGTCAGGGAGACAC

Restriction enzyme =Pst I

G=179+27+123

A=94+85+27+123

CTGCA↑G
G↓ACGTC

Figure (3.10) :The amplified amplicon that was used for rs3842752 genotype.
The sequence with gray shade represents the forward and reverse primer. The (a) colored in red, represents (A) allele of rs3842752 Polymorphisms. The sequence shaded in green represents the restriction site of the(Pst I)enzyme in which the enzyme will cut the amplicon with the (G) allele into 3 pieces(179,27,123 base pair. while in the presence of (A) allele the enzyme will cut the amplicon in to four fragments (94, 85, 27,123) base pairs.

Figure (3.11) :PCR-RFLP genotyping design of rs3842752 in *insulin gene*

3.7.6 The design of PCR-RFLP genotyping technique for (rs360717-G/A) SNP in *interleukin -18 gene*

The primer pairs that are used to amplify the SNP rs360717 in *interleukin -18 gene* are shown in the table (3.8). Figure (3.12) is shown the amplified amplicon that was used for rs360717 genotype. Figure (3.13) represents the PCR-RFLP genotyping design of snp rs360717 in *interleukin gene*.

Table (3.8): Designed primers sets for rs360717(G/A) in *interleukin -18 gene*

Primer gene name	Sequence (5'->3')	Length(bp)	Amplicon Length(bp)
Interleukin-18 gene Forward	GCGATCTGGAAGGTCTGAGG	20	260
reverse complement	TTGCCCTAGGAAAGAGCCTG	20	

Abbreviations: bp: base pair

GCGATCTGGAAGGTCTGAGGTTCCTTTCCTCTTCCCGAAGCTGTGTAGACTGCAGCAGGTGGCAGC/CgCT
TTAGCAGCCAGAGTTGGCAGCCAGGAGGGCAAAATGCACTGGGAGACAATTCCTTGCTGACTGTCCAGGCAG
TCGCCAGGGGAGCAAAGGGCTCAGCAAGCTGGGGAGAGAATGAGGAAGAAGGTGGAGGGAGGAGACTGCTGT
CGGCACTCCTTGGGC/CGCCTTCAAACAGGCTCTTTCCTAGGGCAA

Restriction enzyme =**BspAC I**

G=66+164+30

A=230+30

C↑CGC
GGC↓G

Figure (3.12) :The amplified amplicon that was used for rs360717 genotype.
The sequence with gray shade represents the forward and reverse primer. The (G) colored in red, represents (G) allele of rs360717 Polymorphisms. The sequence shaded in green represents the restriction site of the (BspAC I)enzyme in which the enzyme will cut the amplicon with the (G) allele into 3 pieces(66,164,30)base pair , and (230, 30) base pairs represents A allele .

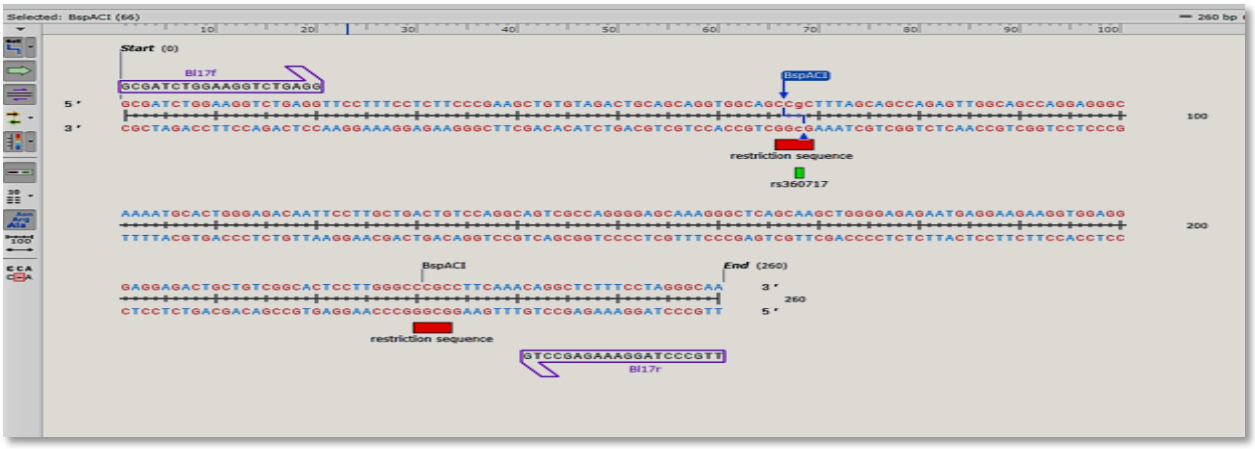


Figure (3.13): PCR-RFLP genotyping design of snp rs360717 in *interleukin gene*

3.7.7 SSCP genotyping technique design of (rs13266634) SNP in *SLC30A8 gene*

The following primers, Table (3.9) represents the primer pairs that are used to amplify the SNP rs13266634 a *SLC30A8 gene* polymorphism. Figure (3.14) represents the position of the targeted rs13266634 SNP within the *SLC30A8 gene*.

Table (3.9): Designed primers sets for rs13266634 a *SLC30A8gene* polymorphism

Primer gene name	Sequence (5'->3')	Length(bp)	Amplicon Length (bp)
SLC30A8 gene Forward	GGAGTCAGAGCAGTCGCC	18	230
reverse complement	ACTGACGGTGTGACTGAGCTA	21	

Abbreviations: bp: base pair

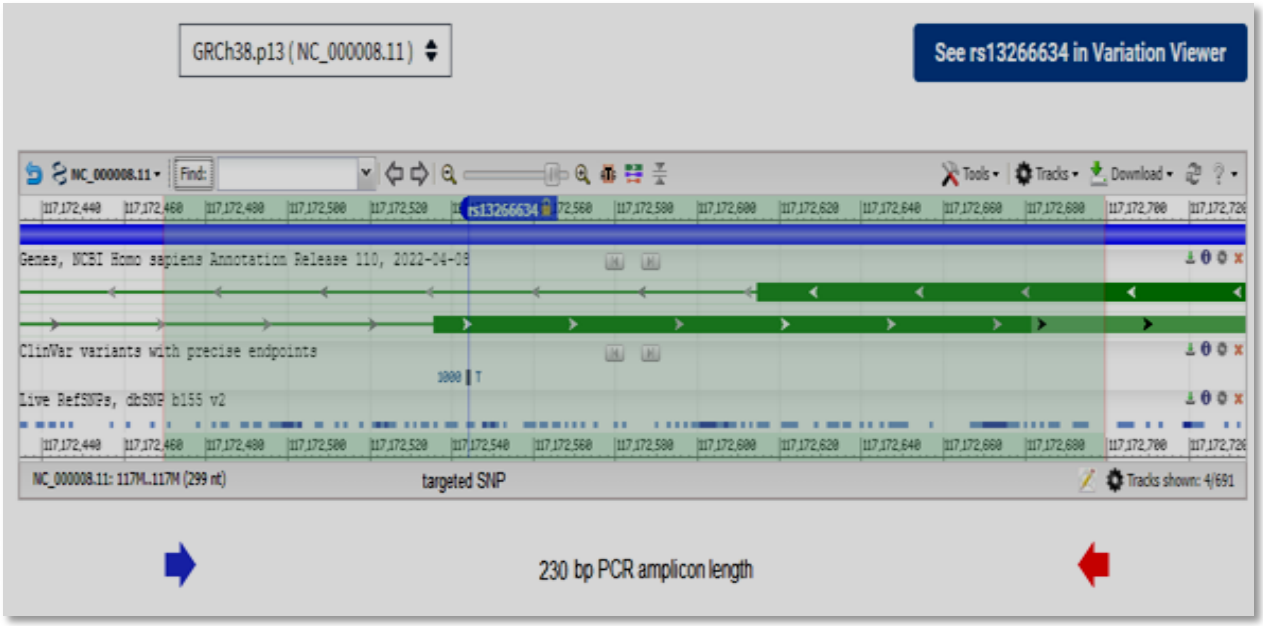


Figure (3.14) :The position of the targeted rs13266634 SNP within the *SLC30A8 gene*.

The exact position of the retrieved 230 bp amplicon spartially covered sequences of the *SLC30A8 gene* within chromosomes 8 (Gen Bank acc. no. NC_000008.11). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

3.7.8 SSCP genotyping technique design of rs12255372 a*TCF7L2 gene* polymorphism

The following primer in table (3.10) represents the primer pairs that are used to amplify the rs11196205 a *TCF7L2 gene* polymorphism. As well as , figure (3.15) shown the position of the targeted rs12255372 SNP within the *TCF7L2 gene*.

Table (3.10) :Designed primers sets for rs12255372 a *TCF7L2 gene* polymorphism

Primer gene name	Sequence (5'->3')	Length (bp)	Amplicon Length (bp)
Forward	TCATAGGGGTCTGGCTTGGA	20	272
reverse complement	TTTGGAGCTGGGGAGAGAGT	20	

Abbreviations: bp : base pair

Figure (3.15) :The position of the targeted rs12255372 SNP within the *TCF7L2 gene*.

The exact position of the retrieved 272 bp amplicon spartially covered sequences of the *TCF7L2 gene* within chromosomes 10 (Gen Bank acc. no. NC_000010.11). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

3.8 DNA extraction from frozen blood samples

DNA was extracted from the frozen blood samples of the two study groups using the extraction kit(Relia Prep™ Blood gDNA Mini prep System-Promega USA) according to the following steps:

1- Blood samples were thawed before mixing for 10 minutes and then mixed well by placing them in the shaker for 10 minutes at room temperature.

2- Twenty microlitres (20 µl) of proteinase K solution was added to a 1.5 ml Eppendorf tube.

3- Two hundred microlitres (200 µl)of blood was added to the tube containing proteinase K solution and mixed briefly .

4-Two hundred microlitres (200 µl) of cell lysis buffer was added to the tube, covered and mixed by vortex for not less than 10 seconds to obtain a good yield.

5- The tube was then placed in the incubator for 10 minutes at 56°C.

6- During the incubation period, the ReliaPrep™ binding column is positioned in an empty collection tube.

7- Two hundred and fifty microlitres (250 µl)of Binding Buffer (BBA) was added to the tube after the incubation period was over. The tube was covered and vortexed for 10 seconds.

8-The contents of the tube were added to the ReliaPrep™ Binding column, capped and placed in a microcentrifuge.

9-The tube was placed in the microcentrifuge for one minute at maximum speed. It was ensured that all the solution had passed through the membrane by checking the absence of lysate at the top of the membrane.

10-The collection tube containing flow through has been removed and the liquid considered to be hazardous waste is disposed of.

11-The binding column was placed inside a new collection tube and 500 µl of washing solution was added to the column and then centrifuged for 3 minutes at maximum speed ,flow through was disposed of. The centrifugation process was repeated for another one minute for tubes containing the remnants of washing solution above the membrane.

12- Step 11 was repeated twice for a total of three washes.

13- The column was put into a new 1.5 ml micro-centrifuge tube.

14-Fifty to two hundred microlitres (50-200 µl) of nuclease-free water was added to the column and centrifuged at maximum speed for 1 minute.

15- The ReliaPrep™ binding Column was discarded and the eluate was saved.

3.9 Spectrophotometry of DNA

The quality and quantity of DNA was measured by the nano-drop device, the quantity and quality of the DNA was determined by calculating the ratios of (260/280) and (260/230). The sample is re-extracted if the sample shows a 260/280 ratio less than (1.8) and/or a (260/230) ratio less than (2).

3.10 Determination of the molecular weight and integrity of the extracted DNA

Electrophoresis on agarose gel was used to determine the DNA integrity and molecular weight, figure (3. 16) according to the steps described by Samboork and Russell (2001)as follows:

1-Agarose powder (0.3 g) was dissolved in 30 ml of 0.5X TBE solution to obtain a 1% gel concentration. Then the solution was heated for 45Sec. using a microwave oven.

2- The agarose gel was cooled using a water bath to 55°C.

3- The gel was mixed with 2µl of Ethidium bromide solution (1 mg/ml) and mixed well by vortexing.

4- The gel was poured into the gel try and left to solidify for 30 minutes.

5- The polymerized gel was then placed into an electrophoresis device containing a 0.5 TBE operating buffer.

6 -Gel wells were loaded with 5 µl of the extracted DNA and2µl of loading dye using a mechanical pipette.

7- The electrophoresis devise was set at 100V, 50 mA for 15-20 min to perform electrophoresis of the loaded samples.

8 -Then the gel template was photographed and the images were analyzed to determine the molecular weight of the extracted DNA(Figure 3.16).

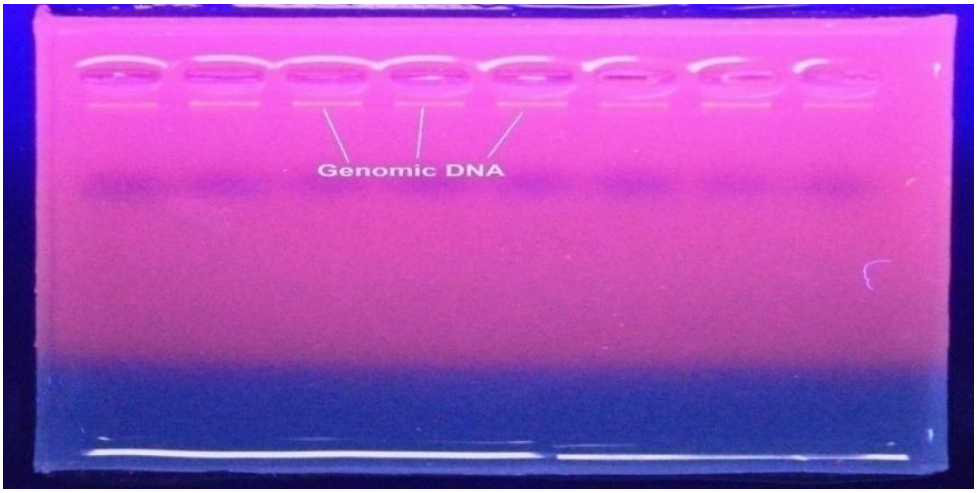


Figure (3. 16):Agarose gel electrophoresis for extracted genomic DNA.
100Volt , 50m- Ampere, 1 hour , 2% Agarose.

3.11 Optimization of polymerase chain reaction (PCR) conditions

The optimization was done for each SNP PCR reaction, include preforming gradient PCR in annealing temperature (55-67 C^0) after that we select the best annealing temperature that produce the best and specific amplicon. Gradient PCR and PCR products were determined using (2%) agarose and horizontal electrophoresis was performed at (100) volts, (50) mA for one hour according to the previously mentioned Samboork and Russell (2001).

3.11.1 Analysis of RFLP products

A (2%) agarose gel was prepared according to the previously mentioned method of Samboork and Russell (2001), and (100-1000) bp DNA ladder was used to identify the size of the resulting RFLP products. A horizontal electrophoresis device was used at (100) volts and (50) mA for one hour. The agarose gel template was then photographed and analyzed In order to obtain results.

3.11.2 Final condition of PCR-RFLP product of (rs1862513) SNP in *resistin gene*

The Optimized or final conditions of the thermal cycles , the reaction ingredients and RFLP reaction mixture for rs1862513 is shown in tables:(3.11) , (3.12) and (3.13) and figures(3. 17), (3. 18),respectively.

Table (3.11) :Optimized PCR condition of (rs1862513) SNP in *resistin gene*.

No	Stage	Temperature (^{0}C)	Time	Number of cycles
1	Initial denaturation	94	5 min	1
2	DNA denaturation	94	30 sec	35
3	Primer annealing	*66	30 sec	
4	Extension	72	30 sec	
5	Final extension	72	5 Min	1

*66=Optimized Primer annealing temperature

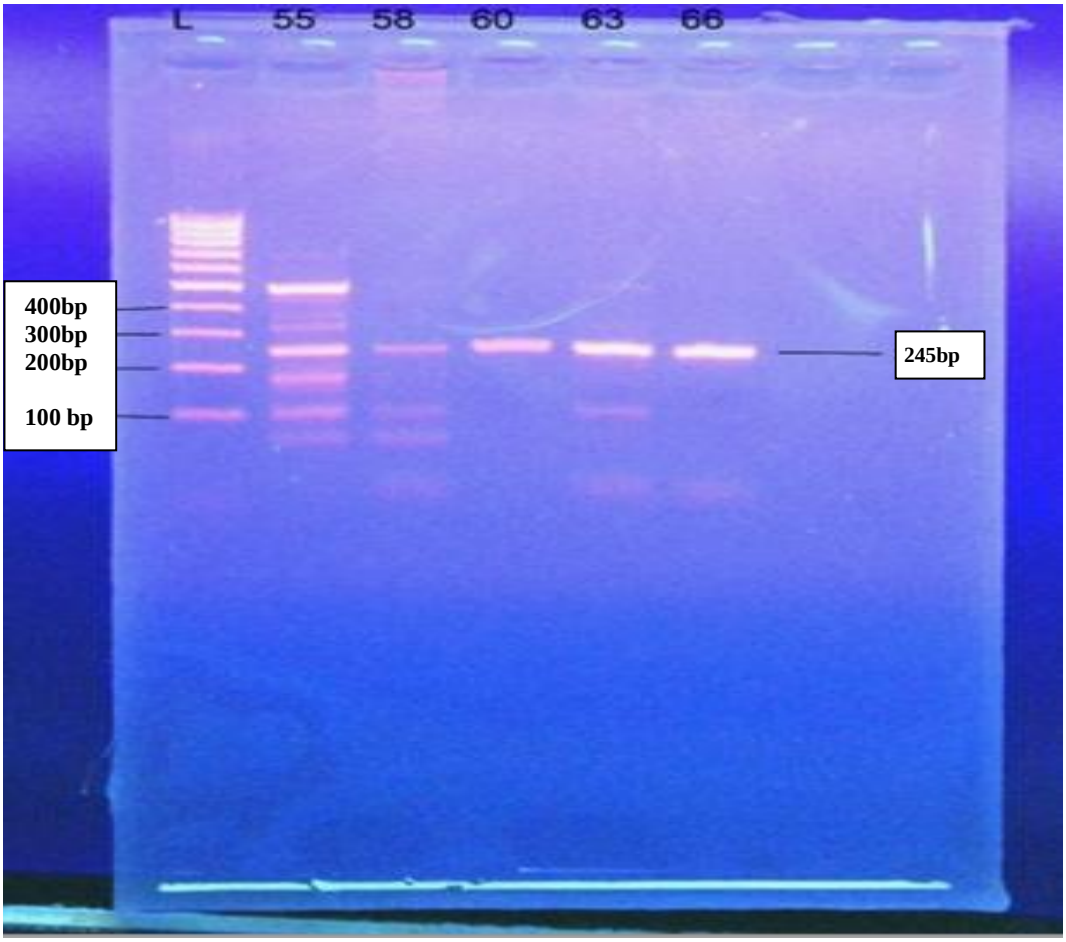


Figure (3. 17): Gradient PCR of(rs1862513) SNP in *resistin gene*.
The 55, 58, 60, 63 and 66: annealing temperature, 66: Optimized Primer annealing temperature, 245bp: Amplicon Length bp, L: Standard DNA ladder (100-1000 base pairs), 100Volt , 50m Ampere, 1 hour, 2%Agarose.

Table (3.12): PCR reaction mixture of (rs1862513) SNP in *resistin gene*.

	Composition	Volume(µl)	Concentration
1	Master mix	8	2.5 X
2	Forward primer	1	10 pmol
3	Revers primer	1	10 pmol
4	DNA sample	2	10- 20 ng/µl
5	Nucleases free water	7.5	
6	Mgcl2	0.5	25 Mm
	Total volume	20	

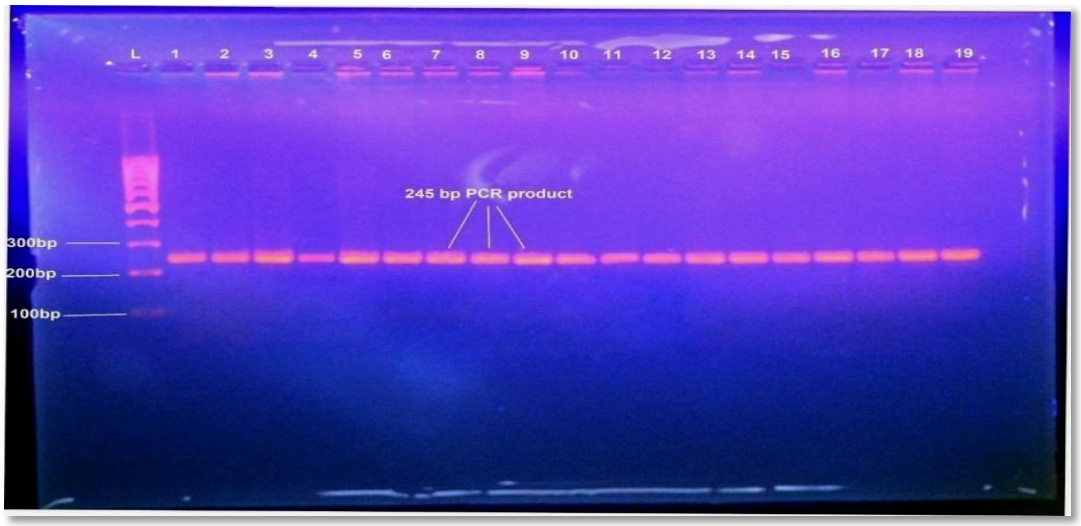


Figure (3. 18): PCR products of (rs1862513) SNP in *resistin gene*.
PCR product (1-19), 245bp: Amplicon Length bp, L:Standard DNA ladder (100-1000 base pairs), 100Volt, 50mAmpere, 1 hour , 2% Agarose.

Table (3.13): RFLP reaction mixture of(rs1862513)SNP in *resistin gene*.

	Composition		Volum(µl)
1	Buffer		1.5
2	BSA		0.1
3	Enzyme Bstv2I		0.2
4	Pcr product		3
5	Nuclease free water		10.2
	Total volume		15
incubation conditions	Temperature(^{0}C)	55	
	Time (hour)	24	

3.11.3 Final condition of PCR-RFLP amplification of (rs11977021) in *visfatin gene*

The Optimized or final conditions of the thermal cycles, the reaction ingredients and RFLP reaction mixture for rs11977021 is shown in tables (3.14),(3.15) and (3.16) and figures(3. 19) ,(3. 20) respectively.

Table (3.14) :Optimized PCR condition of(rs11977021) in *visfatin gene.*

No	Stage	Temperature (^{0}c)	Time	Number of cycles
1	Initial denaturation	94	5 min	1
2	DNA denaturation	94	30 sec	35
3	Primer annealing	*66	30 sec	
4	Extension	72	30 sec	
5	Final extension	72	5 Min	1

*66=Optimized Primer annealing temperature

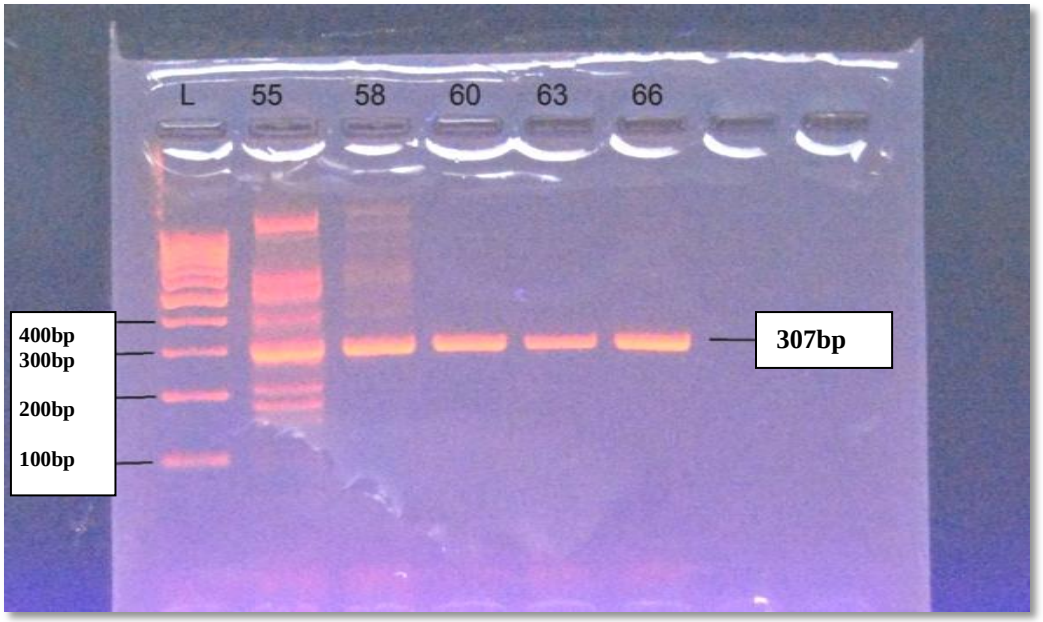


Figure (3. 19) :Gradient PCR of(rs11977021)SNP in *visfatin gene*.
The 55, 58, 60, 63 and 66 : gradient annealing temperature, 66 :Optimized Primer annealing temperature, 307 bp :Amplicon Length bp , L: Standard DNA ladder (100 base pairs) ,100Volt,50mAmpere,1 hour , 2%Agarose.

Table (3.15): PCR reaction mixture for (rs11977021)SNP in *visfatin gene*.

	Composition	Volume(µl)	Concentration
1	Master mix	8	2.5 X
2	Forward primer	1	10 pmol
3	Revers primer	1	10 pmol
4	Dna sample	2	10- 20 ng/µl
5	Nucleases free water	7.5	
6	Mgcl2	0.5	25 Mm
	Total volume	20	

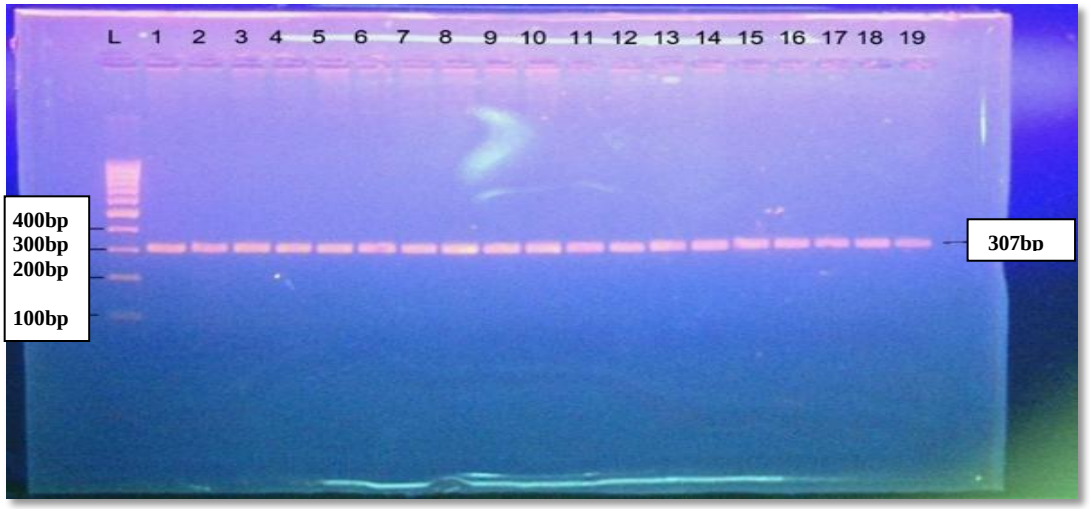


Figure (3. 20) : PCR product of (rs11977021)SNP in *visfatin gene*.
PCR product (1-19), 307 bp: Amplicon Length bp , L : Standard DNA ladder (100 base pairs), 100Volt, 50mAmpere, 1 hour , 2%Agarose.

Table (3.16) :RFLP reaction mixture for(rs11977021)SNP in *visfatin gene*.

	Composition		Volum(µl)
1	Buffer		1.5
2	BSA		0.1
3	Enzyme AcuI		0.1
4	PCR product		5
5	Nuclease free water		6.8
6	SAM		1.5
	Total volume		15
incubation conditions	Temperature(^{0}C)	37	
	Time (hour)	24	

3.11.4 Final condition of PCR-RFLP amplification of (rs3842752) SNP in *insulin gene*

The optimized or final conditions of the thermal cycles, the reaction ingredients and RFLP reaction mixture for rs3842752 is shown in tables (3.17),(3.18) ,(3.19) and figures (3. 21), (3. 22) respectively.

Table (3.17) :Optimized PCR condition of (rs3842752)SNP in *insulin gene*.

No	Stage	Temperature (^{0}c)	Time	Number of cycles
1	Initial denaturation	94	5 min	1
2	DNA denaturation	94	30 sec	35
3	Primer annealing	*60	30 sec	
4	Extension	72	30 sec	
5	Final extension	72	5 Min	1

*60=Optimized Primer annealing temperature

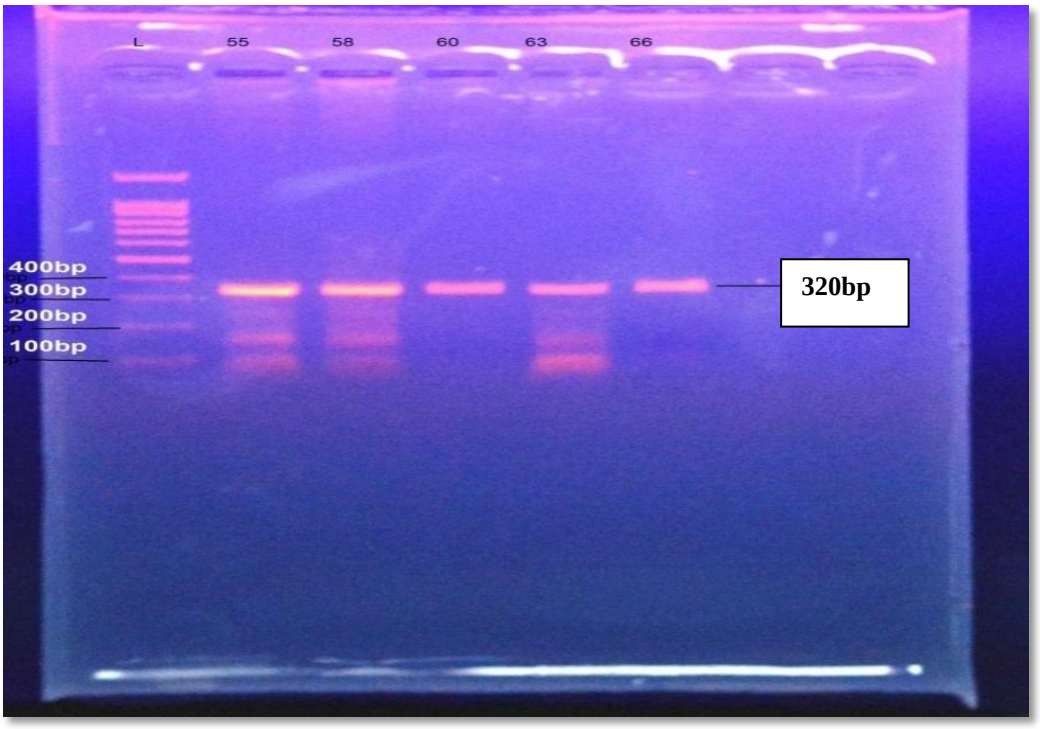


Figure (3. 21) : Gradient PCR of (rs3842752)SNP in *insulin gene*.
The 55, 58, 60, 63 and 66 : gradient annealing temperature, 66 : Optimized Primer annealing temperature, 329 bp : Amplicon Length bp, L : Standard DNA ladder (100-1000 base pairs) , 100Volt , 50mAmpere, 1 hour , 2% Agarose.

Table (3.18): PCR reaction mixture for (rs3842752)SNP in *insulin gene.*

	Composition	Volume (µl)	Concentration
1	Master mix	8	2.5 X
2	Forward primer	1	10 pmol
3	Revers primer	1	10 pmol
4	Dna sample	2	10- 20 ng/µl
5	Nucleases free water	8	
	Total volume	20	

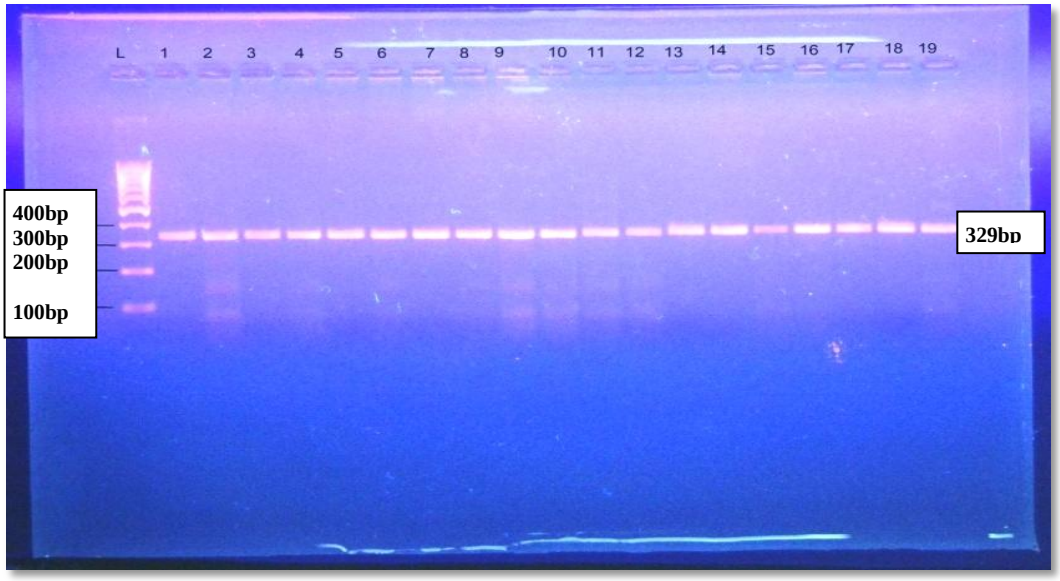


Figure (3. 22) : PCR products of (rs3842752) SNP in *insulin gene*.
PCR product. (1-19), 329 bp = Amplicon Length bp, L =Standard DNA ladder (100 base pairs), 100Volt, 50mAmpere, 1 hour , 2%Agarose.

Table (3.19) :RFLP reaction mixture for (rs3842752) SNP in *insulin gene*.

	Composition		Volum(µl)
1	Buffer		1.5
2	BSA		0.1
3	Enzyme PstI		0.25
4	Pcr product		5
5	Nuclease free water		8.15
	Total volume		15
incubation conditions	Temperature (^{0}C)	37	
	Time (hour)	24	

3.11.5 Final condition of PCR-RFLP amplification of (rs360717) in *interleukin -18 gene*

The Optimized or final conditions of the thermal cycles, the reaction ingredients and RFLPreaction mixture for rs360717 is shown in tables (3.20), (3.21) and (3.22) and figures (3. 23), (3. 24) respectively.

Table (3.20): Optimized PCR condition for (rs360717)SNP-*interleukin-18 gene.*

No	Stage	Temperature (^{0}C)	Time	Number of cycles
1	Initial denaturation	94	5 min	1
2	DNA denaturation	94	30 sec	35
3	Primer annealing	*63	30 sec	
4	Extension	72	30 sec	
5	Final extension	72	5 Min	1

*63=Optimized Primer annealing temperature

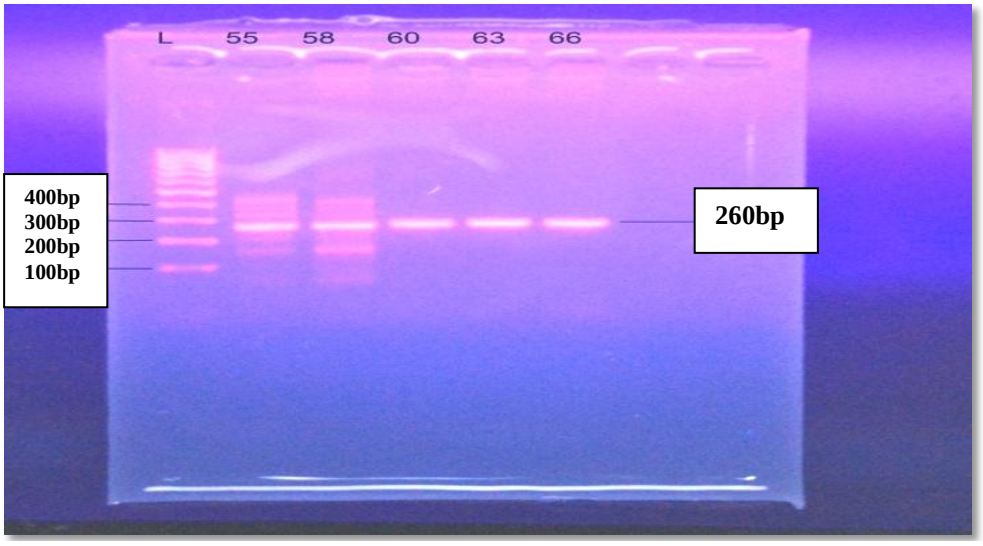


Figure (3. 23): Gradient PCR of (rs360717)SNP in *interleukin-18 gene* .
The 55, 58, 60, 63 and 66 : gradient annealing temperature, 63: Optimized Primer annealing temperature, 260 bp:Amplicon Length, L:Standard DNA ladder (100 -1000 base pairs) , 100Volt, 50mAmpere, 1 hour , 2%Agarose.

Table (3.21): PCR reaction mixture for (rs360717)SNP- *interleukin-18 gene*.

	Composition	Volume(µl)	Concentration
1	Master mix	8	2.5 X
2	Forward primer	1	10 pmol
3	Revers primer	1	10 pmol
4	Dna sample	2	10- 20 ng/µl
5	Nucleases free water	7.5	
6	Mgcl2	0.5	25 Mm
	Total volume	20	

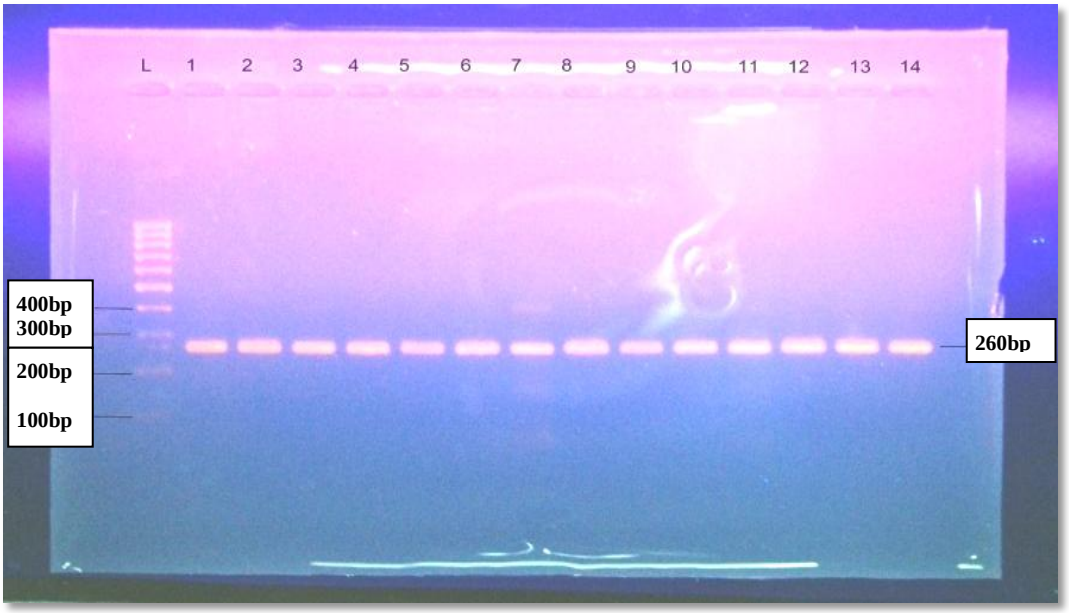


Figure (3. 24):PCR products of(rs360717)SNP in *interleukin-18 gene*.
PCR products (1-14), 260 bp:Amplicon Length bp , L: Standard DNA ladder (100-1000 base pairs),100Volt, 50mAmpere,1 hour , 2%Agarose.

Table (3.22): RFLP reaction mixture for(rs360717) SNP in *interleukin-18 gene*.

	Composition		Volum(µl)
1	Buffer		1.5
2	BSA		0.1
3	EnzymePstI		0.25
4	Pcr product		5
5	Nuclease free water		8.15
	Total volume		15
incubation conditions	Temperature(^{0}c)	37	
	Time (hour)	24	

3.12 Single strand conformation polymorphisms-SSCP genotyping

3.12.1 Poly-acrylamide gel electrophoresis

Acrylamide gel was prepared for electrophoresis of DNA pieces according to (Sambrook & Russell, 2001;Badi *et al*., 2021) as follows:

1- One hundred milliliters(100 ml)of mix acrylamide was prepared by dissolving 29.3 gm acrylamide, 0.7gm Bis -acrylamide, and then the volume was brought to 100 ml by adding double distilled water.

2- twenty milliliters (20 ml)of 15% acrylamide mixture was obtained by mixing 10 ml of mix acrylamide with 6 ml of double water, 4 ml of 5X TBE, 200 µl of ammonium persulfite and 20 µl of TEMED.

3-The electrophoreses device glasses was washed and It was dried well with alcohol, then the tow glasses reassembled to the device according to the manufacturer's instructions.

4-The mixture was poured as quickly as possible between the glassware of the apparatus using a Pasteur pipette. The comb forming the wells was gently inserted into its place, and left for one hour to polymerize.

5-After the polymerization was completed, 0.5X TBE buffer was prepared to submerge the gel, the comb was carefully removed, and the wells were then washed with buffer.

6-A (20mA 200V) constant current pre-run was carried out for 30 minutes.

7-After the pre-run was completed, a mechanical pipette was used to load 5 µl of sample.

8- The electrophoresis run was carried out for 24 hours under a constant current of 20 mA and 150 volts until the bromophenol blue dye reached tow third of the amount of gel.

3.12.2 Sample loading

According to Badi *et al*. (2021) Samples were prepared for loading on acrylamide by taking 2.5 microliters of PCR-product with a similar volume of mixture consisting of (9.4 ml formamid , 1.4 g NaOH , and 0.05g bromophenol blue) and incubated in a water bath at 91 °C for 5 minutes. Then the samples were placed in crushed ice until loading. After the end of the pre- run period, the samples were loaded as quickly as possible into the wells.

3.12.3 Gel staining

The gel was stained and fixed according to the method described by (Byun *et al.*, 2009). Where (3) solutions were used to complete the staining, according to the following steps:

1- The first solution was prepared in a volume of 100 ml by mixing the following components (10 ml ethanol, 0.2 gm sulfur nitrite and 500 microliter acetic acid). Double distilled water was added to bring the volume of the solution to 100 ml.
2- The mixture was placed on the shaker for 30 minutes to mix well.
3- The gel was removed from the device and placed in a container made of inert materials that do not interact with dye solutions.
4- The first solution was poured over the gel and it was placed on the checker for 30 minutes.
5-The first solution was poured out and the washing step was carried out with 100 ml double distilled water for 1 minute.
6-One hundred milliliters(100 ml)of the second solution (development solution) was prepared from (3 g of NAOH, 75 µl of formaldehyde and double distilled water to complete the volume to 100 ml) and then the solution was incubated in a water bath at 55 °C for 20 minutes.
7- The development solution was poured over the gel and the appearance of dark bands was monitored for a period ranging from 1 to 3 minutes.
8-The development solution was discarded and 100 ml of the third solution (fixing or stopping solution) consisting of (10 ml of ethanol and 500 µl of acetic acid and double distilled water in a volume added to get 100 ml) was added for 1 minute.
9- The gel was lifted from the container and placed on the light and photographed.

3.12. 4 Final condition of PCR-SSCP amplification of(rs13266634) in *SLC30A8 gene*

The optimized or final conditions of the thermal cycles and the reaction ingredients for rs13266634 is shown in tables (3.23),(3.24) and figures (3. 25), (3. 26) respectively.

Table (3.23): Optimized PCR condition of (rs13266634)in *SLC30A8 gene*.

No	Stage	Temperature (^{0}C)	Time	Number of cycles
1	Initial denaturation	94	5 min	1
2	DNA denaturation	94	30 sec	35
3	Primer annealing	*60	30 sec	
4	Extension	72	30 sec	
5	Final extension	72	5 Min	1

*60=Optimized Primer annealing temperature

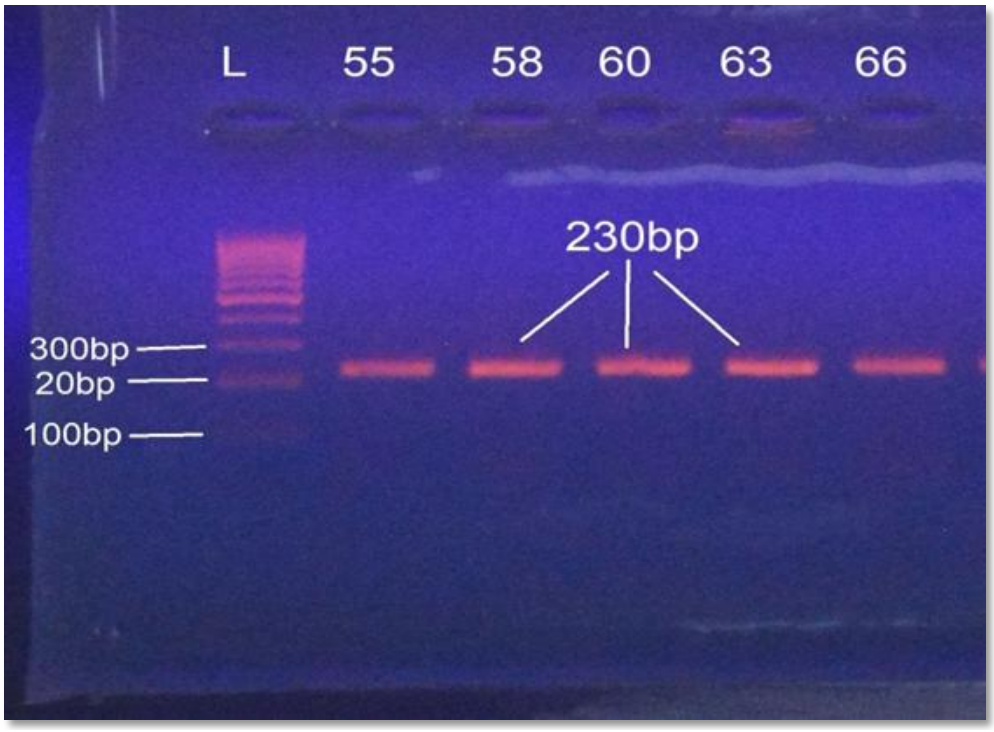


Figure (3. 25) :Gradient PCR of (rs13266634) SNP in *SLC30A8 gene* .
The55, 58, 60, 63 and 66: gradient annealing temperature, 60:Optimized Primer annealing temperature, 230 bp: Amplicon Length bp , L: Standard DNA ladder (100 - 1000base pairs), 100Volt, 50mAmpere, 1 hour , 2%Agarose.

Table (3.24):Optimized PCR reaction mixturefor(rs13266634)SNP in *SLC30A8 gene*.

	Composition	Volume (µl)	Concentration
1	Master mix	8	2.5 X
2	Forward primer	0.5	10 pmol
3	Revers primer	0.5	10 pmol
4	DNA sample	2	10- 20 ng/µl
5	Nucleases free water	7.5	
6	Mgcl2	0.5	25 Mm
	Total volume	20	

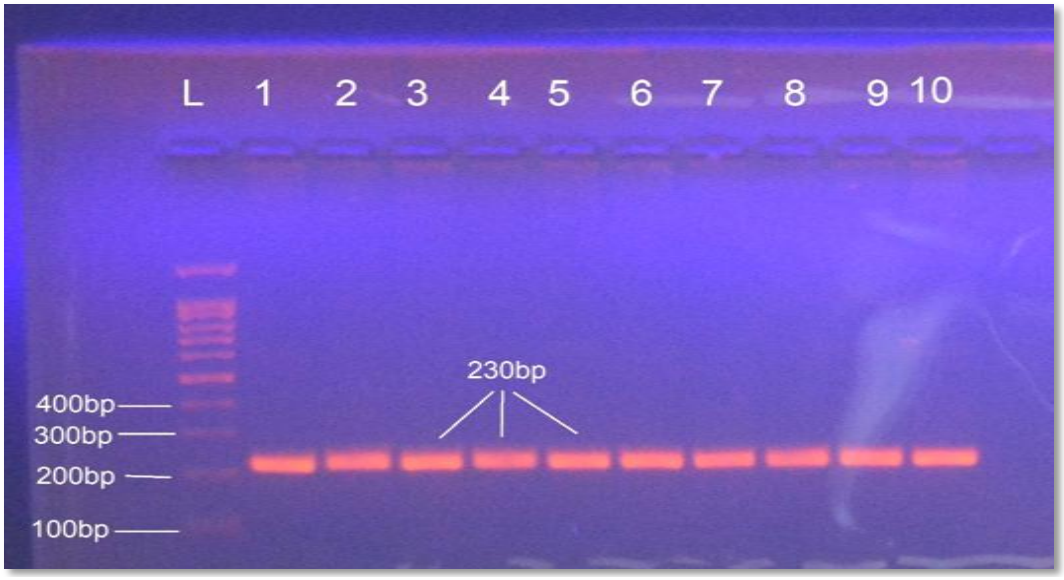


Figure (3. 26) : PCR products of (rs13266634) SNP in *SLC30A8 gene.*
PCR products (1-10), 230 bp:Amplicon Length bp, L:Standard DNA ladder (100-1000 base pairs), 100Volt, 50mAmpere, 1 hour , 2% Agarose.

3.12.5 Final condition of PCR amplification of(rs12255372)SNP in *TCF7L2 gene*

The Optimized or final conditions of the thermal cycles and the reaction ingredients for rs12255372 is shown in tables (3.25) and (3.26) respectively as well as ,figures (3. 27), (3. 28) respectively.

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Table (3.25) :Optimized PCR condition for(rs12255372)SNP in *TCF7L2 gene*.

No	Stage	Temperature (^{0}c)	Time	Number of cycle
1	Initial denaturation	94	5 min	1
2	DNA denaturation	94	30 sec	35
3	Primer annealing	*60	30 sec	
4	Extension	72	30 sec	
5	Final extension	72	5 Min	1

*60=Optimized Primer annealing temperature

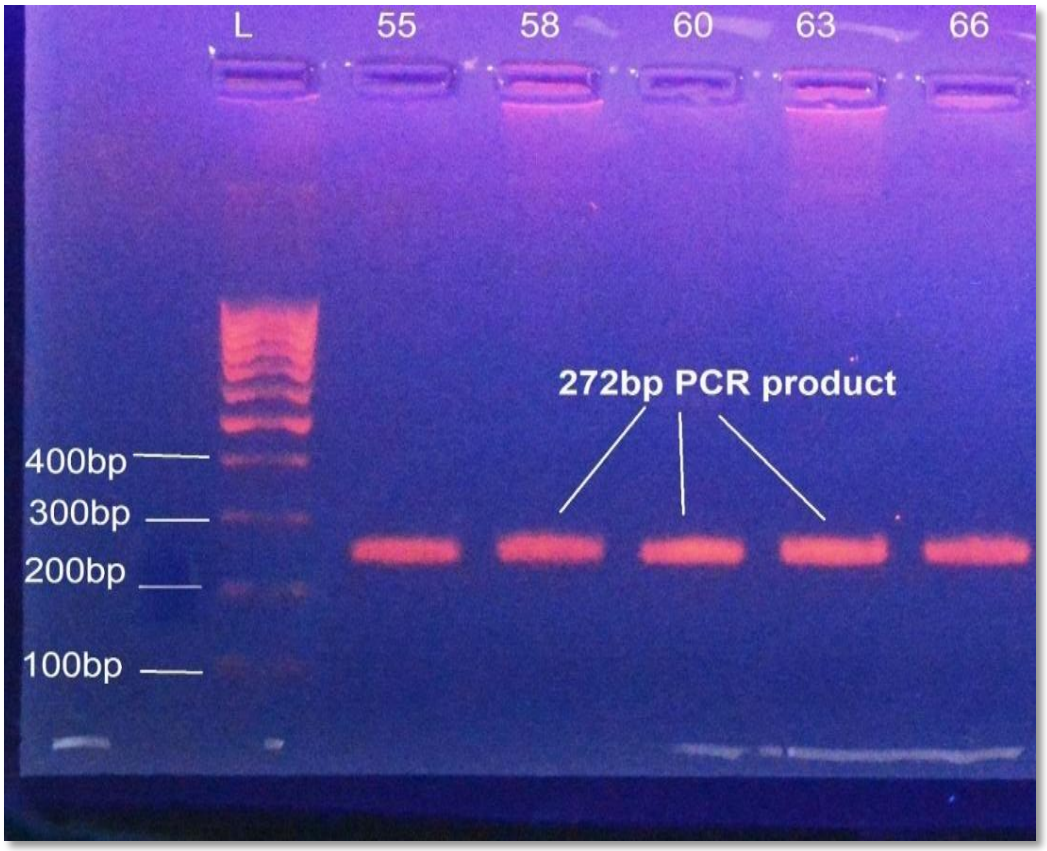


Figure (3. 27):Gradient PCR products of (rs12255372) in *TCF7L2 gene*.
The 55, 58 , 60, 63 and 66 : gradient annealing temperature, 60 : Optimized Primer annealing temperature, 272 bp:Amplicon Length bp, L:Standard DNA ladder (100-1000 base pairs),100Volt, 50mAmpere, 1 hour , 2%Agarose.

Table (3.26) : Optimized PCR reaction mixture of (rs12255372) SNP in*TCF7L2 gene.*

	Composition	Volume (µl)	Concentration
1	Master mix	8	2.5 X
2	Forward primer	0.5	10 pmol
3	Revers primer	0.5	10 pmol
4	Dna sample	2	10- 20 ng/µl
5	Nucleases free water	7.5	
6	Mgcl2	0.5	25 Mm
	Total volume	20	

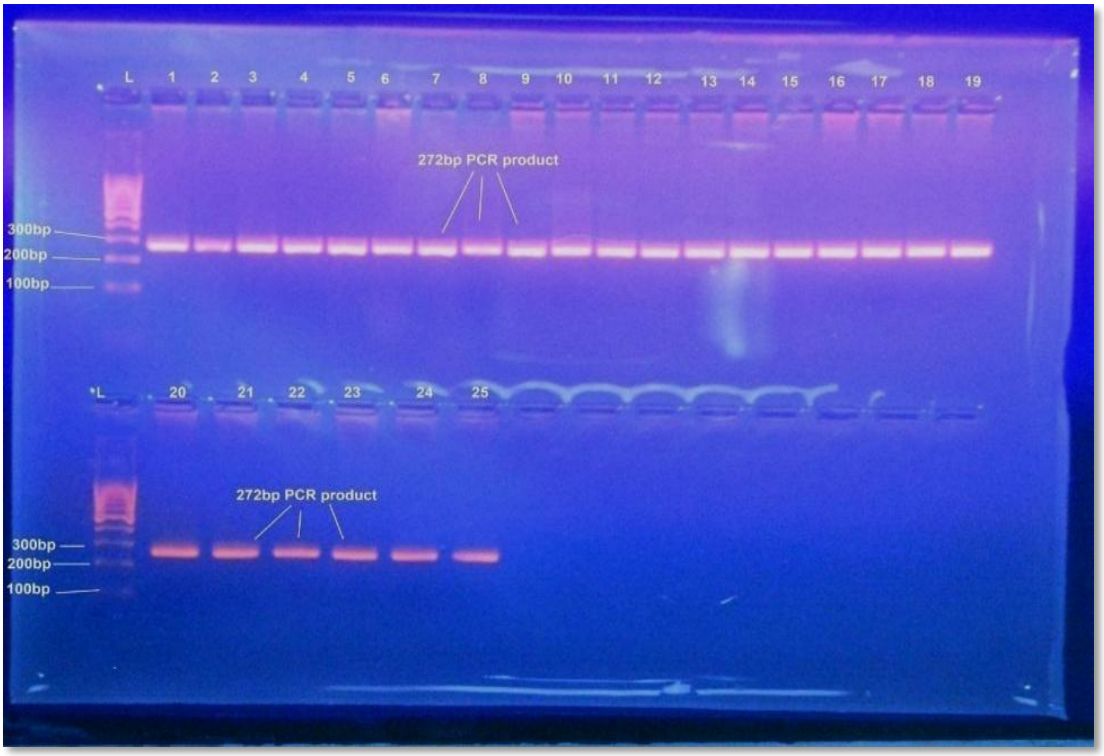


Figure (3. 28) :PCR products of (rs rs12255372) in *TCF7L2 gene*.
PCR products (1-10), 230 bp:Amplicon Length bp, L: Standard DNA ladder (100-1000 base pairs), 100Volt, 50mAmpere, 1 hour , 2%Agarose.

3.13 DNA sequencing

Two amplicons were selected from each DNA pattern were produced by poly-acrylamide gel electrophoresis of rs13266634 a *SLC30A8 gene* polymorphism ,rs12255372 a*TCF7L2 gene* polymorphism. And sent for DNA Sequencing by (Macrogen Inc. Geumchen, Seoul, South Korea).

3.13.1 Sequencing methods

3.13.2 DNA Sequencing of the *SLC30A8* and *TCF7L2*-based PCR amplicons

The resolved PCR amplicons were commercially sequenced from the reverse termini according to the instruction manuals of the sequencing company (Macro-gen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of the investigated samples with the retrieved neighboring DNA sequences of the NCBI Blastn engine, the virtual positions and other details of the retrieved PCR fragments were identified.

3.13.3 Interpretation of sequencing data

The sequencing results of the PCR products of six genotyped samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using Bio Edit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in their corresponding position within the referring genome .The targeted SNP was visualized in the dbSNP database to check its details positioned according to its places in the reference genome.

3.13.4 Checking the details of SNPs

The determinations of the previous depositions of the observed SNPs were performed by viewing their corresponding db SNP positions (https://www.ncbi. nlm.nih.gov/snp/) ,table (3.27). Then, the db SNP positions for the detected SNPs were documented. The observed SNPs were investigated in the dbSNP database to

check the details of their annotations. The targeted SNP positions were checked in their corresponding reference genome to assess their frequency in the same server. Two known frequency calculation parameters were employed in the current study to assess the approximated frequency of each detected SNP, namely GnomAD , TOPMED ,GnomAD_exome, and ExAC records.

Table (3.26) : Forward and reverse primer sequences of rs13266634 and rs12255372

Amplicon	Reference locus sequences (5′ - 3′)	Length
A)rs13266634	*GGAGTCAGAGCAGTCGCCCATGCGTGTGCAATCAGTGCTAATCTC CCTGTGCTTCTTTATCAACAGCAGCCAGCCGGGACAGCCAAGTGGT TCGGAGAGAAATTGCTAAAGCCCTTAGCAAAAGCTTTACGATGCA CTCACTCACCATTCAGATGGAATCTCCAGTTGACCAGGACCCCGAC TGCCTTTTCTGTGAAGACCCCTGTGACTAGCTCAGTCACACCGTCA GT**	230 bp
B)rs12255372	*TCATAGGGGTCTGGCTTGGAAAGTGTATTGCTATGTCCAGTTTAC ACATAAGGATGTGCAAATCCAGCAGGTTAGCTGAGCTGCCCAGGA ATATCCAGGCAAGAATGACCATATTCTGATAATTACTCAGGCCTCT GCCTCATCTCCGCTGCCCCCCCGCCCCCTGACTCTCTTCTGAGTGCC AGATTCAGCCTCCATTTGAATGCCAAATAGACAGGAAATTAGCATG CCCAGAATCCACGTCTTTAGTGCACTCTCTCCCCAGCTCCAAA**	272bp

* refers to the forward primer sequences (placed in a forward direction)
**refers to the reverse primer sequences (placed in a reverse complement direction)

3.14 Statistical analysis

Statistical tests were carried out using the SPSS software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. USA) and Microsoft Excel (2010, Microsoft Corp. USA). Results are represented by mean ± SE. With $p < 0.05$ adopted as a statistical significance. For the values that were not normally distributed, the Mann-Whitney test was used, and for the values that were normally distributed, the T-test was used to compare between two independent samples .

The Kruskal-Wallis test was performed for multiple comparison of values that are not normally distributed, and Spearman's test was used to investigate the correlation in the values that were not normally distributed. Chi-square test to assess categorical association of variables and genetic association (Sole *et al*., 2006).

Chapter Four
Results
and
Discussion

4.Results and discussion

4.1 Comparison and correlation of anthropometric measurements between T2DM and ND groups

The results of the current study, table (4.1), showed a significant increase (p = 0.00) in the means of body mass index (BMI), waist circumference (WC) and neck circumference (NC) in the type II diabetes group (T2DM) compared to the non-diabetic group (ND) or control group.

Table (4.1) :Comparison of anthropometric measurements between T2DM and ND groups.

	ND	T2DM	P-Value
BMI ($kg/m^{2)}$	25.49 ± 1.43	*31.66 ± 4.30	*0.000
WC (cm)	84.88 ±1.71	**96.20 ±1.079	**0.000
NC (cm)	38.95± 2.27	*41.07± 1.51	*0.000
No.	34	54	
Total	88		

Data are presented as (mean± SE, **: indicates the significant increase calculated using the t-test), (mean ± SD, *: indicates the significant increase calculated using the Mann-Whitney test), *p-value ≤0.05 by Mann-Whitney test, **p-value ≤0.05 by T-test, No.: number, T2DM: type II diabetes millets, ND: non-diabetics, BMI: body mass index , WC :waist circumference ,NC: neck circumference.

The current results of anthropometric measurements indicated that there were significant increases in (BMI), (WC), and (NC) in the (T2DM) group compared to the (ND) group. Which may confirm that weight gain is one of the most important environmental factors that increase the chance of developing type II diabetes.

Measuring of body circumferences and body mass index (BMI) is useful in evaluating and assessing the risk of obesity in different age groups (Saka *et al*., 2014).

Body Mass Index ranged from (30-34.9) represent class I obesity that have a high risk factor for diseases, while (BMI) rang from (25-29.9) which represent overweight that perhaps increased the risk of disease susceptibility (Sisodia & Chouhan, 2019). Accordingly, first-degree obesity was identified in patients with T2DM, and the presence of weight gain in the ND group in the current study, which may support the relationship between obesity and susceptibility to type II diabetes.

The above results may indicate the need to determine the normal value of the body mass index among Iraqis, which is not considered a risk factor for developing type II diabetes. Perhaps we must take into consideration that the body mass index may be affected by differences in physical structure between peoples and races. For example, the susceptibility to diabetes increases among South Asians despite the fact that their (BMI) is low (24 kg / m^2), Chinese (25 kg / m^2) and black Africans (26 kg / m^2) compared to whites (30 kg / m^2) (Chiu *et al*., 2011).

These results are consistent with what was concluded by Flegal *et al*. (2009) that (BMI) is more closely related to height than to the percentage of body fat, and diagnosing obesity in the elderly based on (BMI) seems less reliable in determining the level of obesity.

The results of the current study regarding waist circumference showed that there was a significant increase in waist circumference in the T2DM group compared to the ND group, which may indicate the role and importance of central obesity in increasing the chance of developing type II diabetes. These results are consistent with what Venkatrao *et al*. (2020) concluded that waist circumference reflects the level of fat accumulation around and inside the abdomen, and is considered a good predictor of susceptibility to type II diabetes and metabolic diseases compared to general obesity expressed as (BMI). As stated in the study of Kumar *et al*. (2014a) that the normal value of waist circumference for men is less than 94 cm and for women less than 80 cm is associated with a lower risk of disease.

With regard to neck circumference, the results of the current study showed a significant increase in neck circumference in the T2DM group compared to the ND group. This result is consistent with what was stated in the study of Yang *et al*. (2010) in that the normal values for neck circumference in women are less than 34 cm and for men less than 37 cm may be the best cut-off point for determining the incidence of central obesity in individuals that increases the risk of disease (Yang *et al*., 2010).

The significant increase in the body mass index, waist circumference, and neck circumference in the T2DM group may indicate an association between weight gain and the risk of developing type II diabetes, the risks of which can be avoided by losing weight, exercising, and following a healthy eating pattern.

However, the need to determine the normal value of body mass index, which is not considered a risk factor for Iraqis, also requires determining the normal values of waist and neck circumferences, taking into account the characteristics of the physical structure of Iraqis.

The result of correlation analysis in figure (4.1), showed that there was a positive significant correlation(r = 0.695, P=0.000) of (BMI) with (WC), and there was a positive significant correlation, figure (4.2), (r = 0.310, p = 0.023) between (WC) and (NC), while (BMI) was not correlated with (NC),(r = 0.145, P = 0.297).

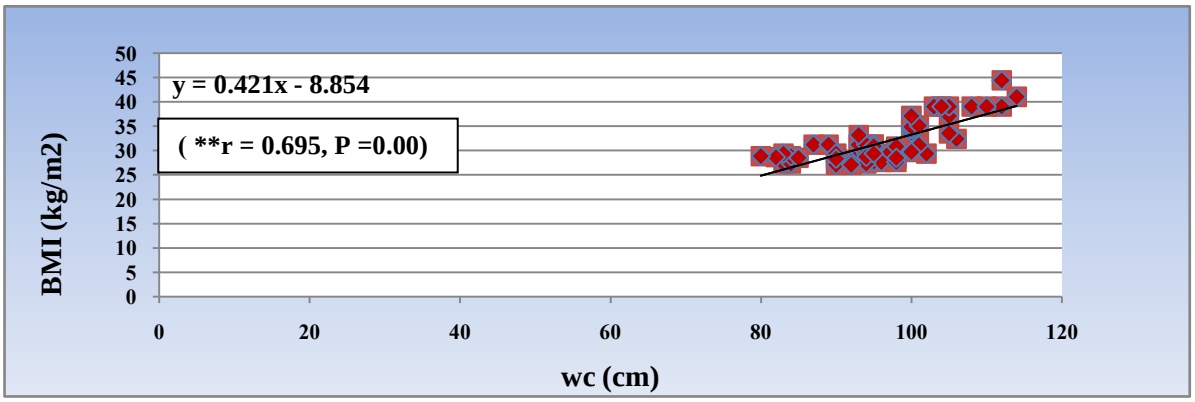


Figure (4.1): Correlation between BMI and WC in T2DM group.
BMI: body mass index ,WC: waist circumference ,T2DM: type II diabetes millets, **Correlation is significant at the 0.01 level (2-tailed)(Spearman's rho Test).

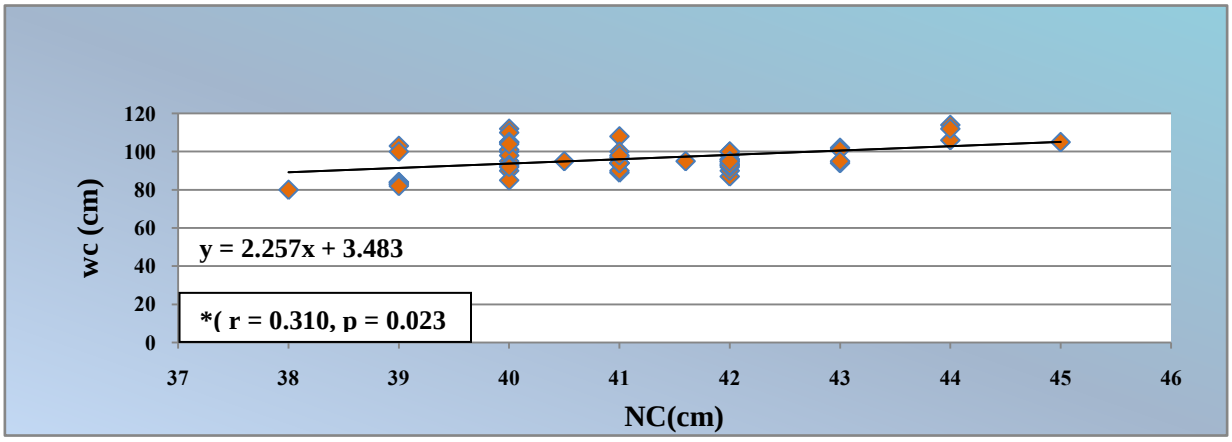


Figure (4.2): Correlation between WC and NC in T2DMgroup
WC: waist circumference ,NC: neck circumference. T2DM: type II diabetes millets,*: Correlation is significant at the 0.05 level (2-tailed) (Spearman's rho Test).

The present results showed a medium positive significant correlation between (BMI) and (WC) in the T2DM group, which is consistent with Chinedu *et al*. (2013) in finding the correlation of (WC) with (BMI) for a large group of individuals from 18 to 75 years of age. And with Gierach *et al*. (2014) of individuals between the ages of 32 to 80 years with metabolic syndrome. Therefore, according to the current findings, the increase in (BMI) may reflect the level of obesity in the body if it is associated with the increase in waist circumference.

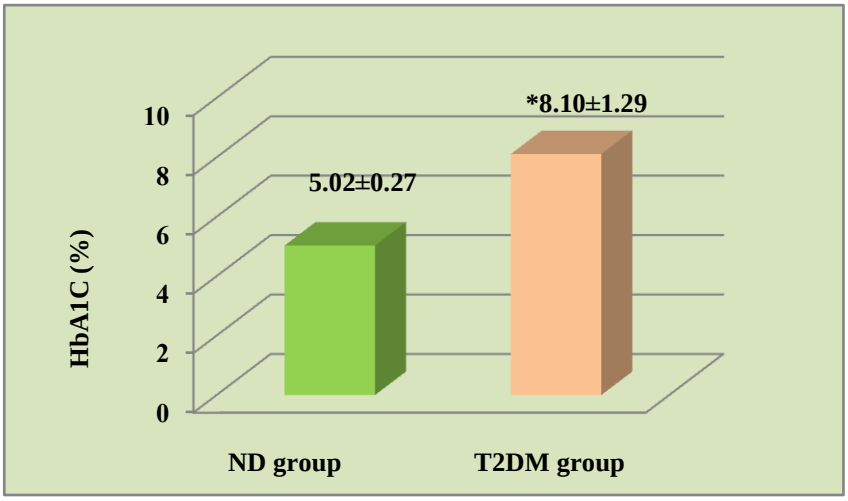
Furthermore, current result showed the presence of a weak positive significant correlation between (WC) and (NC), which agrees with the results of Kumar *et al*. (2014a), and the study of Saka *et al*.(2014) in association of (NC) with (WC) and BMI. We conclude that weight gain is consistent with the increase in body circumferences and body mass index, but the risk of disease may increase with (WC), which is more associated with increased central obesity around the middle.

4.2 Comparison and correlation of glycemic parameters between T2DM and ND groups

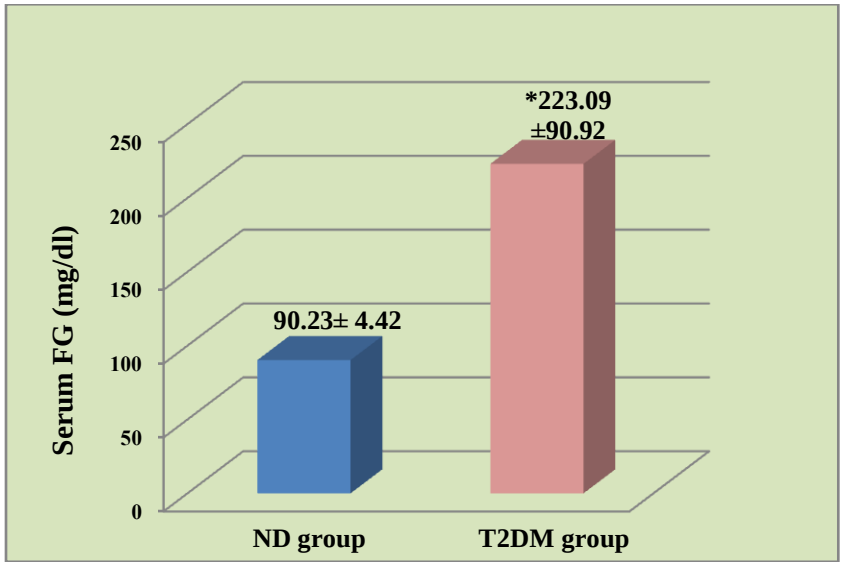
The current results of comparison between glycemic parameters in figure (4.3) showed a significant increase (p=0.000) in the percentage of glycated hemoglobin (HbA1c) analysis in T2DMgroup (8.10 ± 1.29 %) compared to ND group (5.02± 0.27%).

The results of current study in figure (4.4) showed that there was a significant increase (p=0.000) in the fasting serum glucose (FG) level between T2DM group (223.09 ± 90.29mg/dl) and ND group (90.23 ± 4.42mg/dl). Moreover the results in figure (4.5) showed a significant increase (P=0.000) for (FG)level in saliva between T2DM group (18.81 ± 3.92 mg/dl) and ND group (6.82 ±1.15mg/dl).

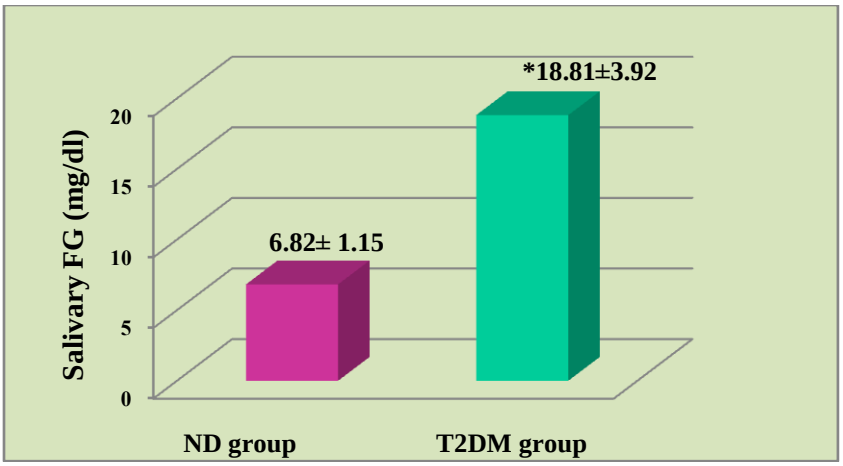
The correlation analysis shown in table (4.2) did not show correlation of (FG) level between serum and saliva, in addition, salivary (FG) was not correlated with (HbA1c),while a positive significant correlation (r = 0.844, p = 0.000) appeared between serum (FG) and (HbA1c),figure (4.6).



Figure(4.3):Comparison of HbA1c ratio between T2DM and ND groups.
Data were expressed with mean ±SD, significant increase noted by(*), p ≤0.05 by Mann-Whitney Test, T2DM: type II diabetes millets, ND: non- diabetics(control) group.



Figure(4.4): Comparison of fasting serum glucose level between T2DM and ND groups.
Data were expressed with mean ±SD, the significant increase noted by (*), p-value ≤0.05 by Mann-Whitney Test , T2DM: type II diabetes millets, ND: non-diabetics(control group), FG: Fasting glucose .

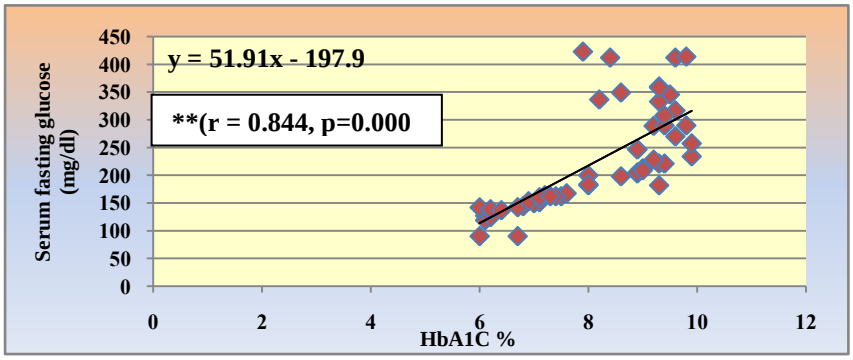


Figure(4.5): Comparison of fasting saliva glucose level between T2DM and ND groups.
Abbreviations: Data were expressed with mean± SE ,the significant deference noted by (*), p ≤0.05 by Mann-Whitney Test, T2DM: type II diabetes millets, ND: non- diabetics,FG: Fasting glucose .

Table(4.2): Correlation between serum and saliva FG and HbA1c in T2DMgroup

		T2DM	
		Saliva-FG (mg/dl)	HbA1c(%)
Serum - FG (mg/dl)	r	0.134	
	P	0.333	
Saliva-FG (mg/dl)	r		0.112
	p		0.420
N	54		

Abbreviations: T2DM: type II diabetics millets, FG: Fasting glucose, N: number, r:correlation coefficient by Spearman's rho test.



Figure(4.6): Correlation between serum –FG and HbA1c in T2DM group.
Abbreviations: FG: fasting glucose ,** Correlation is significant at the 0.01 level (2-tailed) (Spearman's rho Test).

Elevated blood glucose levels are the diagnostic feature of all types of diabetes (Vaziri *et al.*, 2010). Current results showed that the levels of glucose in serum and saliva were significantly higher in (T2DM) group, which is consistent with several studies that were conducted to demonstrate the benefit of saliva, due to its ease of collection and painlessness, in examining glucose levels in patients with type II diabetes (Gupta *et al*., 2014; Satish *et al*., 2014; Lakshmi *et al*., 2015 and Dhanya & Hegde , 2016).

However, in current study, no correlation of glucose between serum and saliva was shown in the T2DM group. These results are consistent with the study of Wang *et al*. (2017) that showed no correlation of glucose between serum and un-stimulated whole saliva, but a strong positive correlation of glucose was found between serum and saliva collected from the parotid gland.

Moreover, many studies showed a correlation in the levels of glucose between serum and saliva in the type II diabetic group and non-diabetic group (Naik *et al*., 2011; Abhikshyeet *et al*., 2012 and Gupta *et al*., 2014). On the other hand, other studies showed a correlation between saliva and serum glucose level in the type II diabetes group only (Darwazeh *et al*.,1991and Hegde *et al*.,2010).

There are many factors that affect the composition of saliva collected in the oral cavity, unlike saliva that is collected directly from the salivary gland, whose composition is not affected by the biological conditions of the oral cavity (Dhanya & Hegde, 2016). In addition, Murrah *et al*. (1989) explained that the increase glucose concentration in saliva may be due to a change in the permeability of the basement membrane of the parotid glands in type II diabetic patients.

It can be concluded that the environment of the oral cavity may affect the concentration of some components that are secreted within the saliva ,like oral normal flora, and on the other hand, it is possible that the difference in the type of secretions of the salivary glands may also affect the concentrations of proteins derived from plasma, and in the case of diabetic patients, chronic high blood glucose may affect the permeability of salivary gland tissues.

Current results also showed that there was a medium positive correlation between the HbA1c with the glucose level in the serum of the T2DM group, These results agree with the study of Sashikumar & Kannan (2010) and Kumar *et al*. (2014 b).

The chronic high blood glucose levels in the T2DM group may explains the association with HbA1c, which represents the state of blood glucose levels during the last three months.

In the current study, the HbA1c was not correlated with the level of glucose in saliva, unlike what was stated in a study of López *et al*. (2003), and the study of Abikshyeet *et al*. (2012). The difference in results between previous studies and the current study may be due to the difference in the techniques used for saliva collection, managements and measurement or the number of participants.

4.3 Comparison and correlation of insulin level in serum and saliva between T2DM and ND groups

The current results of fasting insulin level, figure (4.7) showed that there was a significant increase ($p \leq 0.000$) in serum fasting insulin levels between T2DM group (3.86 ± 5.01 μIU/ml) and ND control group (1.09 ±0.28μIU/ml).

In addition, there was a significant increase ($p=0.043$ μIU/ml) in the level of salivary fasting insulin between T2DM group (0.72 ± 0.32 μIU / ml)and ND group (0.36 ± 0.12 μIU/ml) as shown in figure (4.8).

Moreover, the correlation analysis, figure (4.9) showed that there was a positive significant correlation ($r = 0.741$, $p= 0.00$)of fasting insulin level between serum and saliva in the T2DM group.

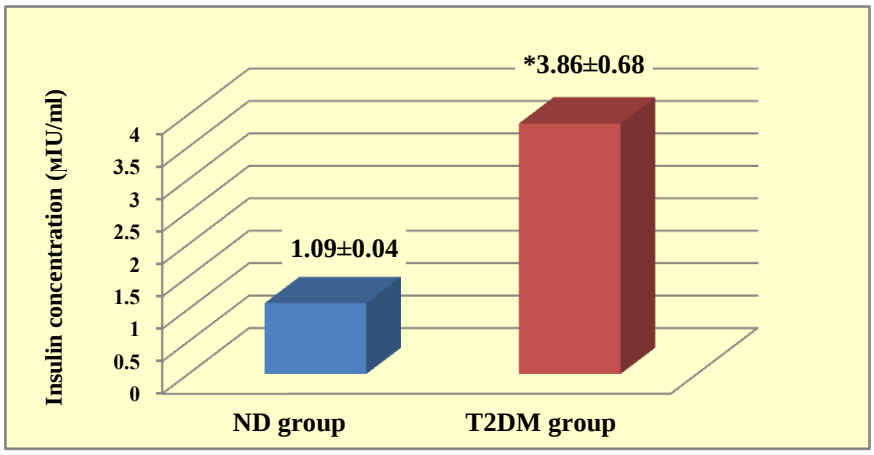
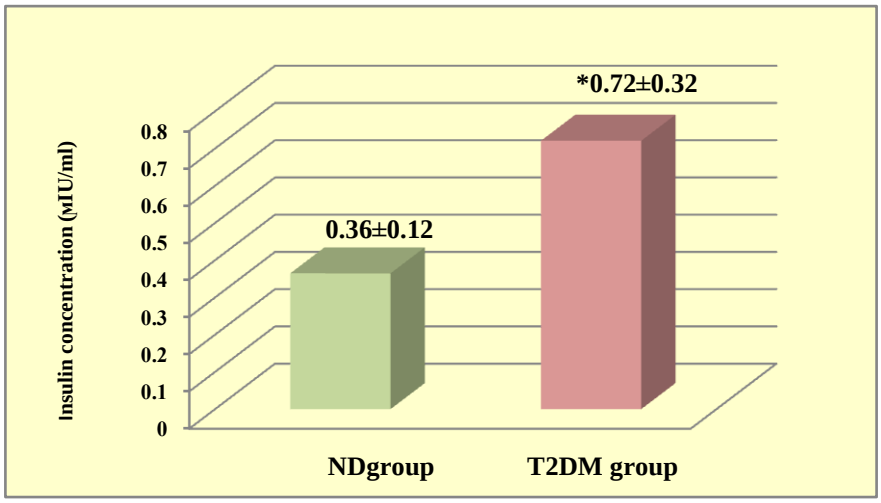


Figure (4.7): Comparison of Serum Fasting insulin level between T2DM and ND groups.
Abbreviations : data were expressed with mean ±SD, significant increase noted by(*), $p \leq 0.05$ by Mann-Whitney Test , T2DM: type II diabetics millets, ND: non- diabetics, F: Fasting



Figure(4.8): Comparison of Saliva Fasting insulin level between T2DMandND groups.
Abbreviations: data were expressed with mean ±SD, significant increase noted by(*), p ≤0.05 by Mann-Whitney Test ,T2DM: type II diabetes millets, ND: non- diabetics, F: Fasting

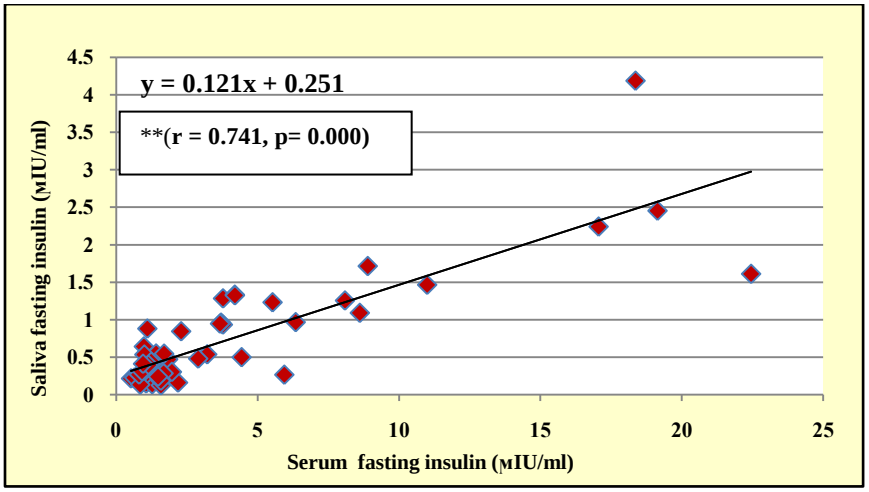


Figure (4.9): Correlation between serum and saliva Fasting insulin in T2DM group.
** Correlation is significant at the (0.01 level , Spearman's rho Test).

The use of saliva can be of great benefit in the tests that serve to detect biomarkers in type II diabetes (Naseri *et al*., 2018). Insulin measurement may be required as a necessity in case of diabetes and metabolic diseases (Velicu & Henkin, 2006).

The current results showed that insulin levels in serum and saliva were significantly increase (p=0.00, p=0.043) respectively in T2DM group compared to ND group, which was consistent with the results of Marchetti *et al*. (1986) in

patients with type II diabetes and healthy obese patients compared to healthy non-obese subjects.

Frequently, there were high levels of insulin in obese individuals and patients with type II diabetes, whether these high levels of insulin are a cause of insulin resistance or were a consequence of it (Corkey, 2012 and Sbraccia *et al*., 2021).

All of these results in the current study and previous studies above indicate the fact that obesity is involved in the occurrence of insulin resistance and the subsequent attempt of beta cells to increase insulin production to compensate for the insensitivity of tissues to insulin.

Moreover, the current study showed that there was a significant positive correlation between fasting serum and salivary insulin levels in T2DM group, which is consistent with previous studies in the existence of an association of insulin levels between serum and saliva, for example in healthy subjects (Marchetti *et al*., 1988). And in patients with type 1 diabetes, after taking insulin as an injection (Marchetti *et al*., 1990).

Other studies have shown that the level of insulin in saliva correlates with insulin levels in serum after about 30 minutes, and that the level of insulin in saliva is lower than insulin in serum by a ratio of (1: 2) (Fekete *et al*.,1993; Messenger *et al*., 2003). Furthermore, another study found that the insulin level in saliva is (10:100) of the insulin level in plasma (Fabre *et al*., 2012).

All these results may be explained by the nature of insulin secretion during fasting and after eating, its spread through the tissues, the effect of removal and breakdown of the insulin in the liver, despite that and although insulin may take time to appear in saliva, perhaps measuring the level of insulin in saliva in a fasting state can be considered a good indicator for diagnosing type II diabetes.

It is worth noting that, in clinical diagnosis, insulin measurement is not a routine examination (Janssen, 2021). It may be due to the association of serum insulin measurement with many clinical and analytical problems, although there are many test kits with continuous improvements that are commercially available (Clark , 1999 and Taylor *et al*., 2016).

The difficulties in accurately determining insulin concentration may be due to the pulsating nature of insulin secretion associated with glucose concentrations (Porksen *et al*., 2002 and Crofts *et al*.,2015). Also, the secreted insulin is distributed among the tissues and inside the blood vessels, and therefore the amount of insulin in the blood vessels is less than its amount outside the vessels

(De León & Stanley, 2013). Moreover, 80% of the secreted insulin is removed by the liver (Najjar & Perdomo, 2019).

4.3.1Genetic analysis of SNPs related to insulin levels

4.3.1.1Genotyping of SNP rs3842752G>A in *insulin gene*

The current results of the genotype analysis of rs3842752 by PCR-RFLP technique, figure (4.10) showed that the DNA pieces of size (179 + 123 + 27) bp represent the allele (G), while the size of the pieces (123 + 94 + 85 + 27) bp represents the allele (A), and accordingly, the DNA pieces of size (179 + 123) bp represent the genotype (G/G), (123 + 85 + 94) bp represent the genotype (A/A) and (179 + 123 + 85 + 27) bp represents the genotype (G/A).

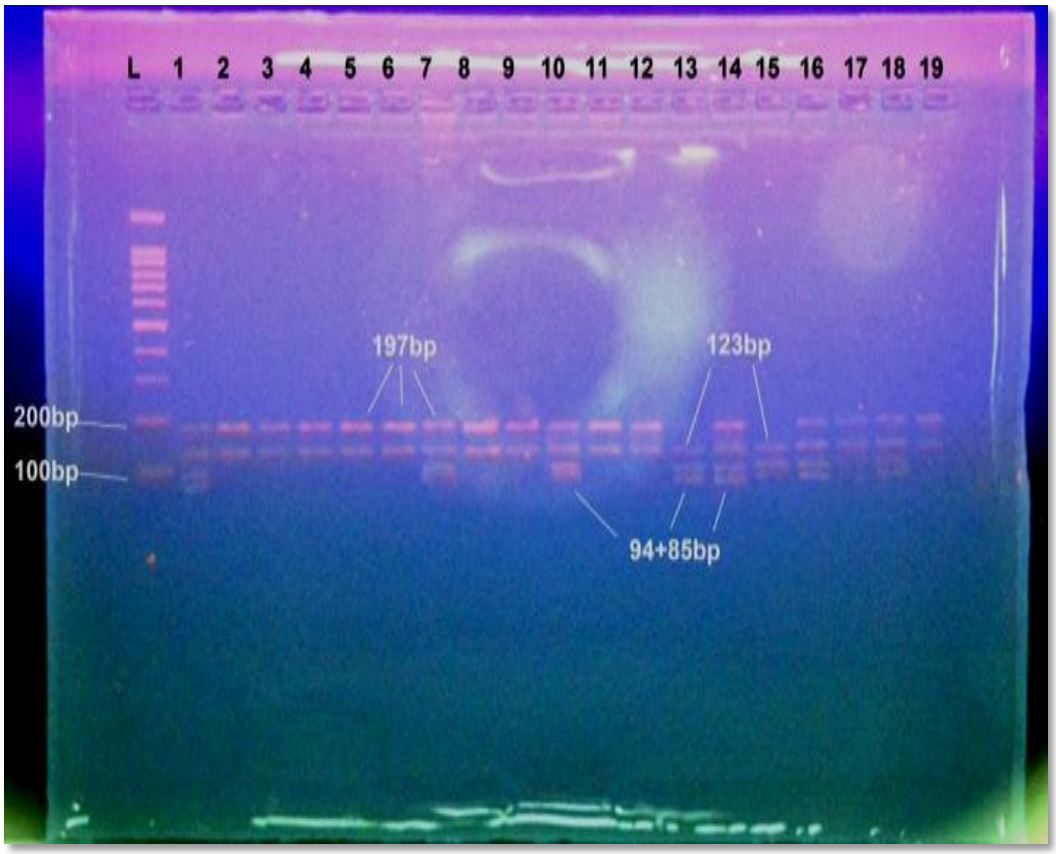


Figure (4.10):Gel electrophoresis of the(rs3842752-*insulin gene*) genotyping by PCR-RFLP.
lane L :DNA ladder100-1000bp ; lanes 2,3,4,5,6,8,9,11,12,17 and 19 G/G genotype; lanes 1,8,10,14,16 and 18 G/A genotype; lanes 13 and 15 A/A genotype,100V ,50mA-1h., 2%Agarose.

4.3.1.2 Genotyping of SNPs(rs12255372 - *TCF7L2gene*) and (rs13266634- *SLC30A8 gene*)

The results of the genotyping of rs12255372 by Single stranded conformation polymorphism (SSCP-PCR) technique ,figure (4.11) showed the presence of an A, B, and C pattern. DNA sequencing. Figure(4.13) reveal that A,B and C pattern represent A/A, G/G and G/A genotypes of rs12255372 respectively. In addition, the results of the SSCP genotyping of rs13266634, figure (4.12) showed the presence of A, B, and C pattern. DNA sequencing reveal that A, B and C patterns represent T/T, C/C and C/T genotype of rs13266634 respectively (figure4.13).

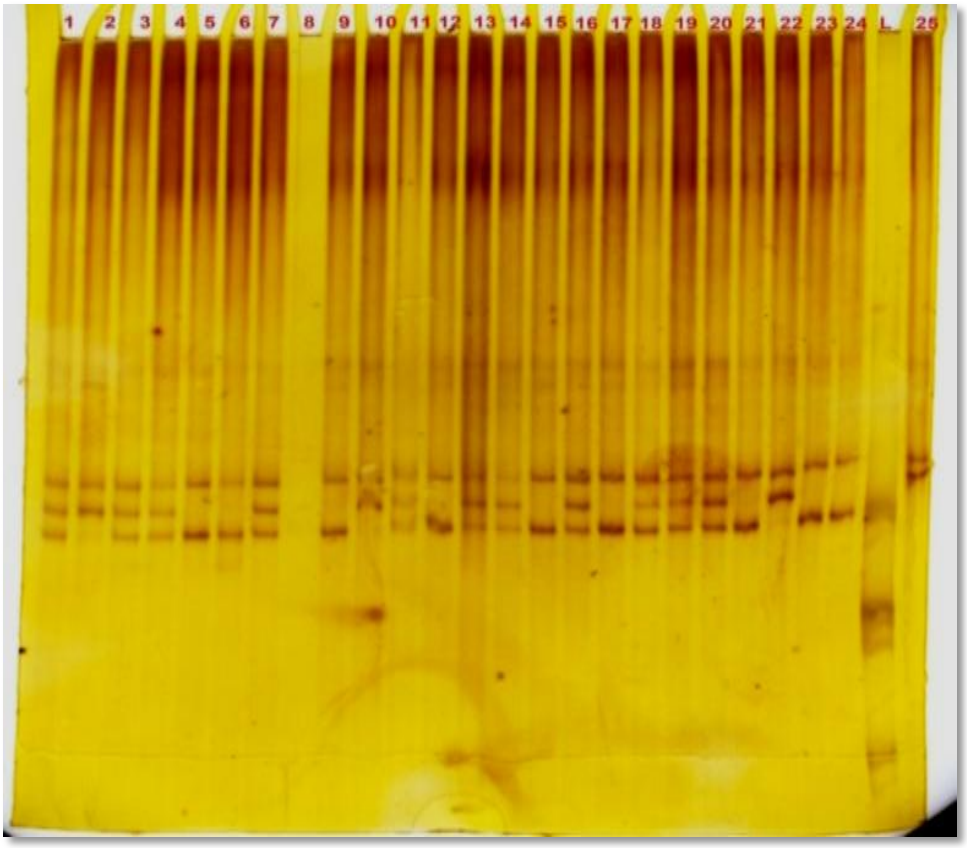


Figure (4.11):Gel electrophoresis of rs12255372-*TCF7L2 gene* by PCR-SSCP technique.
lane L: DNA ladder , lanes 2,10,22 and 25 A pattern; lanes 5,6,9,12,15,17,21,23 and 24 B pattern; lanes 1,3,4,7,11,13,14,16,18,19 and 20 C pattern .DNA sequencing reveal that A,B and C pattern represent A/A, G/G and G/A genotypes respectively, 150v, 24h.<10C°, 15% acrylamide gel.

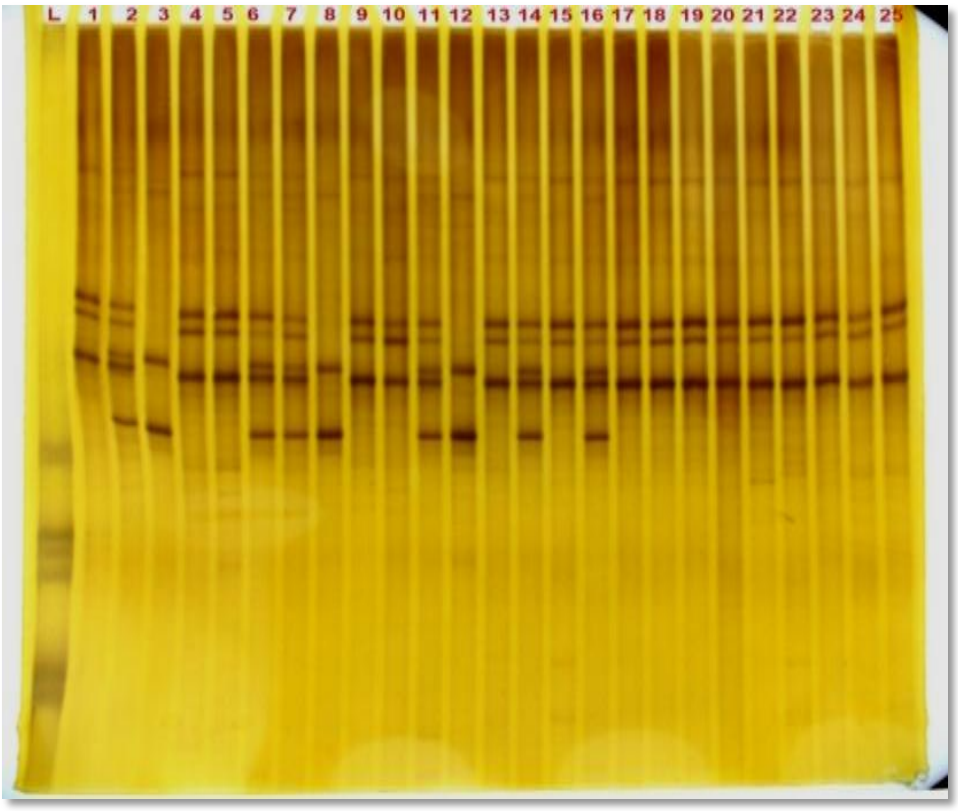


Figure (4.12): Gel electrophoresis of rs13266634-*SLC30A8 gene* by PCR-SSCP technique.
lane L:DNA ladder ; lanes 3,8,and 12 A pattern; lanes 1,4,5,9,10,13,15,17,18,19,20,21,22,23,24 and 25 B pattern; lanes 2,6,7,11,14 and 16 C pattern .DNA sequencing reveal that A,B and C patterns represent T/T, C/C and C/T genotypes respectively,150v, 24h.<10C°,15%acrylamide gel.

4.3.1.3 Sequencing results of SNPs(rs12255372-*TCF7L2gene*) and (rs13266634 - *SLC30A8 gene*)

The results of the sequencing analysis for the single nucleotide polymorphism rs13266634 showed the presence of the genotype (C/T) heterozygous, homozygous (T/T), homozygous (C/C).

As for the single nucleotide polymorphism rs12255372, the results of the sequencing analysis showed the presence of heterozygous (G/A), homozygous (G/G), and homozygous (A/A), moreover recording the appearance of the allele (A) in Iraqis as a substitute for the minor allele (T) according to sequence analysis, figure (4.14).

In the present study, the *SLC30A8* and *TCF7L2 gene* sequences in chromo-somes 8 and 10 respectively. Concerning the targeted rs13266634 SNP within the

SLC30A8gene, the alignment results of the 230 bp samples confirmed the polymerphism of this variant in the analyzed samples in comparison with the referring reference DNA sequences. In this SNP, cytosine is replaced with thymine in the 75th position of the amplified fragment, namely75C>T, figure (4.13A). Concerning the targeted rs12255372 SNP within the *TCF7L2gene*, the alignment results of the 272 bp samples confirmed the polymorphism of this variant in the analyzed samples in comparison with the referring reference DNA sequences. In this SNP, guanine is replaced with adenine in the 107th position of the amplified fragment, namely 107G>A, figure (4.13B). The sequencing chromatogram of the identified variations, as well as their detailed annotations, were documented, and the chromatogram of this sequence was shown according to its position in the PCR amplicon.

Concerning the targeted rs13266634 SNP within the *SLC30A8gene*, one nucleic acid substitution was confirmed in the targeted locus.

The observed substitution SNP wasfound in a heterozygous (C/T) form in S1 and S2, a homozygous (T/T) form in S3 and S4, and another homozygous (C/C) form in both S5 and S6 samples, figure (4.14A).

The observed SNP was documented well in its corresponding position in the chromatogram, and the absence of any possible technical error was confirmed in the analyzed sequencing files.

Concerning the targeted rs12255372SNP within the *TCF7L2 gene*, one nucleic acid substitution was confirmed in the targeted locus. The observed substitution SNP was found in a heterozygous (G/A) form in S1 and S2, a homozygous (G/G) form in S3 and S4, and another homozygous (A/A) form in both S5 and S6 samples , figure (4.14 B).

The observed SNP was also documented well in its corresponding position in the chromatogram, and the absence of any possible technical error was confirmed in the analyzed sequencing files.

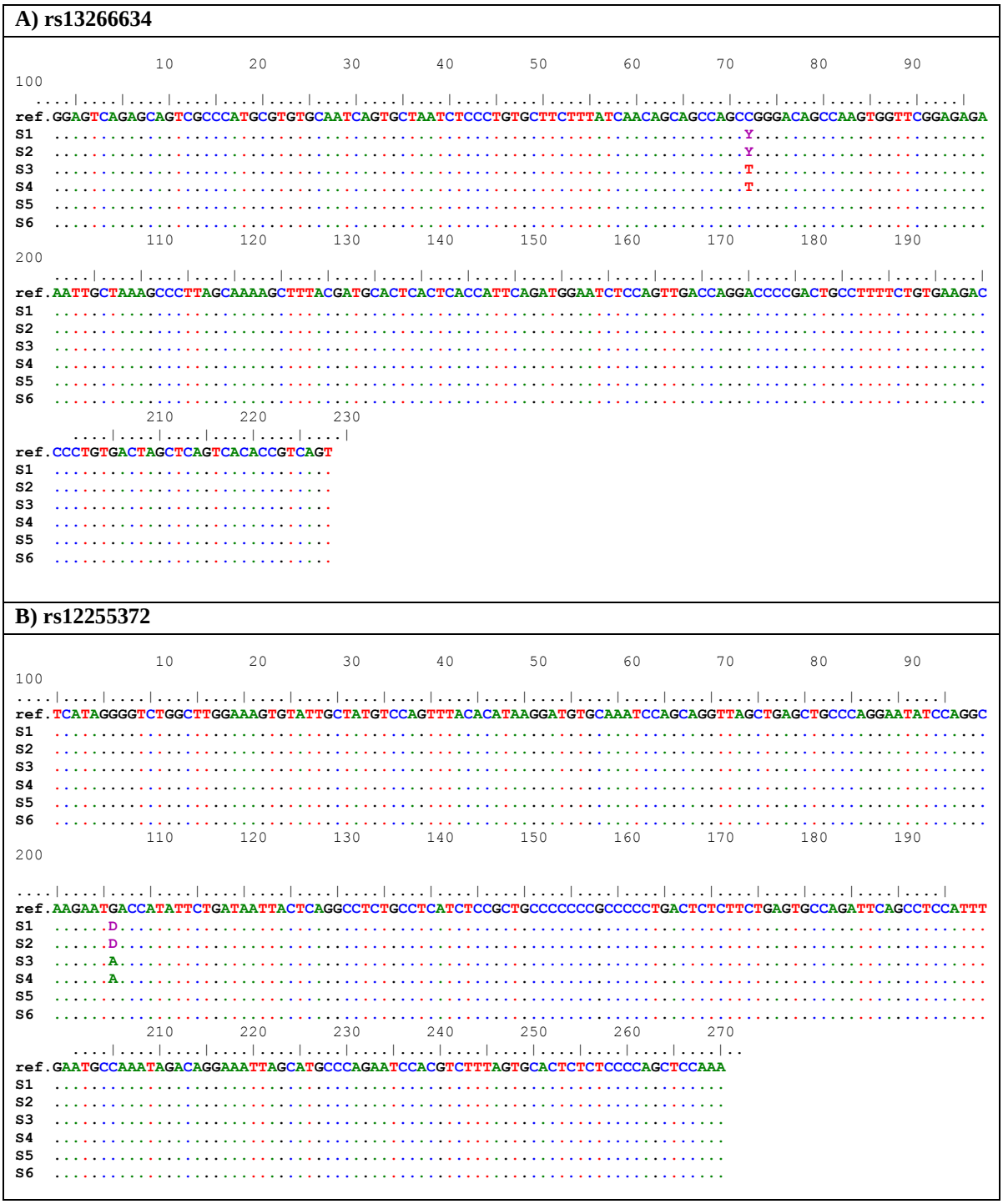


Figure (4.13): DNA sequence alignment of six genotyped samples with their corresponding reference sequences of the *SLC30A8* and *TCF7L2 gene*.
as shown in branches A and B, respectively. The symbol "ref." refers to the NCBI referring sequence, and "S1-S6" refers to the genotyped samples 1 to 6, respectively.

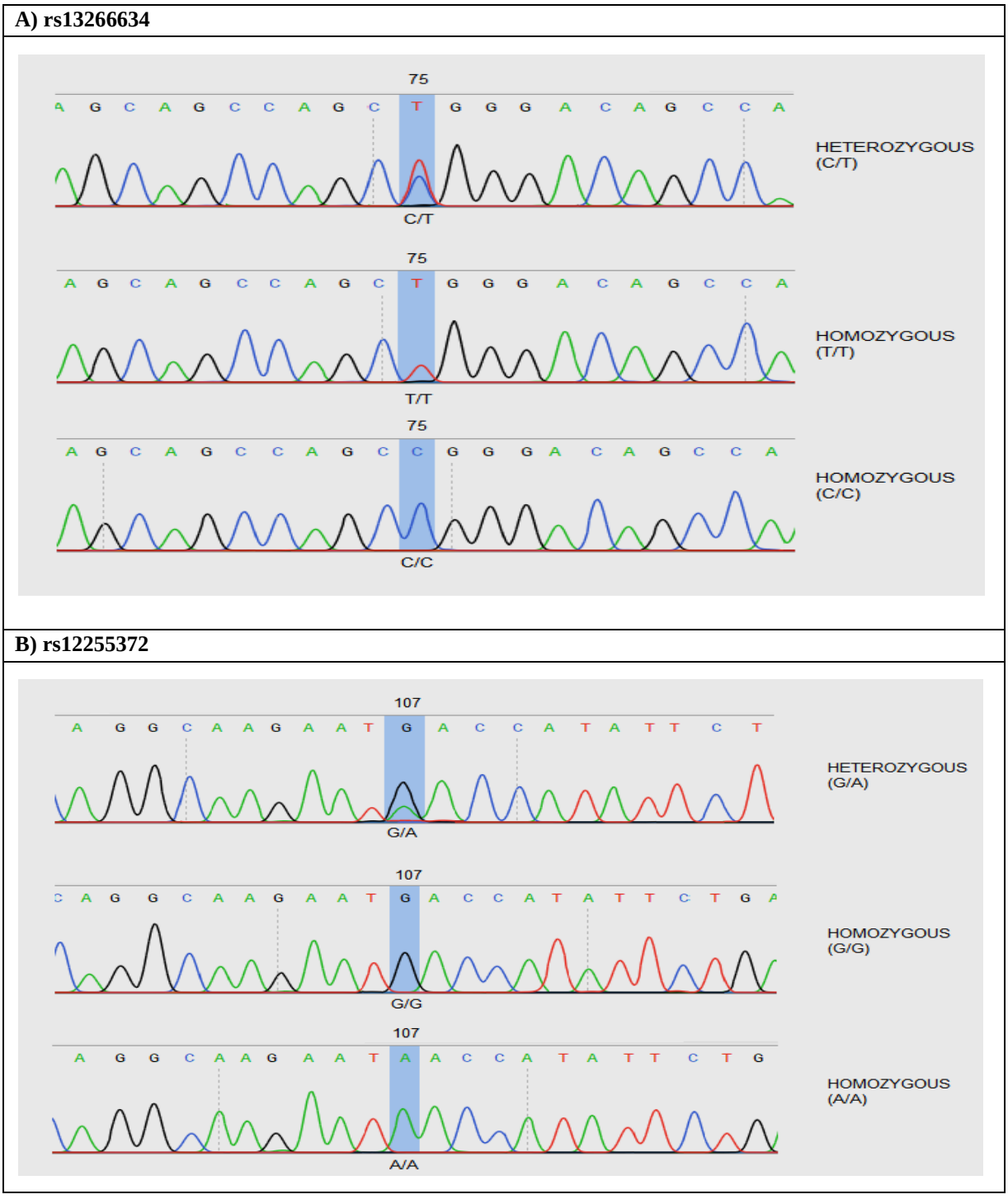


Figure (4.14): The pattern of the detected SNPs within the DNA chromatogram of the *SLC30A8* and *TCF7L2 gene* as shown in branches A and B, respectively.

The identified SNPs were highlighted according to their positions in the PCR amplicons.

Concerning the targeted rs13266634 SNP within the *SLC30A8gene*, the position of the targeted SNP concerning its deposited database of the sequenced 230 bp fragment was retrieved from the dbSNP server (https:// www. ncbi. nlm. nih. gov/ projects/SNP/).

To find out the details of this detected SNP, a graphical representation was performed concerning the *SLC30A8* dbSNP database within chromosome 8 (Gen Bank Acc. No. NC_000008.11.figure(4.15 A). By reviewing the dbSNP engine, it was found that the detected SNPswere found to be deposited with a relatively high frequency within the exon-12 of the *SLC30A8gene*. This is due to the deposited frequency of the allele T, which was accounted to be 0.290653 (https:// www. ncbi. nlm.nih.gov/snp/rs13266634).

However, this SNP was reported in many publications regarding the potential effect of the rs13266634 SNP locus on many variations in different metabolic syndromes, such as diabetic Mellitus type-1 and type-2, Hyperglycemia, and fasting plasma glucose and lipid levels and many other disorders in various metabolic dysfunctions worldwide (https:// www.ncbi. nlm.nih.gov/ snp/ rs1326 6634 #publications).

Concerning the targeted rs12255372 SNP within the *TCF7L2*gene, the position of the targeted SNP concerning its deposited database of the sequenced 272 bp fragment was retrieved from the dbSNP server. To find out the details of this detected SNP, a graphical representation was performed concerning the TCF7L2 dbSNP database within chromosome 10 (GenBank Acc. No. NC_000010.11) figure (4.15, B).

By reviewing the dbSNP engine, it was found that the detected SNPs were found to be deposited with a relatively high frequency within the intronal sequences of the *TCF7L2gene*. This is due to the deposited of the relatively high frequency of the allele T, which was accounted to be 0.284413 (https:// www. ncbi. nlm.nih.gov/snp/rs12255372).

However, this SNP was reported in many publications regarding the potential effect of the rs12255372 SNP locus on many variations in different metabolic syndromes, such as diabetic Mellitus type-II, colorectal cancer, and cardiovascular disease, and many other disorders in various metabolic dysfunctions worldwide (https:// www.ncbi.nlm.nih.gov/snp/rs12255372# publications).

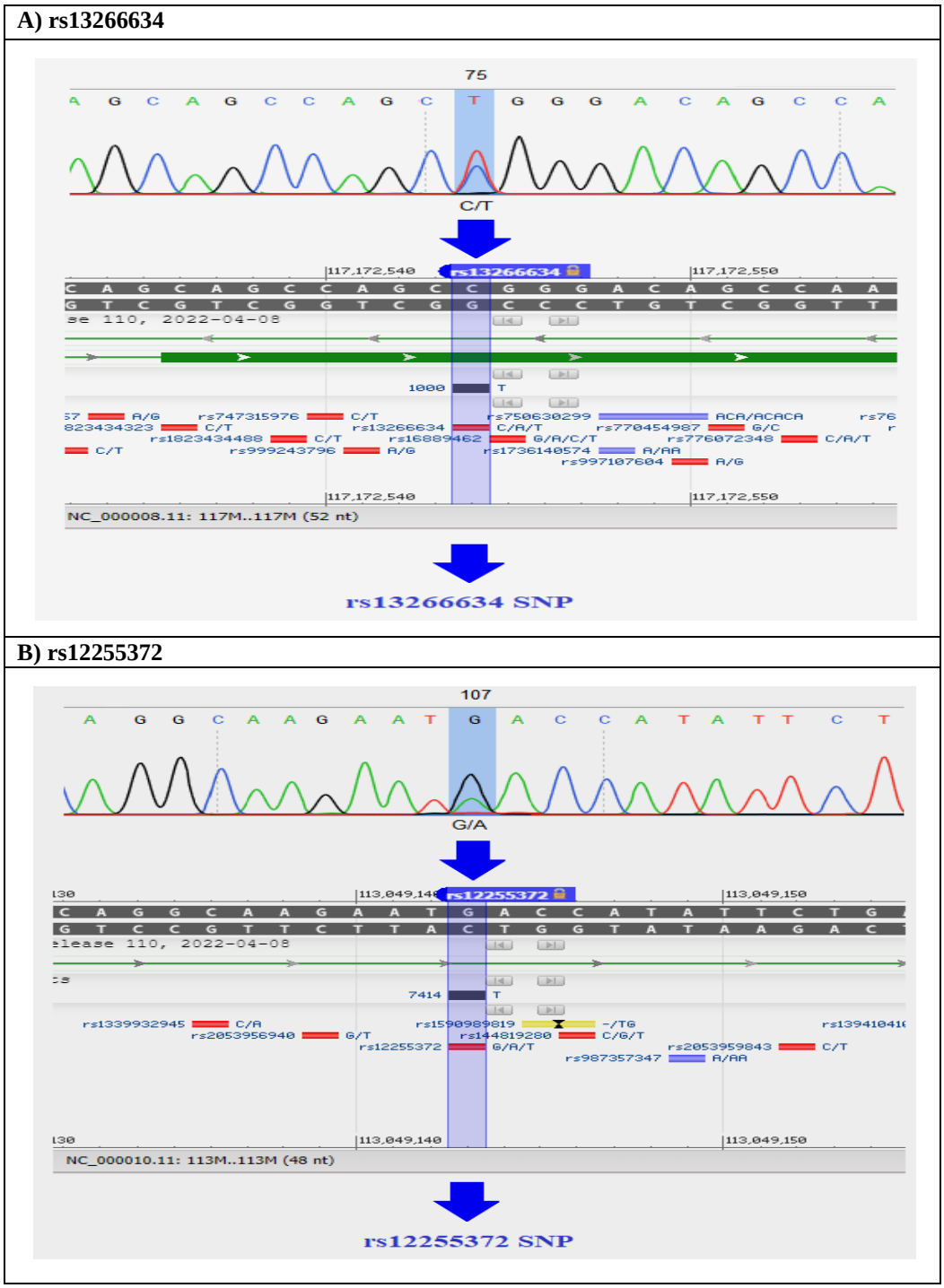


Figure (4.15): SNP's annotations checking of *SLC30A8* and *TCF7L2*genetic single nucleotide polymorphisms using the db SNP server.

The identified known SNPs were marked with a blue color. Gen Bank acc. no. NC_00008.11 and NC_00010.11 was used in the positioning of the highlighted rs13266634 and rs12255372 SNPs in branch A and B, respectively. The positions of the targeted sequences were found in the positive strand.

4.3.1.4 Distribution of alleles and genotypes of (*insulin gene* - rs3842752), (*TCF7L2 gene*- rs12255372), and (*SLC30A8 gene*-rs13266634) SNPs

The results of the genetic analysis in table (4.3) related to the rs3842752-*insulin gene* showed that there was no significant difference (p> 0.05) between genotypes (G/G) (G/A) (A/A) and between alleles (G,A) (P> 0.0). In addition, the study group appeared within Hardy-Weinberg equilibrium (p> 0.05). On the other hand, the results of genetic analysis in table (4.4) did not show a significant difference (p > 0.05) between the genotypes (G/G) (A/G) (A/A) nor between the two alleles (A and G) (p >0.05) in the rs12255372-*TCF7L2gene*, whereas the study group was within Hardy-Weinberg equilibrium (p > 0.05).

Table(4.3): Distribution of alleles and genotypes in *insulin gene*- rs3842752 SNP

Model	Genotype	ND		T2DM		OR (95% CI)	P-value
Codominant	G/G	18 (52.9%)		27 (50%)		1.00	0.83
	G/A	13 (38.2%)		20 (37%)		1.03 (0.41-2.57)	
	A/A	3 (8.8%)		7 (13%)		1.56 (0.35-6.82)	
Dominant	G/G	18 (52.9%)		27 (50%)		1.00	0.79
	G/A-A/A	16 (47.1%)		27 (50%)		1.12 (0.48-2.66)	
Recessive	G/G-G/A	31 (91.2%)		47 (87%)		1.00	0.55
	A/A	3 (8.8%)		7 (13%)		1.54 (0.37-6.41)	
Overdominant	G/G-A/A	21 (61.8%)		34 (63%)		1.00	0.91
	G/A	13 (38.2%)		20 (37%)		0.95 (0.39-2.30)	
		ND		T2DM		OR (95% CI)	P-value
Allele		Count	Proportion	Count	Proportion		
G		49	0.72	74	0.69	0.84 (0.43-1.65)	0.62
A		19	0.28	34	0.31	1.19 (0.61-2.31)	
(*Insulin gene*- rs3842752 SNP) exact test for Hardy-Weinberg equilibrium (n=88)							
		GG	GA	AA	G	A	P-value
All subjects		45	33	10	123	53	0.31
ND		18	13	3	49	19	0.69
T2DM		27	20	7	74	34	0.34

Abbreviations : T2DM: type II diabetes millets, ND: non-diabetes, OR: odds ratio

Table(4.4): Distribution of alleles and genotypes in the *TcF7L2 gene* -rs12255372 SNP

Model	Genotype	ND		T2DM		OR (95% CI)	P-value
Codominant	G/G	13 (38.2%)		12 (22.2%)		1.00	0.15
	G/A	15 (44.1%)		35 (64.8%)		2.53 (0.94-6.81)	
	A/A	6 (17.6%)		7 (13%)		1.26 (0.33-4.84)	
Dominant	G/G	13 (38.2%)		12 (22.2%)		1.00	0.11
	G/A-A/A	21 (61.8%)		42 (77.8%)		2.17 (0.84-5.57)	
Recessive	G/G-G/A	28 (82.3%)		47 (87%)		1.00	0.55
	A/A	6 (17.6%)		7 (13%)		0.70 (0.21-2.28)	
Overdominant	G/G-A/A	19 (55.9%)		19 (35.2%)		1.00	0.056
	G/A	15 (44.1%)		35 (64.8%)		2.33 (0.97-5.61)	
		ND		T2DM		OR (95% CI)	P-value
Allele		Count	Proportion	Count	Proportion		
G		41	0.6	59	0.55	0.79 (0.43-1.47)	0.46
A		27	0.4	49	0.45	1.26 (0.68-2.34	
(*TcF7L2 gene*-rs12255372 SNP) exact test for Hardy-Weinberg equilibrium (n=88)							
		GG	GA	AA	G	A	P-value
All subjects		25	50	13	100	76	0.19
ND		13	15	6	41	27	0.72
T2DM		12	35	7	59	49	0.052

Abbreviations: T2DM: type II diabetes millets, ND: non-diabetics(control group),OR: odds ratio

Table(4.5): Distribution of alleles and genotypes in the *SLC30A8gene*- rs13266634 SNP

Model	Genotype	ND		T2DM		OR (95% CI)	P-value
Codominant	C/C	20 (58.8%)		27 (50%)		1.00	0.44
	C/T	8 (23.5%)		11 (20.4%)		1.02 (0.35-3.00)	
	T/T	6 (17.6%)		16 (29.6%)		1.98 (0.66-5.95)	
Dominant	C/C	20 (58.8%)		27 (50%)		1.00	0.42
	C/T-T/T	14 (41.2%)		27 (50%)		1.43 (0.60-3.40)	
Recessive	C/C-C/T	28 (82.3%)		38 (70.4%)		1.00	0.2
	T/T	6 (17.6%)		16 (29.6%)		1.96 (0.68-5.66)	
Overdominant	C/C-T/T	26 (76.5%)		43 (79.6%)		1.00	0.73
	C/T	8 (23.5%)		11 (20.4%)		0.83 (0.30-2.34)	
		ND		T2DM		OR (95% CI)	P-value
Allele		Count	Proportion	Count	Proportion		
C		48	0.71	65	0.6	0.63 (0.33-1.2)	0.1624
T		20	0.29	43	0.4	1.59 (0.83-3.04)	
(*SLC30A8gene*- rs13266634 SNP)exact test for Hardy-Weinberg equilibrium (n=88)							
		CC	CT	TT	C	T	P-value
All subjects		47	19	22	113	63	<0.0001
ND		20	8	6	48	20	0.014
T2DM		27	11	16	65	43	<0.0001

Abbreviations:T2DM: type II diabetes millets, ND: non-diabetics(control group),OR: odds ratio

With regard to the comparison of insulin level between the three genotypes in the studied genes, the current results showed in table (4.6) that there was no signi-fycant difference (p>0.05) for insulin levels between the genotypes carriers G/G, G/A and A/A for SNP rs3842752G>A- *insulin gene*.

Also, there was no significant difference (p > 0.05) for insulin levels between the genotypes carriers G/G, G/A, A/A for SNP rs12255372 G>T in *TcF7L2 gene*.

In addition ,The results of the current study did not show a significant difference (p > 0.05) for insulin levels between the genotype carriers C/C, C/T, T/T for rs13266634T>C in *SLC30A8 gene* with insulin levels.

Table(4.6): Comparison of insulin levels among genotype carriers of T2DM group

Insulin gene - rs3842752G>A SNP			
Genotype	N	Insulin level (MIU/ml) (Mean ± SE)	P-value
GG	27	3.481± .988	0.390
GA	20	4.411±.969	
AA	7	3.765±2.568	
TCF7L2 gene -rs12255372 G>T SNP			
Genotype	N	Insulin level (MIU/ml) (Mean ± SE)	P-value
GG	12	7.257±2.302	0.809
GA	35	2.899±.560	
AA	7	2.857±1.366	
SLC30A8 gene- rs13266634T>C SNP			
Genotype	N	Insulin level (MIU/ml) (Mean ± SE)	P-value
CC	27	4.107±1.008	0.580
CT	11	3.99±1.581	
TT	16	3.361±1.181	
T2DM (n)	54		

Abbreviations: insulin levels has been displayed as the mean ± SE, N: number,T2DM: type II diabetes millets, p≤0.05 using Kruskal Wallis test.

Some studies related to determinie the role of the SNP rs3842752 in the *insulin gene* showed that it was not associated with type II diabetes (Cervin *et al*., 2008 ; Massarenti's *et al.*, 2022 and Cook *et al*., 2023).

Moreover, Meigs *et al*. (2008) indicated that the presence of this rare SNP in (3' UTR) mRNA sequences may be the reason why it did not affect mRNA translation and protein production.

However, the results of the current study did not show a relationship of the rs3842752G>A SNP with type II diabetes, and insulin level were not affected by the inheritance of the allele (G or A), which indicates that there is no functional role for this SNP in the occurrence of type II diabetes or in the insulin production process. This opinion may support the presence of this SNP In the (3'UTR) mRNA region, which is removed later to produce pro-insulin protein. Anyway, these results need future studies to support them and to clarify the functional role of this SNP in Iraqis.

The results related to the sequence analysis of rs12255372 in *TCF7L2 gene* and the chromatographic report, figure (4.14) showed that the alternative allele of rs12255372is (A) in Iraqis instead of the alternative allele (T) which previously appeared in several countries and races, which had a role in the susceptibility to diabetes type II (Xi & Ma, 2020).

The replacement of thiamine with adenosine in the results of the current study needs other studies to clarify the effect of this replacement on the process of expressing transcription factor TCF7L2 among Iraqis. It may be preferable to investigate this exchange among Iraqis from other cities in Iraq, or perhaps according to nationalities (Arabs, Kurds, Turkmen).

The absence of a significant difference in the frequency of alleles (G) and (A) and the distribution of genotypes between the two study groups and its association with the susceptibility to diabetes and its lack of influence on insulin levels among the genotypes carriers may indicate that the occurrence of adenosine substitution changed the function of rs12255372 in *TCF7L2 gene*, and it did not appear It has an effect on the expression and production of transcription factor TCF7L2in Iraqi type II diabetic patients.

However, more studies on this allele (A) and with a relatively larger number of participants may clarify the importance and role of the allele (A) in Iraqis. It is noteworthy that the sample size in genetic studies may have an influence on the

results of genetic analyses (Politi *et al.*, 2023), where the sample size was significantly larger in the studies that showed association of rs12255372SNP as a risk factor for type II diabetes (Acharya *et al*., 2015; Ibrahim *et al*., 2015; Yüzbaşıoğulları *et al*., 2020 and Elhourch *et al*., 2021) than in our sample size.

Finally, with regard to the rs13266634 SNP in the *SLC30A8 gene*, although there was no significant difference between the frequency of (C) and (T) alleles or in the distribution of genotypes between the two study groups, and there was no significant difference in insulin levels between the carriers of the genotypes. But both study groups were within Hardy-Weinberg equilibrium (P<0.05), which may indicate the relationship of the (C) allele with type II diabetes among Iraqis.

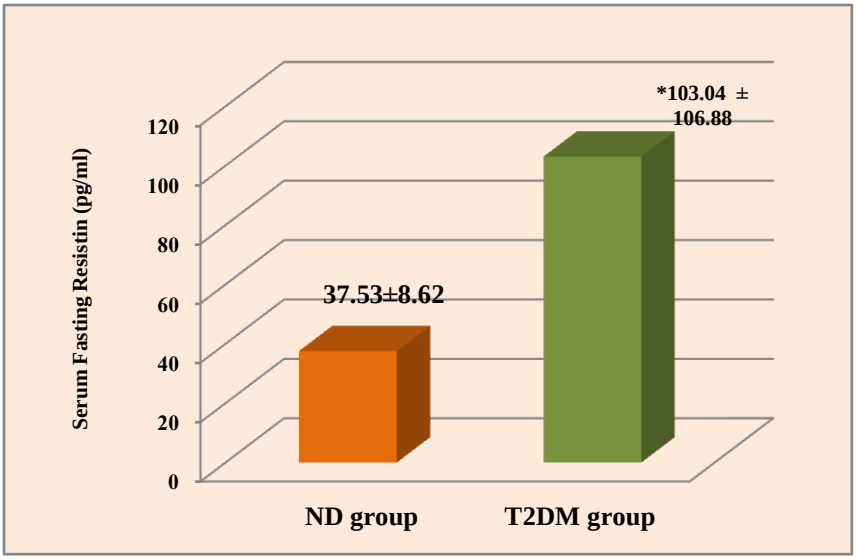
In some previous studies the (C) allele at the rs13266634 SNP has been associated with susceptibility to type II diabetes (Xu *et al*., 2011; Cheng *et al*., 2015and Fan *et al*., 2016). However, the number of participants in these studies was relatively larger than what is in the current study. Therefore, additional studies with larger participants may be needed to explain the relationship of this SNP to insulin levels and susceptibility to type II diabetes among Iraqis.

4.4 Comparison and correlation of resistin level in serum and saliva between T2DM and ND groups

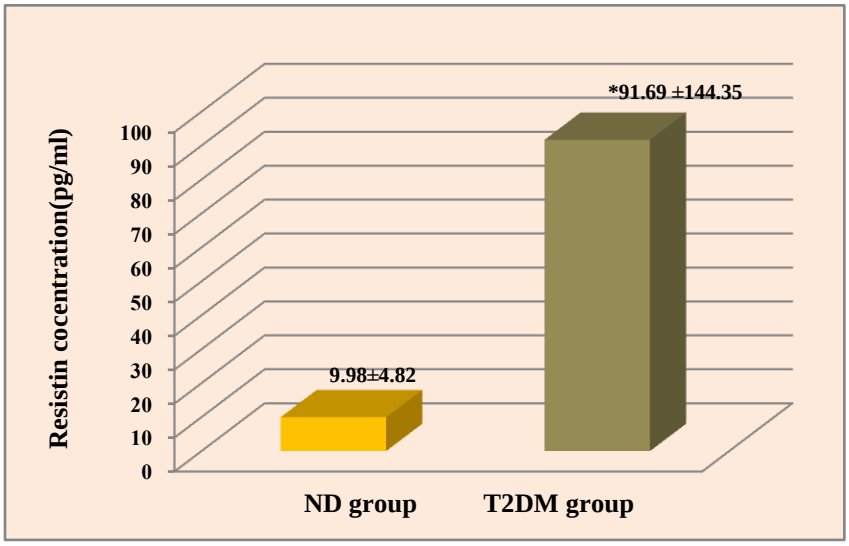
The results of the current study showed a significant increase ($p < 0.05$) in serum fasting resistin levels between T2DM group (103.04±106.88 pg/ml) and ND group (37.53±8.621pg/ml) figure (4.16).

Moreover, the current study figure (4.17) showed that there was a significant increase ($p<0.05$) in the salivary fasting resistin level between T2DM group (91.69 ± 144.35pg/ml) and ND group (9.98 ± 4.82 pg/ml) at ($p \leq 0.05$).

On the other hand, the correlation analysis, table (4.7) showed that no correlation between serum and saliva fasting resistin in T2DM group.



Figure(4.16): Comparison of serum fasting resistin level between T2DM and DN groups
Abbreviations: Data are presented as the mean ±SD, T2DM: type II diabetes millets, ND: non-diabetics, significant increase noted by(*), p≤0.05 - Mann-Whitney test.



Figure(4.17):Comparison of Saliva fasting resistin levels.
Abbreviations: Data are presented as the mean and ±SD, T2DM: type II diabetes millets, ND: non-diabetics, significant increase noted by(*) , p≤0.05 - Mann-Whitney test.

Table(4.7): Correlation between serum and saliva resistin levels inT2DM group

T2DM		
		Serum resistin (pg/ml)
Saliva resistin (pg/ml)	r	- 0.043
	p	0.759
N		54

Abbreviations : Correlation by Spearman's rank (2-tailed) , T2DM: type II diabetes millets, r :correlation coefficient, p:p-value, N:number

In the current study, T2DM group had higher values in body mass index, waist circumference, and neck circumference, which indicated the presence of obesity compared to the control group. They also had higher levels of glucose and insulin in serum and saliva compared to the control group, which may indicate the presence of insulin resistance.

This indicates that the increase in visceral fat in the abdominal region and the chronic elevation of blood glucose levels, as well as the elevation of insulin, greatly enhance the inflammatory state and thus increase the levels of resistin, which competes with insulin for insulin receptors in the target cells, thus increasing the state of insulin resistance, the common condition in patients with type II diabetes.

These opinions are consistent with study of Choudry *et al*. (2015), that patients with type II diabetes often suffer from insulin resistance and obesity, which are associated with increased levels of inflammatory factors. And with the study of Stan *et al*.(2011) in that the expression of resistin increases with hyperglycemia. As well as, with the study of Takata *et al*. (2008), which indicated that the level of resistin in the blood is always high in patients with type II diabetes who suffer from obesity.

Moreover, the current results are consistent with the results of Rathwa *et al*. (2019) in the elevation of fasting glucose and resistin levels in the serum and saliva of the T2DM group, and with the results of Choudhry *et al*.(2020), Srinivasan *et al*.(2018) and Yin *et al*. (2012), that related to elevated resistin level in serum and saliva of type II diabetes mellitus group compared to the control group.

On the other hand, the absence of a correlation in the levels of resistin between blood and saliva in the T2DM group may be attributed to the changes that occur in the tissues of the salivary glands due to chronic high levels of glucose in the blood, which may change the permeability of these tissues, allowing the passage of some proteins and other substances, or Perhaps because the blood takes some time to reach the salivary glands.

However, this result is not consistent with that of Choudhry *et al*. (2020) that showed a strong correlation in resistin levels between the serum and saliva of a group of type II diabetic patients. But it is consistent with other previous studies (Loo *et al*., 2010; Zhang *et al*., 2010 and Williamson *et al*., 2012), that indicated the possible mechanism for the increased passage of proteins and other metabolites from the exocrine glands, in addition to the enhanced extravasation of serum components into saliva. It may be due to the increase in basement membrane permeability of the salivary glands that is often associated with diabetes.

It also agrees with other studies (Furugen *et al*., 2010 and Devanoorkar *et al*., 2014) that referred to the contribution of macrophage in the oral cavity, may increases the secretion of resistin and its excretion in saliva. Or, may due to an immune response against lipopolysaccharides of the gram positive bacteria wall that are common in the oral cavity of obese people with type II diabetes and non-diabetics (Al-Rawi & Al-Marzooq, 2017).

4.4.1 Genotyping of rs1862513 SNP in *resistin gene*

Genotype analysis of rs1862513 SNP in *resistin gene* using PCR-RFLP, figure (4.18) showed that the size of the DNA segment 245bp represents the (G) allele, while the allele (C) is represented by the two segments 124 bp and 121bp.

Accordingly, the appearance of the 245bp DNA segment in electrophoresis represents homozygote (G/G), and segments (245+124+121) bp represent heterozygote (G/C), while for homozygote (C/C), they are represented by segments (124 +121) bp.

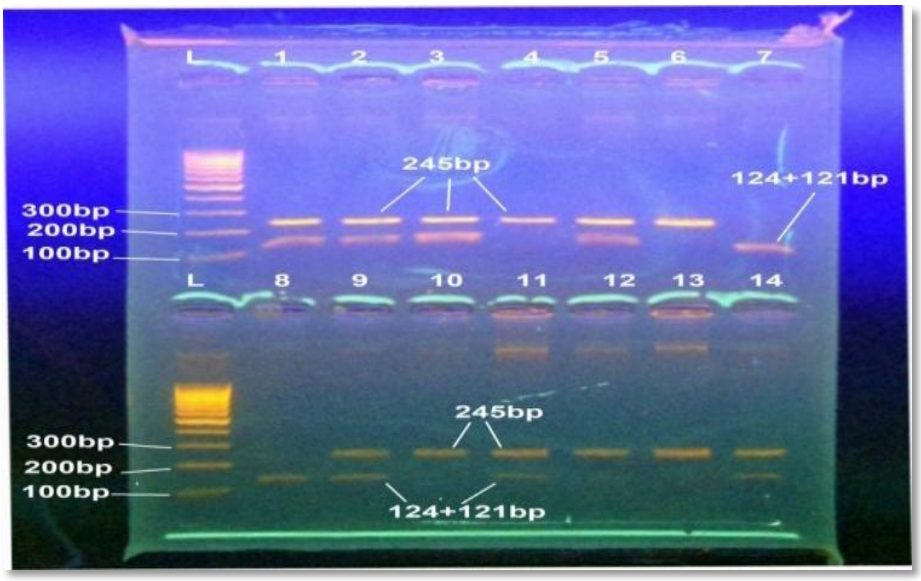


Figure (4.18):Gel electrophoresis of rs1862513SNP in *resistin gene* and genotyping by PCR-RFLP.
lanes L DNA ladder ; lanes 7,8 C/C genotype ; lanes 1,2,3,5,9,11 and 14 G/C genotype; lanes 4,6,10,12 and 13 G/G genotype, 100V , 50mA-1h. , 2%Agarose.

4.4.2 Distribution of alleles and genotypes ofrs1862513SNP in *resistin gene*

The results of the current study table (4.8) showed that the genotype (C/C) was increased by 8 times in the T2DM group, compared to the ND group, OR = 8.00, CI (1.74-36.71), P= 0.014. Moreover, the frequency of the allele (C) was twice as high as the allele (G) OR=2.32, CI(1.24-4.32), P= 0.008, which indicates that it is a risk factor for Iraqis to be susceptible to type II diabetes.

Whereas the allele (G) represents a protective factor against type II diabetes, OR = 0.43, CI (0.23-0.8), P= 0.008, moreover the study group was within Hardy-Weinberg equilibrium ($p > 0.05$).

The current results presented in table (4.9) regarding the comparison of the fasting serum resistin level between the genotypes carriers of the *resistin gene* showed that there was no significant difference for the resistin level between the three genotypes.

Table (4.8): Distribution of alleles and genotypes of SNP rs18 62 513 - *resistin gene*

Model	Genotype	ND N (%)	T2DM N (%)	OR (95% CI)	P-value
Codominant	G/G	12 (35.3%)	8 (14.8%)	1.00	0.014
	C/G	19 (55.9%)	30 (55.6%)	2.37 (0.82-6.86)	
	C/C	3 (8.8%)	16 (29.6%)	8.00 (1.74-36.71)	
Dominant	G/G	12 (35.3%)	8 (14.8%)	1.00	0.027
	C/G-C/C	22 (64.7%)	46 (85.2%)	3.14 (1.12-8.77)	
Recessive	G/G-C/G	31 (91.2%)	38 (70.4%)	1.00	0.015
	C/C	3 (8.8%)	16 (29.6%)	4.35 (1.16-16.31)	
Allele					
G		43(0.63)	46(0.43)	0.43(0.23-0.8)	0.008
C		25(0.37)	62(0.57)	2.32(1.24-4.32)	

(*Resistin gene* -rs1862513 SNP) exact test for Hardy-Weinberg equilibrium (n=88)

	GG	CG	CC	G	C	P-value
All subjects	20	49	19	89	87	0.39
ND	12	19	3	43	25	0.46
T2DM	8	30	16	46	62	0.41

Abbreviations: T2DM: type II diabetes millets group, ND: non-diabetics, OR: Odds ratio, SNP: single nucleotide polymorphism, n: number.

Table (4.9): Comparison of fasting serum resistin level among rs1862513 genotypes

Genotypes of rs1862513	No.	Serum fasting resistin (pg/ml)	Mean rank	P-value
GG	8	59.87±25.735	20.0	0.186
CG	30	121.324±21.334	30.68	
CC	16	90.347±24.57	25.28	
Total	54			

Abbreviations : Data are expressed as mean ±SE and mean rank, ($p \leq 0.05$) Kruskal Wallis test, No.= number.

There are several genetic variants that affect the expression of resistin (Wang *et al*., 2017). With regard to SNP (rs1862513-Resistin Gene), the odds ratio analysis showed that the allele (C) is a risk factor while the allele (G) is a protective factor among the study group.

Although there was no significant difference in insulin concentrations in the three genotypes, but the average insulin levels (Table 4.9) in the genotypes (C/G and C/C) seemed significantly higher than it was in the genotype (G/G), which indicates the risk of the allele (C) may lie in the increased expression of resistin

and thus the increased production that causes insulin resistance as a common characteristic of type II diabetes.

However, previous studies have shown that the genotype (G/G) is associated with increased resistin production and susceptibility to type II diabetes, as in Japan (Osawa *et al*., 2004), in Iranian women (Takhshid & Zare, 2015), and in Indians (Rathwa*et al*., 2019), but not in the Italians and Caucasians (Menzaghi *et al*., 2006 ; Hivert *et al*., 2009).

The difference in the results between studies related to (rs1862513) SNP may be due to genetic differences between races (Wang *et al*., 2017). Or to the conditions of the study itself, for example, the number of participants in the study is too small to give a realer image of the distribution of genotypes among the population (Politi *et al*., 2023).

Therefore, the results of this study may need additional studies in which the participation of a larger number of Iraqis from Baghdad governorate and perhaps from other Iraqi cities (Taking into account the ethnic diversity) to clarify the role of each allele (G) and allele (C) in the susceptibility to type II diabetes among Iraqis.

4.5 Comparison and correlation of visfatin concentration in serum and saliva between T2DM and ND groups

The results of the current study related to vistatin are shown in figure (4.19), a significant increase ($P<0.05$) in the fasting serum visfatin level between T2DM group (14.19± 4.52 ng/ml) and ND control group (2.19± 1.18 ng/ml).

Moreover, the results showed in figure (4.20), a significant difference ($P<0.05$) in the level of fasting salivary visfatin between T2DM group (0.99± 0.60 ng/ml) and ND group (0.69±0.53 ng/ml)at ($p\leq0.05$).

In addition, the results of the correlation analysis, figure (4.21) showed that there was a medium positive significant correlation ($r = 0.528$, $p= 0.000$) of fasting visfatin level between serum and saliva in T2DM.

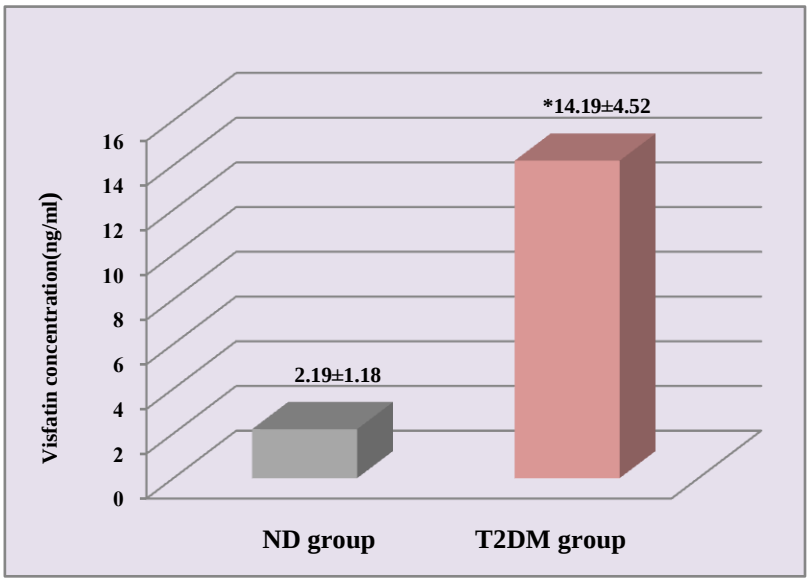


Figure (4.19): Serum fasting visfatin level of T2DM and ND groups.
Abbreviations : Data are presented as the mean ± SD, T2DM: type II diabetes millets, ND: non-diabetics, significant increase noted by(*), ($p \leq 0.05$) Mann-Whitney test.

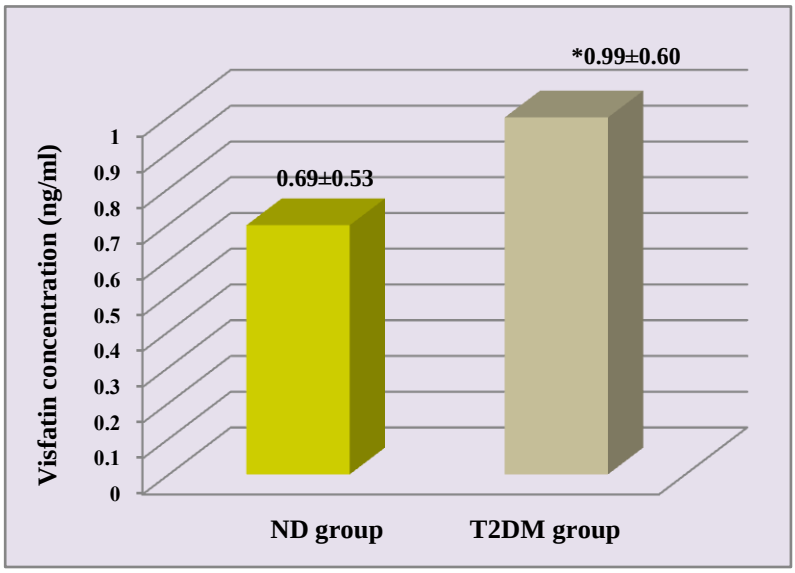


Figure (4.20):Comparison of Saliva fasting visfatin level betweenT2DM and ND groups.
Abbreviations : Data are presented as the mean ±SD, T2DM: type II diabetics millets, ND: non-diabetics, significant increase noted by(*), ($p \leq 0.05$) Mann-Whitney test.

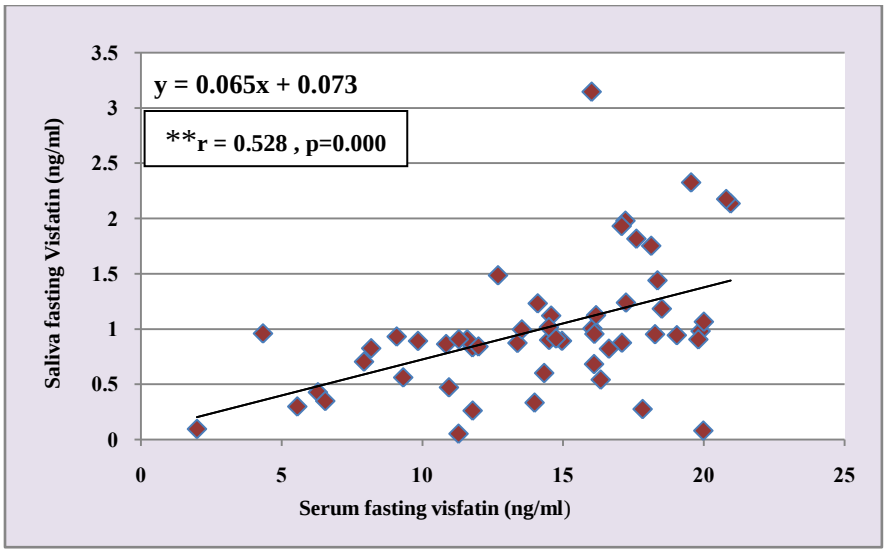


Figure (4.21): Correlation between serum and saliva fasting visfatin in T2DM group.
Abbreviations: ** Correlation is significant at the (0.01 level , Spearman's rho test, T2DM: type II diabetes millets.

The present results regarding the comparison of visfatin levels showed that there was a significant increase of visfatin levels in the serum and saliva of the T2DM group compared to the ND group. Similar results have been shown in previous studies (Stofkova, 2010; Pradeep *et al*., 2012; Abd Rabo *et al*.,2013 and Srinivasan *et al*., 2018).

High values of body circumferences and body mass index, fasting insulin, fasting blood glucose along with high levels of fasting visfatin in the T2DM group in the current study may indicates the strong relationship of visfatin with high weight gain. This is similar to what was reported in the study by Adeghate (2008).

In addition, high blood glucose levels may affect the visfatin signaling pathway with the target cells and thus the production of visfatin is increased as compensation (Chen *et al*., 2006).

Moreover, higher fasting insulin and glucose levels in the serum of T2DM group than ND group in the current study may indicate the presence of insulin resistance, which was correlated positively with an increase in fasting serum visfatin levels as in previous studies conducted by Sandeep *et al*.(2007) and Abd Rabo *et al*.(2013).

Regarding the investigation of the correlation of serum and salivary visfatin levels in the T2DM group in the current study, the results showed that there was a

significant positive correlation of visfatin levels between serum and saliva in the T2DM group, which is consistent with the other previous studies conducted by Abd Rabo *et al*. (2013) and Srinivasan *et al*.(2018). These results may indicate that the measurement of visfatin in saliva can be a substitute for the measurement in serum. Also may indicates that the measurement of visfatin in saliva can be considered a good diagnostic indicator for type II diabetes.

4.5.1 Genotyping of (rs11977021) SNP in *visfatin gene*

The result of determining the genotypes, figure (4.22) of (rs11977021) in *visfatin gene* by PCR-RFLP technique, showed , that the size of DNA segment 307bp represents the (C) allele, and two DNA segments of size (56 and 242) bp represent (T) allele.

Accordingly, the size of DNA segment 307bp represents the genotype (C/C), 242bp represents the genotype (T/T), and finally the DNA segments of size (242 and 307) bp represent the genotype (C/T).

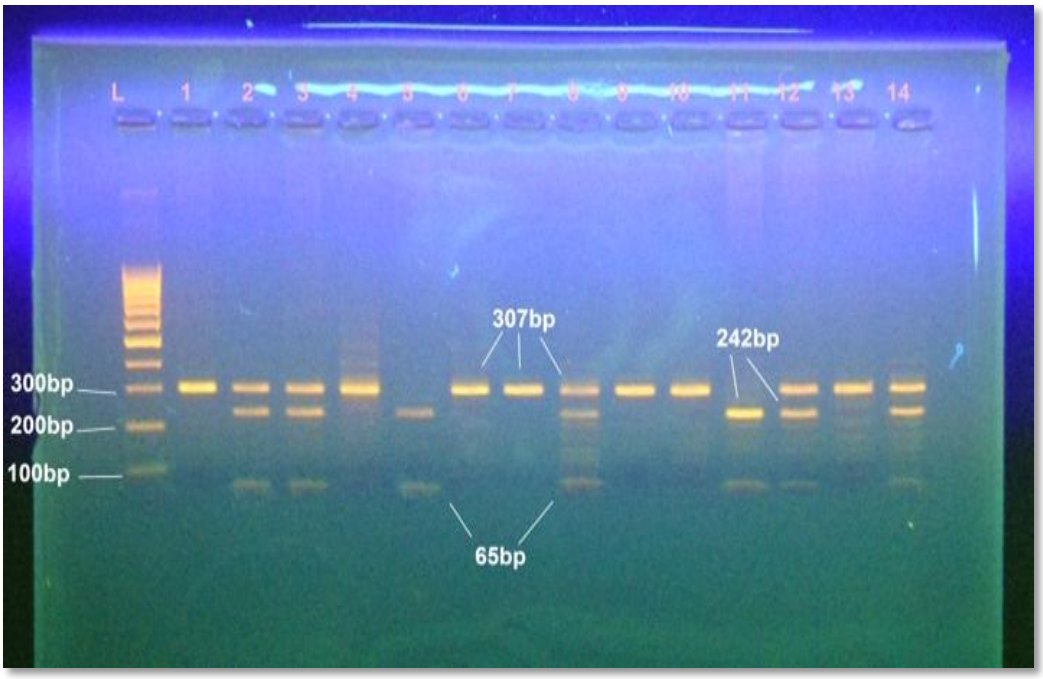


Figure (4.22):Genotyping of rs11977021SNP using PCR-RFLP technique .
lane L: DNA ladder100-1000 bp, lanes 5 and 11 T/T genotype , lanes 1,4,6,7,9,10 and 13 C/C genotype , lanes 2,3,8,12 and 14 C/T genotype, 100V , 50mA-1h. , 2%Agarose.

4.5.2 Distribution of alleles and genotypes of rs11977021 SNP in *visfatin gene.*

With regard to testing the distribution of alleles and genotypes, there was no significant difference ($p > 0.05$) between the three genotypes of all models, table (4.10) in the analysis of the odds ratio between genotypes (C/C), (C/T) and (T/T) for (rs11977021) SNP in *visfatin gene.*

On the other hand, the odds ratio analysis did not show a significant difference ($p=1.000$) ,OR=1 (0.5-2.02) between the two alleles (C and T). Also, no significant difference ($p >0.05$) appeared in the H-WE test, indicating that the study group was within the Hardy-Weinberg equilibrium.

Table(4.10): Distribution of alleles and genotypes of rs11977021 SNP in *visfatin gene.*

Model	Genotype	ND		T2DM		OR (95% CI)	P-value
Codominant	C/C	20 (58.8%)		30 (55.6%)		1.00	0.74
	C/T	11 (32.4%)		21 (38.9%)		1.27 (0.51-3.20)	
	T/T	3 (8.8%)		3 (5.6%)		0.67 (0.12-3.64)	
Dominant	C/C	20 (58.8%)		30 (55.6%)		1.00	0.76
	C/T-T/T	14 (41.2%)		24 (44.4%)		1.14 (0.48-2.72)	
Recessive	C/C-C/T	31 (91.2%)		51 (94.4%)		1.00	0.56
	T/T	3 (8.8%)		3 (5.6%)		0.61 (0.12-3.20)	
Overdominant	C/C-T/T	23 (67.7%)		33 (61.1%)		1.00	0.53
	C/T	11 (32.4%)		21 (38.9%)		1.33 (0.54-3.28)	
		ND		T2DM		OR (95% CI)	P-value
Allele		Count	Proportion	Count	Proportion		
C		51	0.75	81	0.75	1 (0.5-2.02)	1.000
T		17	0.25	27	0.25		
(rs11977021 SNP - *Visfatin gene*) exact test for Hardy-Weinberg equilibrium (n=88)							
		CC		CT	TT	C	T	P-value
All subjects		50		32	6	132	44	0.78
ND		20		11	3	51	17	0.39
T2DM		30		21	3	81	27	1

Abbreviations :T2DM: type II diabetes millets group, ND: non-diabetics, OR: Odds ratio, SNP: single nucleotide polymorphism.

Moreover, no significant difference was shown between the levels, in the results presented in table (4.11) regarding the comparison of visfatin levels among carriers of genotypes (C/C, C/T, T/T) in T2DM group.

Table (4.11): Comparison of fasting serum visfatin level among rs11977021 SNP genotype carriers of T2DM group

Genotype of rs11977021	No.	Fasting Serum visfatin (ng/ml)	Mean Rank	P-value
CC	30	14.23± 0.83	27.77	0.999
CT	21	14.11± 0.98	27.19	
TT	3	14.42±3.42	27.00	
Total	54			

Abbreviations:Data are expressed as mean ±SE , and mean Rank by Kruskal Wallis test at $p \leq 0.05$.

The (rs11977021) SNP is located upstream of the promoter of the *visfatin gene*, which may influence the transcription step of the *visfatin gene*. As In a study of Ooi *et al*. (2016) on obese children, the results indicated the role of this SNP in promoting transcription and thus raising the levels of visfatin.

However, the current results regarding the (rs11977021) SNP did not show an association with type II diabetes, nor did they show an effect on visfatin levels. Which may indicate that there is no role for this SNP in susceptibility to type II diabetes among Iraqis, and it does not affect the expression level and thus the production of visfatin.

As far as we know, few studies have examined this SNP for instance, the study of Martínez Larrad *et al*. (2016) showed that obese (C/C) carriers had high levels of pro-insulin in obese subjects. Also, the study of Bailey *et al*.(2006) showed a strong association of the (rs11977021) SNP with total cholesterol and LDL cholesterol. While the study of Chen *et al*. (2022)among Taiwanese (C/C) carriers showed a high predisposition to oral squamous cell carcinoma.

It is clear from previous studies and the current study that there is a functional role for the (C) allele with obesity and its complications. Perhaps it is possible to clarify this role by conducting future studies on a larger group of diabetic patients and healthy people to confirm the presence or absence of an effect of the (C and T) alleles on the level of visfatin expression and susceptibility to type II diabetes.

4.6 Comparison and correlation of interleukin-18 level in serum and saliva between T2DM and ND groups

The results related to the comparison of Interleukin-18 levels are presented in figure (4.23), as it showed that there was a significant increase (p =0.000) in the level of fasting interleukin-18 in serum between (T2DM) group (219.39 ± 123.89 pg/ml) and (ND) control group (103.92 ± 27.65pg/ml).

Moreover, the results presented in figure (4.24) related to the fasting interleukin -18 levels in the saliva of the T2DM group (16.78±18.33 pg/ml) showed a significant increase (p =0.000) from that of ND group (8.35±6.196 pg/ml).

No correlation was shown (r = 0.014, p= 0.922)for fasting interleukin-18 level between serum and saliva of T2DM group, table (4.12).

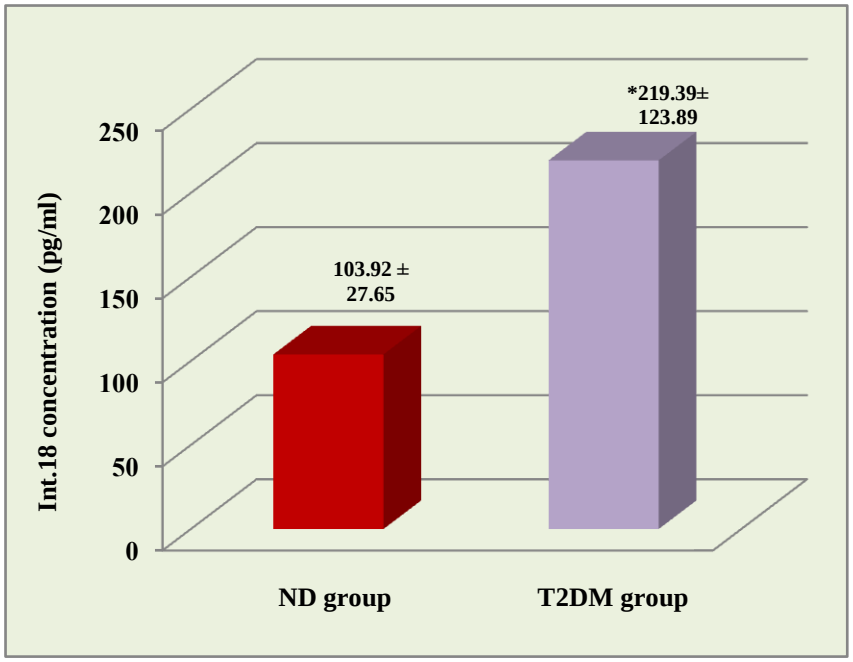


Figure (4.23): Comparison of Serum fasting interleukin-18 levels betweenT2DM and ND groups.
Abbreviations: Data were expressed with mean ±SD, T2DM: type II diabetes millets, ND: non-diabetics, significant increase noted by(*) , (p≤0.05) Mann-Whitney test.

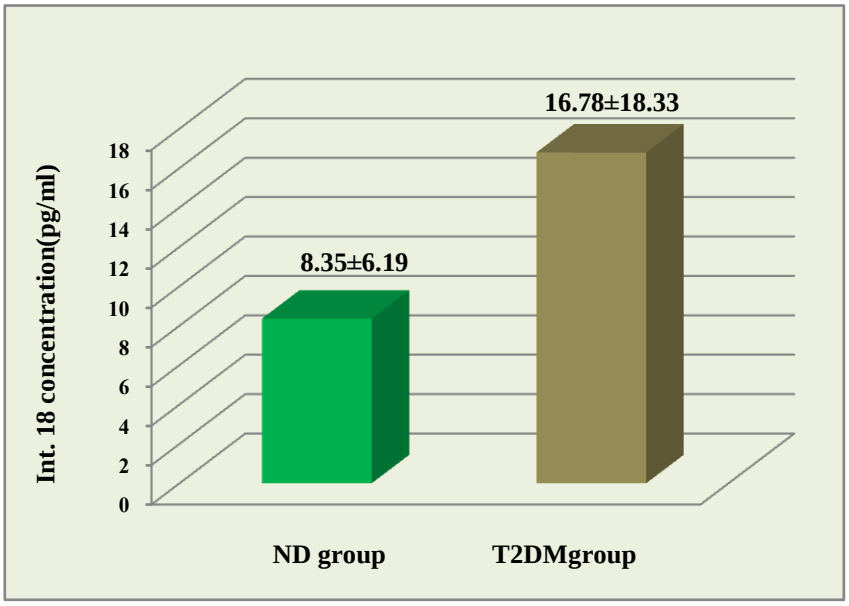


Figure (4.24): Comparison of saliva fasting iInterleukin-18 levles between T2DM and ND group .
Abbreviations: Data were expressed with mean ±SD , significant increase noted by(*), ($p \leq 0.05$) Mann-Whitney test, T2DM: type II diabetes millets, ND: non- diabetics.

Table (4.12): Correlation between serum and saliva fasting interleukin-18 levels inT2DM group.

T2DM		
		Saliva interleukin-18
Serum interleukin-18	r	0.014
	p	0.922
No.	54	

Abbreviations : Correlation by Spearman's rank (2-tailed) , T2DM: type II diabetes millets, r:correlation coefficient, p:p-value.

Many studies have shown the role of inflammation as a cause of diabetes (Wellen & Hotamisligil, 2005; Donath & Shoelson , 2011). In addition several inflammatory cytokines have been shown to have high levels in many clinical complications of diabetes (Nakamura *et al*., 2005;Wong *et al*., 2007 and Jha *et al.*, 2014).

In the current study, the levels of interleukin-18 were higher in the serum of the (T2DM) group, with a higher body mass index and body circumferences indicating obesity, higher fasting glucose, higher fasting insulin levels, than the (ND) group. These results were similar to the findings regarding a high level of interleukin-18

in the serum of a type II diabetes group, conducted by Zaharieva *et al*. (2018), who also reported the association of interleukin-18 with glycemic parameters, inflammation and lipids.

High levels of interleukin-18 were also observed in individuals with metabolic syndrome, which is considered a risk factor for type II diabetes (Troseid *et al*., 2010). Moreover , another study found that the level of interleukin-18 increased in healthy individuals after inducing hyperglycemia and in individuals with impairment in a glucose tolerance test (Esposito *et al*., 2002), also patients with type II diabetes at onset of diagnosis (Aso *et al*., 2003 and Esposito *et al*., 2003).

It should be noted that there are many factors were associated with this increase in interleukin-18 levels, such as resistance to insulin action, lipid profile, fasting glucose and insulin, body mass index, waist circumference, and blood pressure (Aso *et al*., 2003; Hung *et al*., 2005; Fischer *et al*., 2005 and Evans *et al*., 2007).

The current study showed an increase in the levels of interleukin-18 in the saliva of T2DM group compared to the ND group, this finding is not consistent with the study of Techatanawat *et al*. (2020), who found no significant difference for the level of interleukin 18 in the saliva of a group of type 2 diabetes patients (with and without gingivitis) compared to the control group.

On the contrary, Ozcaka *et al*. (2011) found that interleukin-18 was elevated in the saliva of subjects without systemic disease but with chronic gingivitis, compared to healthy subjects without chronic gingivitis, but it didn't rise in the serum.

While another study conducted on patients with chronic gingivitis and people without chronic gingivitis showed an increase in the level of interleukin-18 in plasma in those with chronic gingivitis by 46% compared to healthy subjects, and the increase in Interleukin-18 in saliva was about 5 times higher in the saliva of subjects with chronic gingivitis than in healthy subjects (Banu *et al*., 2015).

It is clear through previous studies and the current study that the levels of interleukin-18 rise as a complication of obesity and chronic high blood glucose in patients with type II diabetes, which is often associated with the occurrence of gum problems of various degrees in patients with diabetes.

Measuring interleukin-18 in saliva may be a challenge because of these complications and may not reflect the concentrations of interleukin-18 in serum, this may explain the lack of correlation between serum and salivary interleukin-18 levels in patients with type II diabetes in the current study.

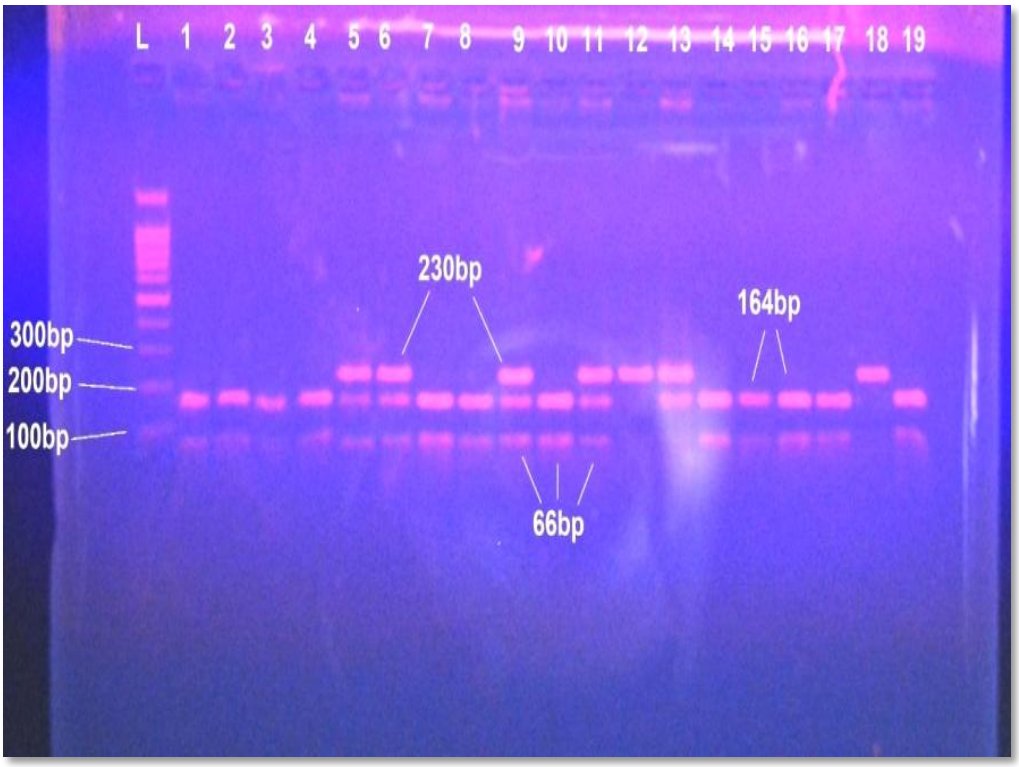
These results are consistent with what Techatanawat *et al*. (2020) found in the absence of a correlation for interleukin-18 levels between serum and saliva. These discrepancies in the results possibly due to the different physiological conditions of the participants, or the difference in the methods used in collecting and analyzing samples, or the statistical methods used in analyzing the data. However, estimating salivary interleukin-18 levels in the type II diabetes group may requires more studies to find a correlation of interleukin -18 levels between serum and saliva.

4.6.1Genotyping of SNP(rs360717) in *interlukin-18 gene*

Genotyping of the (rs360717) SNP - *IL-18gene,* was done by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) technique.

Where it appears in figure (4.25) that the size of the DNA pieces (164 +66 +30) bp represents the (G) allele, while the pieces of size (230 +30) bp represent the allele (A), therefore the appearance of the piece (230) bp alone, represents the genotype (A/A) while, the appearance of the segments (164 + 66) bp represents (G/G).

The genotype (A/G) is represented by the size of the segments (230 +164 + 66 + 30) bp. Note that the size of the original piece of DNA before cutting with the restriction enzyme is (260) bp.



Figure(4.25):Genotyping of rs360717 SNP in interlukin-18gene by PCR-RFLP technique.

Lane L : DNA ladder 100-1000 bp, lanes 1,2,3,4,7,8,10,14,15,16,17 and 19(G/G) genotype ; lanes 5,6,9,11 and 13(G/A) genotype ; lanes 12 and 18 (A/A) genotype , 100V ,50mA-1h. , 2%Agarose.

4.6.2 Distribution of alleles and genotypes of rs360717 SNP in *interleukin-18 gene*

The result of the genetic analysis between the genotypes of (rs360717)SNP in the *interleukin-18 gene* presented in table (4.13) showed that there was no significant difference between the three genotypes (P> 0.05), nor between the alleles (A and G) (P >0.05). While the results showed that the study group was within Hardy-Weinberg equilibrium (P > 0.05).

Moreover, the results of the current study in table (4.14) did not show a significant difference in the levels of interleukin-18 among the carriers of genotypes (A/A, A/G, G/G) (p>0.05) of T2DM group.

Table(4.13):Distribution of alleles and genotypes of rs360717 SNP in *interleukin-18 gene*

Model	Genotype	ND		T2DM		OR (95% CI)	P-value
Codominant	A/A	18 (52.9%)		32 (59.3%)		1.00	0.35
	A/G	12 (35.3%)		20 (37%)		0.94 (0.37-2.35)	
	G/G	4 (11.8%)		2 (3.7%)		0.28 (0.05-1.69)	
Dominant	A/A	18 (52.9%)		32 (59.3%)		1.00	0.56
	A/G-G/G	16 (47.1%)		22 (40.7%)		0.77 (0.33-1.84)	
Recessive	A/A-A/G	30 (88.2%)		52 (96.3%)		1.00	0.15
	G/G	4 (11.8%)		2 (3.7%)		0.29 (0.05-1.67)	
Overdominant	A/A-G/G	22 (64.7%)		34 (63%)		1.00	0.87
	A/G	12 (35.3%)		20 (37%)		1.08 (0.44-2.64)	
		ND		T2DM		OR (95% CI)	P-value
Allele		Count	Proportion	Count	Proportion		
A		48	0.71	84	0.78	1.46 (0.73-2.91)	0.285
G		20	0.29	24	0.22	0.69 (0.34-1.37)	
(rs360717 SNP -*interlukin18 gene*) exact test for Hardy-Weinberg equilibrium (n=88)							
		A/A	A/G	G/G	A	G	P-value
All subjects		50	32	6	132	44	0.78
ND		18	12	4	48	20	0.41
T2DM		32	20	2	84	24	1

Abbreviations :T2DM: type II diabetes millets group , ND: non-diabetics(control group), OR: odds ratio, SNP: single nucleotide polymorphism.

Table (4.14): Comparison of serum fasting interlukin-18 levels among(rs360717) SNP genotype carriers.

Genotype of rs360717	No.	Fasting Serum interlukin-18 (pg/ml)	Mean Rank	P-value
A/A	32	237.73±22.09	29.48	0.416
A/G	20	190.47±28.17	23.60	
G/G	2	215.11±10.57	29.00	
T2DM	54			

Abbreviations : Data are expressed as mean ± SE, and mean Rank by Kruskal Wallis test at $p \leq 0.05$, T2DM: type II diabetes millets group.

The presence of variants in the *interleukin-18 gene*, specifically within the promoter region, may affect the expression and production of cytokines (Barbaux *et al*., 2007 and Pavlovna *et al*., 2008).

The results in table (4.14) indicate that the carriers of the genotype (G/G) are higher in the levels of interleukin-18 despite the difference in the number of carriers of the genotype (G/G) compared to the carriers of the genotypes (A/A and

A/G), which may indicate the role of the allele (G) in increasing the expression of the *interleukin-18 gene*, however the participation of a larger group of patients with type II diabetes and healthy individuals may determine the presence or absence of an effect of the allele (G) in susceptibility to type II diabetes and the effect on increased expression.

The absence of a relationship of the (rs360717)SNP with susceptibility to type II diabetes and its effect on interleukin-18 levels in the current study, may indicate that there is no role or effect for this SNP among Iraqis, or it may have another functional role. However, these results need extensive studies in order to clarifying the role of this SNP among Iraqis.

The SNP (rs360717) has been studied to identify its association with diseases and its effect on the expression and production of IL-18 in many previous studies; as a risk factor in Tunisian women and the women of Bahrain with repeated pregnancy loss due to unknown causes (Al-Khateeb *et al*., 2011 and Messaoudi *et al*., 2012), as a risk factor in acute myocardial infarction (Koch *et al*., 2011), in susceptibility to infection with gastric ulcer bacteria in Koreans (Myung *et al*., 2015).

4.7 Comparison and correlation of vitamin D3 level in serum and saliva between T2DM and ND groups

The results related to the fasting serum vitamin D3 level ,figure (4.26) showed a significant increase (P=0.000) between ND group (38.93±12.81ng/ml) compared to T2DM group (24.72 ±13.20 ng/ml) at $p \leq 0.05$.

The results in figure (4.27) showed a significant increase (p=0.000) in the fasting vitamin D3 level in the saliva of the T2DM group (58.29± 21.76 ng/ml) compared to the ND group (5.17± 3.60 ng/ml).

With regard to correlation analysis, the current study (figure 4.28) showed a positive significant correlation (r = 0.544, p =0.000) of vitamin D3 level between serum and saliva of T2DM group.

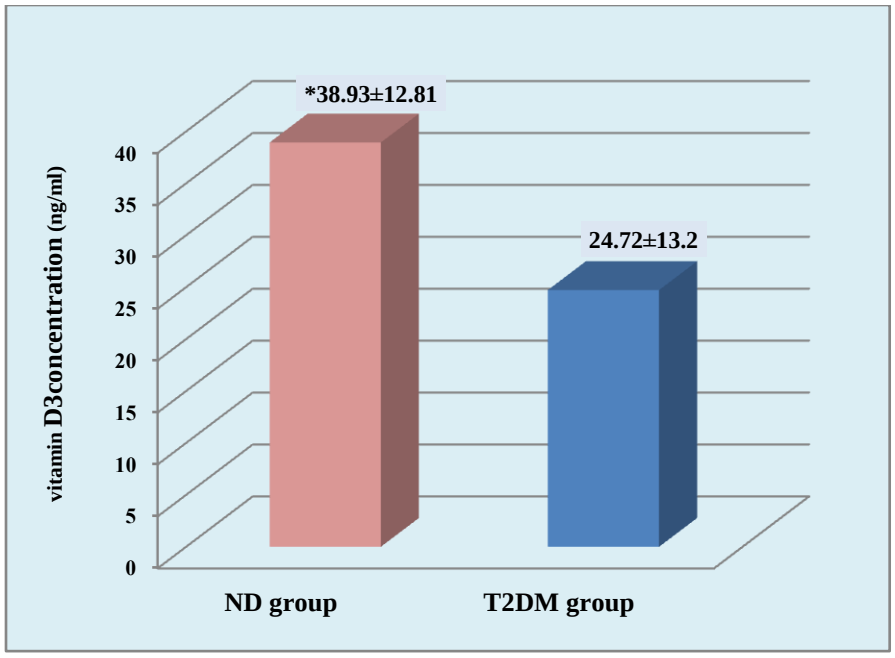


Figure (4.26): Serum fasting Vitamin D3 levels of T2DM and ND groups.
Abbreviations : Data were expressed with mean ±SD, significant deference noted by (*), ($p \leq 0.05$) by Mann-Whitney Test, T2DM: type II diabetes millets, ND: non- diabetics group.

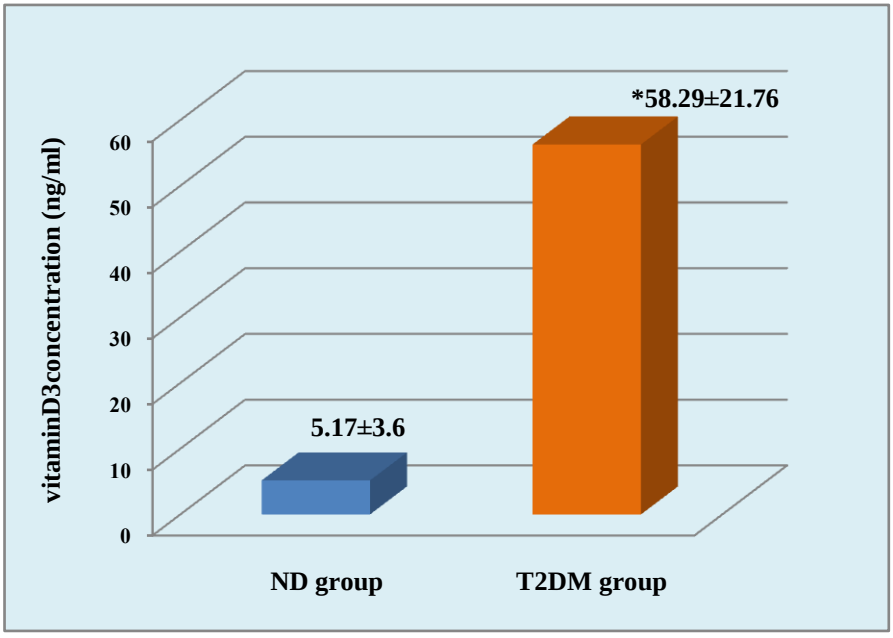


Figure (4.27): Saliva fasting vitamin D3 levels of T2DM and ND groups .
Abbreviations : Data were expressed with mean and ±SD, significant deference noted by(*), ($p \leq 0.05$) by Mann-Whitney Test, T2DM: type II diabetes millets, ND: non- diabetics group.

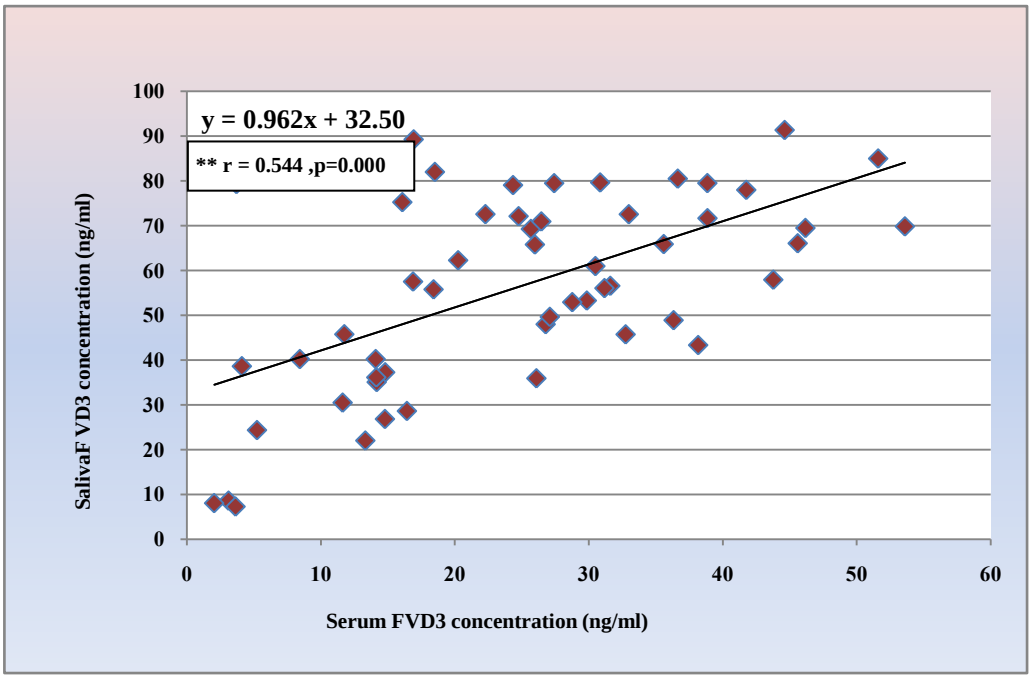


Figure (4.28): Correlation between serum and saliva Vitamin D3 inT2DM group.
Abbreviations : ** :Correlation is significant at the 0.01 level (2-tailed) spearman rho test , T2DM: type II diabetes millets, FVD3: fasting Vitamin D3, r:correlation coefficient ,p:p- value.

Vitamin D3 has many functions in all interactions that occur in the body, with regard to diabetes, Vitamin D3 regulates insulin secretion and sensitivity, as well as works to reduce the level of inflammation throughout the body (Mitri & Pittas , 2014). Un-stimulated saliva is much preferred than stimulated saliva for the assessment of biomarker status in general, as with vitamin D3 (Higashi *et al*., 2013).

However, the current results of the vitamin D3 analysis showed a significant decrease in the levels of vitamin D3 in the serum of T2DM group compared to the control group, which may explain that high levels of blood glucose may hinder the absorption of many minerals and vitamins in the gastrointestinal tract and in tissues.

While the analyzes conducted on saliva showed the exact opposite, the levels of Vitamin D3 were significantly higher in the saliva of the T2DM group than the control group. These findings are consistent with the study of Abdolsamadi *et al*.(2018). As well as, it is consistent with previous studies in the presence of

reduced levels of serum vitamin D3 in patients with type II diabetes (Boucher, 2011; Rahimi *et al*., 2016).

The elevated levels of vitamin D3 in the saliva of T2DM in the current study may be explained by changes in the permeability of the salivary gland membranes due to complications of chronic hyperglycemia, which may allow the entry of proteins that bind vitamin D3 into saliva. As stated in the study of Higashi *et al*. (2013), these proteins that bind to vitamin D3 cannot pass easily from the salivary glands to the oral cavity in healthy people.

In addition, salivary vitamin D3 levels showed a moderate positive correlation with serum levels in the T2DM group, although there was a decrease in serum vitamin D3 levels. This result needs other studies to explain this association, which may be largely related to the levels of secretion of proteins that are associated with vitamin D3 and the mechanism of its transfer from the salivary glands to saliva.

In the study of Abdolsamadi *et al*. (2018), correlation was found between serum and saliva vitamin D3 levels in a healthy control group only. Where these results indicate that the possibility of reliable measurement of vitamin D3 in saliva. It also indicates the need to understand the mechanism of transmission of proteins that are associated with vitamin D3 into saliva in healthy subjects.

Finally, the evaluation of vitamin D3 is possible in saliva, but it is not comparable to the evaluation in blood serum, and it may be needs more studies to adopt it in assessing the level of vitamin D3.

4.8 Correlations of biomarkers with each other in saliva and serum of T2DM group

The results of the correlation analysis for the markers with each other, table (4.16), which were measured in the saliva of T2DMgroup, showed a weak negative correlation (r = -0.272*) (p = 0.047) of fasting interleukin-18 levels with fasting blood glucose. In addition, there is a moderate positive correlation of fasting vitamin D3 with fasting insulin in saliva (r = 0.428, p= 0.001), while other biomarkers did not correlate with each other.

With regard to the correlations of biomarkers with each other in serum, the results presented in table (4.16) showed a weak positive correlation (r = 0.303)(p= 0.026) of fasting vitamin D3 with fasting insulin in the serum of the T2DM group, while no correlations were shown between the biomarkers with each other in the serum of the T2DM group.

Table (4.15):Correlations of biomarkers with each other in the saliva of T2DM group

		Saliva F-Insulin (µIU/ml)	Saliva F-Resistin (pg/ml)	Saliva F-Visfatin (ng/ml)	Saliva F-Interlukin-18 (pg/ml)	Saliva F-Vitamin D3 (ng/ml)
Saliva FG (mg/dl)	r	0.263	-0.197	-0.123	-0.272*	0.143
	P	0.055	0.153	0.374	0.047	0.303
Saliva F-Insulin (µIU/ml)	r		0.199	-0.077	0.005	0.428**
	P		0.149	0.578	0.973	0.001
Saliva F-Resistin (pg/ml)	r			0.099	0.250	0.198
	P			0.474	0.068	0.151
Saliva F-Visfatin (ng/ml)	r				0.004	0.052
	P				0.977	0.710
Saliva F-Interlukin-18 (pg/ml)	r					0.237
	P					0.084
No.		54				

Abbreviations : *Correlation is significant at the 0.05 level, **Correlation is significant at the 0.01 level (2-tailed) spearman rho test, T2DM: type II diabetes millets, F: fasting ,FG: fasting glucose, N: number, r:correlation coefficient , p: p-value.

Table (4.16):Correlations of biomarkers with each other in the serum of T2DM group

		Serum F-Insulin (µIU/ml)	Serum F-Resistin (pg/ml)	Serum F-Visfatin (ng/ml)	Serum F-Interlukin18 (pg/ml)	Serum F-Vitamin D3 (ng/ml)
Serum FG (mg/dl)	r	0.041	-0.104	-0.111	-0.085	-0.031
	p	0.767	0.453	0.426	0.540	0.824
Serum F-Insulin (µIU/ml)	r		0.172	-0.136	-0.086	0.040
	p		0.213	0.325	0.537	0.777
Serum F-Resistin (pg/ml)	r			-0.131	-0.236	0.303*
	p			0.344	0.085	0.026
Serum F-Visfatin (ng/ml)	r				0.081	0.070
	p				0.561	0.614
Serum F-Interlukin-18 (pg/ml)	r					-0.155
	p					0.262
N		54				

Abbreviations: *Correlation is significant at the 0.01 level (2-tailed) spearman rho test ,T2DM: type II diabetes millets, F: fasting , FG: fasting glucose, N: number, r:correlation coefficient , p: p-value.

With regard to the correlations between biomarkers with each other in saliva, glucose levels were associated with interleukin-18 levels with a weak inverse relationship, this result is not consistent with what was found in previous studies of interleukin-18 elevated levels with chronically elevated blood glucose in type II diabetic patients (Aso *et al*., 2003; Esposito *et al*., 2003 and Zaharieva *et al*., 2018). This may be explained by the change in the permeability of the salivary glands in patients with type II diabetes.

In the investigation of the relationship of vitamin D3 with the other biomarkers in the current study, the levels of vitamin D3 in saliva were moderately and positively correlated with the levels of the insulin hormone in the T2DM group.

Perhaps this result supports the fact that vitamin D3 is important in all vital processes, for example insulin production.

However, these relationships are likely to be useful in assessing the status of salivary glands and periodontal tissues in patients with type II diabetes , because it reflects the increased permeability of the salivary gland membranes that allows the passage of large substances such as proteins into the saliva (Loo *et al*., 2010 ; Zhang *et al*.,2010 and Williamson *et al*., 2012).

In addition, the lack of correlation between the rest of the biomarkers with each other in saliva may be due to a change in the permeability of the salivary gland membranes, the mechanism of transmission of the biomarker from the blood to the saliva, or to the conditions of the oral cavity environment, or all of these factors combined.

Regarding the correlations of biomarkers with each other in the serum ,the lack of correlation of glucose levels with insulin levels in serum may be due to the nature of insulin secretion in the form of bursts and the submission of 80% of the secreted insulin to removal by the liver and association with target cells, which also explains the lack of association of insulin with the rest of the biomarkers.

In addition the levels of vitamin D3 in the serum were correlated weakly and positively with the levels of resistin. These relationships may clearly indicate a role for vitamin D3 in insulin secretion, insulin sensitivity. This opinion is consistent with what Lips *et al*. (2017) mentioned about the role of vitamin D3 in increasing insulin secretion. In addition the effects of vitamin D3 on the inflammatory and glucose homeostasis in patients with type II diabetes (Wallace *et al*., 2016).

However, the lack of association between the remaining biomarkers with each other may be due to physiological differences between diabetic patients themselves and the nature and conditions of secretion of each marker.

All the biomarkers included in the current study are useful in predicting type II diabetes, and their appearance in measurable quantities in saliva encourages the adoption of the use of saliva in measuring these biomarkers, taking into account the technical aspects in choosing the appropriate saliva collection method for each marker, which accurately reflects the level associated with the level of biomarker in the serum.

This study is a small contribution to scientific research attempts to find alternative vital fluids for blood in conducting routine and diagnostic tests for diseases that are less expensive and less painful for the patient and perhaps more secure for the laboratory staff in terms of reducing infection when drawing blood samples.

Conclusion & Recommendations

Conclusions

Based on the results of the current study, the following has been concluded:

1- Saliva can be replaced by serum in measuring (blood glucose, insulin, resistin, visfatin, interleukin-18, and vitamin D3), but adopting saliva as an alternative to serum in routine tests requires more future studies.

2- The waist circumference measurement reflects well the central obesity related to the development of type II diabetes.

3- Serum and saliva fasting biomarkers: blood glucose, insulin, resistin, visfatin, interleukin-18,vitamin (D3) are good indicators for predicting or diagnosing type II diabetes.

4- The presence of correlations between serum and saliva in insulin and visfatin, strongly indicates the efficiency of saliva as a biological fluid as a substitute for serum, and the absence of associations with other markers indicates the need for more studies to know the factors that affect secretion and that limit the possibility of using saliva as an alternative to serum in analysis.

5- The genetic variants of single nucleotide polymorphisms may differ according to races and societies, and their functional role may also differ.

6- The allele (C) at SNP(rs1862513-in *resistin gene*) is a risk factor for type II diabetes among Iraqis.

Recommendations

Based on what was concluded in the current study, the following was recommended:

1-Studying further salivary biomarkers like C-reactive protein, adiponectin, liptin and ghrelin in serum and saliva of type II diabetes patients.

2- Studying the level of a single biomarker in unstimulated saliva, stimulated saliva, and through direct collection from the parotid gland to determine the best saliva collection method in which the level of the biomarker in saliva correlates with the serum.

3-Conducting further research of type II diabetes in children and adolescents with family history of diabetes.

4- Performing genome wide association scan studies in Iraq for type II diabetes.

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الخلاصة

داء السكري النوع الثاني، مرض متنوع الأسباب ويسبب مضاعفات معقدة . يعاني منه الكثير من البشر في البلدان المتقدمة والنامية. ارتبط داء السكري النوع الثاني ارتباطًا وثيقًا بتطور نمط حياة الفرد والنظام الغذائي غير الصحي. يظهرغالبا عند كبار السن، وقد بدأ مؤخرًا في الظهور في فئة الشباب. تختلف أسبابه البيئية والجينية بين المجتمعات والأعراق في العالم.

أشتملت الدراسة الحالية ٥٤ مريضًا يعانون من النوع الثاني من مرض السكري (27 ذكور و ٢٧ إناث) و ٣٤ أصحاء غير مصابين بمرض السكري النوع الثاني (17 ذكور و ١٧ إناث). تراوحت الأعمار لكلا المجموعتين من ٤٠ إلى ٥٥ عامًا ، تم إجراء الدراسة في مختبر العيادة الاستشارية في مستشفى بغداد التعليمي في بغداد - العراق ، من كانون الأول ٢٠٢١ إلى شباط ٢٠٢٢.

أخذت الموافقة الشفوية من المرضى والأصحاء لجمع عينات الدم واللعاب والمعلومات المتعلقة بالعمرو الوزن والطول لحساب مؤشر كتلة الجسم وقياس محيط الخصر و محيط العنق .

اعتُمدت طريقة أوكسيديز- بيروكسيديز باستخدام مجموعة الفحص من شركة راندوكس-المملكة المتحدة لتحديد مستوى جلوكوز الدم الصيامي في اللعاب ، وتم الحصول على نتائج اختبار كلوكوز الدم الصيامي في المصل والهيموغلوبين السكري للمرضى والأصحاء من سجلات مختبر العيادة الاستشارية في مستشفى بغداد التعليمي، بالإضافة إلى ذلك استخدمت تقنية المقايسة الماصة المناعية المرتبطة بالإنزيم (أليزا) لتحديد مستويات الدلائل الحيوية لمرض السكري النوع الثاني (الأنسولين ، الريزسستين ،الفزفاتين ، إنترلوكين - ١٨ و فيتامين D3) في دم ولعاب المرضى والأصحاء.

استخدمت تقنية تفاعل البلمرة التسلسلي- تمايز طول قطع التقييد لتحديد الأليلات والطرزالوراثية لتعدد أشكال النوكليوتيدات المفردة (rs1862513- جين الريزسستين، rs11977021- جين الفزفاتين ، rs3842752 - جين الأنسولين، rs360717 -جين انترلوكين١٨) استخدمت تقنية تفاعل البلمرة التسلسلي- تمايز اشكال الشريط المفرد لتحديد الطرز الوراثية لتعدد أشكال النوكليوتيدات المفردة (rs 122255372- جين *TCF7L2* و rs13266634-جين *SLC30A8*). أُجري تحليل تسلسل النيوكليوتيدات بواسطة شركة ماكروجين - كوريا الجنوبية.

أظهرت نتائج الدراسة الحالية زيادة معنوية ($p < 0.05$) في مؤشر كتلة الجسم ومحيط الخصر ومحيط الرقبة في مجموعة السكري النوع الثاني مقارنة بالمجموعة الضابطة. كما أظهرت النتائج زيادة معنويةً ($p < 0.05$) في النسبة المئوية للهيموغلوبين السكري ومستويات الدلائل الحيوية الصيامية (الجلوكوز ، الأنسولين ، الريزيستين ،الفزفاتين ،الإنترلوكين- ١٨ وفيتامين D3) في المصل واللعاب لمجموعة السكري النوع الثاني مقارنة بمجموعة الأصحاء.

بالإضافة إلى ذلك، أظهرت النتائج الحالية وجود علاقة ارتباط موجبة متوسطة معنوية ($r = 0.695$) بين مؤشر كتلة الجسم ومحيط الخصر، وارتباط معنوي موجب ضعيف($r = 0.31$) بين محيط الخصر ومحيط الرقبة في مجموعة السكري النوع الثاني.

وُجد إرتباط موجب قوي (r = 0.844) بين الهيموغلوبين السكري وكلوكوز الدم الصيامي في المصل ، كما وجد ارتباط موجب قوي (r = 0.741) لمستويات الأنسولين ، و ارتباط معنوي موجب متوسط (r = 0.528) لمستويات الفزفاتين ، وارتباط معنوي موجب متوسط (r = 0.544) لمستويات فيتامين D3، بين المصل واللعاب لمجموعة السكري النوع الثاني.

أظهر توزيع الأليلات والطرزالوراثية أن الأليل (C) لتعدد أشكال النوكليوتيدات المفردة (rs1862513) في جين الريزستين كعامل خطورة للإصابة بمرض السكري من النوع الثاني بين العراقيين. بينما لم يكن هناك فرق معنوي في مستويات الرزيستين بين حاملي الطرز الوراثية (G / G، G / C ،/ C / C). من جهة اخرى لم تظهر فروق ذات دلالة إحصائية(P> 0.05) في توزيع الأليلات والطرز الوراثية بين حاملي الطرز الوراثية الثلاثة بين المرضى والأصحاء للنيوكليوتيدات المفردة متعددة الاشكال: (rs3842752-جين الأنسولين، rs12255372- جينTCF7L2، rs13266634 - جينSLC30A8، rs 11977021- جين الفزفاتين، rs 360717 - جين انترلوكين -١٨). كشف تحليل التسلسل الجيني للنيوكليوتيدة المفردة rs12255372 - جين TCF7L2 عن وجود ألاليل (A) كبديل للاليل (T) عند مجموعة المرضى.

استتنتجت الدراسة الحالية قد يكون ممكنا استخدام اللعاب كبديل للمصل في تقييم مستويات بعض الدلائل الحيوية المتعلقة بمرض السكري النوع الثاني . بالإضافة إلى ذلك ، يعد قياس محيط الخصر مؤشرًا جيدًا للسمنة المركزية التي تزيد من قابلية الأصابة بمرض السكري النوع الثاني ، كما تعد الدلائل الحيوية التي يتم قياسها في حالة الصيام (الجلوكوز ، الأنسولين ، الريزيستين ، الفيزفاتين ، إنترلوكين- ١٨) مؤشرات جيدة للتنبؤ والتشخيص لمرض السكري النوع الثاني في المصل واللعاب.

كما استنتجت الدراسة الحالية ،ان علاقات الارتباطات بين المصل واللعاب للدلائل الحيوية (الأنسولين ، الفيزفاتين) اشارت بقوة إلى كفاءة اللعاب كبديل للمصل ، على العكس من ذلك، اشارعدم وجود الارتباطات بين الدلائل الحيوية الأخرى إلى الحاجة للمزيد من الدراسات لتوضيح العوامل المسببة للتغيير في مستوى الإفراز.

كما استنتجت الدراسة الحالية أيضًا، أن المتغيرات الجينية لتعدد أشكال النوكليوتيدات المفردة قد تختلف بين الأعراق والمجتمعات ، وقد يختلف دورها الوظيفي أيضًا. واستنتج أيضًا أن الأليل (C) الموجود في (rs1862513) في جين الريزستين هوعامل خطورة للإصابة بمرض السكري النوع الثاني بين العراقيين. وقد كان من بين التوصيات ، قياس معلمات حيوية أخرى ذات علاقة بمرض السكري من النوع الثاني مثل الجريلين واللبتين في اللعاب والتحري عن المزيد من أشكال النوكليوتيدات المفردة التي من المتوقع أن يكون لها دور وظيفي في حدوث مرض السكري النوع الثاني بين العراقيين.

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جامعة بابل /كلية العلوم
قسم علوم الحياة

دراسة مقارنة لبعض الدلائل الحيوية في الدم واللعاب لداء السكري النوع الثاني

أطروحة

مقدمة إلى مجلس كلية العلوم- جامعة بابل

كجزء من متطلبات نيل درجة الدكتوراه فلسفة في العلوم/

علوم الحياة/علم الحيوان

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