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Association between Dipeptidyl Peptidase-4 Polymorphism (rs1861978) and Incretin Level in Type 2 Diabetic Patients in Holy Kerbala

A Thesis

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بسم الله الرحمن الرحيم

﴿وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّنَ الْعِلْمِ إِلَّا قَلِيلًا ﴿٨٥﴾﴾

صدق الله العلي العظيم

(الاسراء ٨٥)

Dedication

To my parents, and all people who

might get benefit from this research

I dedicate this work.

Asaad

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Asaad

Supervisors' Certification

We certify that this M.Sc. thesis entitled:-

Association between Dipeptidyl Peptidase-4 Polymorphism (rs1861978) and Incretin Level in Type 2 Diabetic Patients in Holy Kerbala

Was prepared under our supervision in laboratories at the Department of Biochemistry – College of Medicine / University of Kerbala as a partial fulfillment of the requirements of Master Degree in Clinical chemistry.

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In the view of the available recommendation, I forward this M.Sc. thesis for debate by the examining committee.

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Abstract

Background

Type2 Diabetes Mellitus is associated with progressively increasing morbidity and mortality across the globe. It is a complex multigenic disease with severe consequences.

As several genes involved in Type2 Diabetes Mellitus regulations, Single Nucleotide Polymorphism rs1861978 in dipeptidyl peptidase-4 has been suggested to be associated with this disease.

On the other hand, Incretin hormone like Glucose-Dependent Insulinotropic Polypeptide (GIP) in recent researches has been found to be linked to Type2 Diabetes Mellitus in different circumstances.

Aim of the Study

This study aimed to investigate the possible association of single nucleotide polymorphisms rs1861978 of the DPP4 gene in kerbala subjects with Type2 Diabetes Mellitus and evaluate whether they had an effect on the serum levels of GIP levels.

Objectives

This study designed to find out the association between Incretin hormones, single nucleotide polymorphisms rs1861978 dipeptidyl peptidase-4 gene polymorphism in Type2 Diabetes Mellitus.

Subjects and Method

This is case control study for two hundred and forty Iraqi subjects. One hundred and twenty patients who were diagnosed previously with type 2 diabetes mellitus based on physicians diagnosis prior to their current visit to Al-Hassan diabetic and endocrinology care center in Imam Hussein medical city/holy Kerbala city/Iraq. Some of them were taking medications and others were not. They all have no obvious renal and liver problems. One hundred and twenty apparently healthy individual candidates as control group matched in age and BMI with patients, They have neither symptoms nor signs of diabetes and they are apparently healthy.

Fasted blood samples were collected from all patients and health subjects during the period from January to April 2018 for measuring the level of Incretin hormones (GIP) in addition to fasting serum glucose, and lipid profile. Two mls of these samples were collected in EDTA tubes for DNA extraction and polymerase chain reaction.

The DNA extraction and PCR were done at the research laboratory of the biochemistry department of the Medicine College of Kerbala University.

Results

There was an elevation in serum fasting glucose, Triglyceride, total cholesterol and low density lipoprotein, and decrease in serum high density lipoprotein in Diabetic patient as compared to those of the control group.

The Diabetic patient in this study showed a significant association between single nucleotide polymorphisms (rs1861978) in dipeptidyl peptidase-4 gene (genotype G/G) and incretin hormone (GIP).

Conclusion

The DPP4 allelic distributions and their association with incretin hormones (GIP) were investigated. In Iraqi type 2 diabetic patients, which may have clinical significance.

The association between SNP rs1861978 in dipeptidyl peptidase-4 (DPP-4) gene and incretin (GIP) was significant.

Incretin Levels in Diabetic patients showed no significant association with control.

Incretin hormones glucose-Dependent Insulinotropic Polypeptide (GIP), fasting serum glucose and HbA1c level were increase significantly in diabetic patients in comparison with healthy subjects.

List of Abbreviations

Abbreviation	Words
ADA	American Diabetes Association
ANOVA	Analysis Of Variance
apos	Apolipoproteins
ARMS	Amplification Refractory Mutation System
BMI	Body Mass Index
bp	Base Pair
CAD	Coronary Artery Disease
CE	Cholesterol Esters
CLB	Cell Lysis Buffer
CVD	Cardiovascular Disease
CVA	Cerbrovascular Accident
CWS	Column Wash Solution
DM	Diabetes Mellitus
DNA	Deoxy ribonucleic Acid
dNTPs	Deoxy nucleotide tri-p
DPP4	Dipeptidyl-Peptidase 4
EDTA	Ethyline Diamine Tetra Acetic Acid
EECs	Enteroendocrine Cells
ELISA	Enzyme-Linked Immune Sorbent Assay
FAs	Fatty Acids
FC	Functional Connectivity
FPG	Fasting plasma glucose
GIP	Glucose-Dependent Insulinotropic Peptide
GLP	Glucagon-Like Peptide
GWAS	Genome-Wide Association Studies

List of Abbreviations

Abbreviation	Words
Hb	Hemoglobin
HbA1c	Glycated Hemoglobin
HDL	High-Density Lipoprotein
HIF-1α	Hypoxia Inducible Factor-1α
HNF-1α	Hepatocyte Nuclear Factor-1 A
IDF	International Diabetes Federation
IDL	Intermediate Density Lipoprotein
JDS	Japanese Diabetes Society
LCAT	Lecithin-Cholesterol Acyltransferase
LDL	Low-Density Lipoprotein
LPL	Lipoprotein Lipase
Lps	Lipoproteins
NEFA	Nonesterified Fatty Acids
NGSP	National Glycohemoglobin Standardization Program
OGTT	Oral Glucose Tolerance Test
PCR	Polymerase Chain Reaction
PK	Proteinase K
PLs	Phospholipids
PVD	Peripheral Vascular Disease
R	Ratio
RCT	Reverse Cholesterol Transport
S.	Serum
S.TG	Serum Triglycerides
SGLT2	Sodium-Glucose Cotransporter
SNPs	Single Nucleotide Polymorphisms
SPSS	Statistical Package For The Social Sciences
T2DM	Type 2 Diabetes Mellitus

List of Abbreviations

Abbreviation	Words
TBE	Tris/Borate/EDTA
TC	Total Cholesterol
TGs	Triglycerides
TZD	Thiazolidinedione
UV	Ultraviolet
VLDL	Very Low-Density Lipoprotein
WHO	World Health Organization

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Chapter One

Introduction

&

Literature Review

Introduction & Literature Review

1.1-Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Two major forms of diabetes were identified; type 1 and type 2. Lack of or severe reduction in insulin secretion due to autoimmune or viral destructions of cells is responsible for type 1 diabetes, which accounts for 5-10% of diabetic patients. The more prevalent form, type 2 diabetes mellitus (T2DM), accounts for more than 90% of cases (Olefsky 2001). T2DM usually begins as insulin resistance, a disorder in which the cells do not use insulin properly. As the need for insulin rises, the pancreas gradually loses its ability to produce it (Cohen 2006).

In the human body, the liver is the main site of gluconeogenesis. Increased gluconeogenesis in the liver of patients with T2DM is considered a major contributor to hyperglycemia and subsequent diabetic organ damage. Insulin is a key hormone that inhibits gluconeogenesis, and insulin resistance is a hallmark of T2DM. Understanding the regulation of gluconeogenesis and the role of insulin signaling in this pathway is important to developing new therapies for T2DM (Hatting, Tavares *et al*. 2018).

Both La Barre and Heller suggested that the tentative substance could be used in the treatment of diabetes, which was rather farsighted considering that it was only demonstrated in the early 1990s that the main incretin hormone had glucose-lowering actions in T2DM (Ahren 2013).

Genetic factors and lifestyle play a critical role in the development of T2DM (Vazquez, Klimentidis *et al.* 2015). The Asian population has strong genetic susceptibility to T2DM, developing diabetes at younger ages and at a lower degree of obesity (Yoon, Lee *et al*. 2006)

Currently the incidence of T2DM has reached epidemic levels in Asia .Despite knowledge of the critical role of genetic factors; these have not been incorporated into the clinical assessment of T2DM risk (Ahmed, Huri *et al.* 2016).

Nonetheless, genetic polymorphisms of DPP4 and their association with T2DM have rarely been investigated (Rohrborn, Wronkowitz *et al*. 2015).

However, no previous study investigated the relationship between (DPP4) Polymorphism and Incretins Levels in Patients with (T2DM). Therefore, this study will be assessing the relationship between (DPP4) Polymorphism and Incretins Levels in patients with (T2DM) patients from Kerbala Governorate. Understanding the status of (DPP4) polymorphism and other parameters could be useful in the management of the disease.

1.1.1- Objective

The general objective of the present study is to assess incretins hormones and some biochemical parameters in T2DM patients and study the Relationship between Dipeptidyl-Peptidase 4 (DPP4) polymorphism and incretins levels from Kerbala Governorate.

1.1.2-Significance

1. Diabetes mellitus is prevalent in Kerbala governorate, as well as worldwide.

2. Several studies have been carried out on biochemical parameters of T2DM in Kerbala governorate, without speculation of the role of relationship between (DPP4) polymorphism and incretins levels in the T2DM. Therefore, this study to assess (DPP4) Polymorphism in T2DM patients in Holy Kerbala.

3· It is important to give a detailed picture on the Dipeptidyl-Peptidase 4 (DPP4) Polymorphism as well as on other biochemical features of diabetes among type 2 diabetic patients in Holy Kerbala.

4-Genetic polymorphisms of the Dipeptidyl Peptidase 4 (DPP4) gene may play a role in the etiology of type 2 diabetes mellitus (T2DM)

1.2-Literature Review

1.2.1-Diabetes Mellitus

Diabetes mellitus (DM) is a chronic disorder that can alter carbohydrate, protein, and fat metabolism. It is caused by the absence of insulin secretion due to either the progressive or marked inability of the β-Langerhans islet cells of the pancreas to produce insulin, or due to defects in insulin uptake in the peripheral tissue (Al-Goblan, Al-Alfi *et al*. 2014).

This metabolic disorder is due to insulin secretion deficiency or a resistance to insulin action, or both (Edrees, Elbehiry *et al*. 2017).

Diabetes is a chronic disease, which if not treated properly, generates serious complications that reduce patient quality of life and raises the cost of their care (Rios, Francini *et al*. 2015).

Persistent hyperglycemia leads to damage and dysfunction of various organs (kidneys, heart, eyes, blood vessels or nerves) (Wojciechowska, Krajewski *et al.* 2016). Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia (Agrawal and Hiwale 2018).

1.2.2-Classification of Diabetes Mellitus

1.2.2.1-Type 1 Diabetes Mellitus

(T1DM) is a chronic immune-mediated disease with a subclinical prodromal period, characterized by selective loss of insulin-producing-β cells in the pancreatic islets of genetically susceptible individuals (Knip and Siljander 2016).

T1DM can be acquired at any age and accounts for about five to ten percent of all diabetes mellitus cases (Fullerton, Jeitler *et al*. 2014).

T1DM was largely considered a disorder in children and adolescents, but this opinion has changed over the past decade, so that age at symptomatic onset is no longer a restricting factor Polydipsia, polyphagia, and polyuria (Atkinson, Eisenbarth *et al*. 2014).

People with T1DM have a higher rate of mortality compared with the general population, with variations across countries (Sandahl, Nielsen *et al*. 2017).

Although intensive glycemic control has reduced the incidence of micro-vascular and macro-vascular complications, the majority of patients with T1DM are still developing these complications. Major research efforts are needed to achieve early diagnosis, prevent β-cell loss and develop better treatment options to improve the quality of life and prognosis of those affected (Katsarou, Gudbjornsdottir *et al*. 2017).

1.2.2.2-Type 2 Diabetes Mellitus

(T2DM) is a major health concern. The total number of Diabetics is expected to reach 366 million by 2030 (SV, Kumar *et al.* 2017).

(T2DM) is a very complex and multifactorial metabolic disease characterized by insulin resistance and β cell failure leading to elevate blood glucose level (Nowotny, Jung *et al*. 2015).

(DM) is a common metabolic disorder predisposing to diabetic cardiomyopathy and atherosclerotic cardiovascular disease (CVD), which could lead to heart failure through a variety of mechanisms, including myocardial infarction and chronic pressure overload (De Rosa, Arcidiacono *et al*. 2018).

Depressive symptoms are associated with worse outcome in the natural history of T2DM and are associated with an approximately 60% increased risk of incident disease; conversely, (T2DM) increases the risk of incident depressive symptoms by approximately 20% (Ismail, Moulton *et al*. 2017).

(T2DM) can co-occur with other medical conditions, such as gestational diabetes occurring during the second or third trimester of pregnancy or pancreatic disease associated with cystic fibrosis. T2DM may also be induced, e.g., by use of glucocorticoids in the inpatient setting or use of highly active antiretroviral agents like protease inhibitors and nucleoside reverse transcription inhibitors in HIV-positive individuals (Chaudhury, Duvoor *et al*. 2017).

(T2DM) is associated with increased morbidity and mortality due to micro-vascular (e.g. retinopathy, nephropathy, and neuropathy) and macro-vascular complications (e.g. myocardial infarction, peripheral vascular disease, and stroke) (Wilson, Willis *et al*. 2017).

(T2DM) epidemic is driven by the global increase in obesity rates, linked to decreased physical activity and altered dietary habits, as well as longevity. Indeed, obesity is associated with impaired insulin action on various tissues, including muscle, adipose and liver. However, failure of pancreatic beta cells to match insulin secretion to the increased insulin demand is the driving force behind the development of severe hyperglycemia (van Raalte and Verchere 2017).

(T2DM) patients manifest functional changes in certain brain regions, and these changes are different from those associated with normal aging. For instance, the abnormal amplitude of low-frequency fluctuation, regional homogeneity, and functional connectivity (FC) in T2DM patients have been associated with poor performance in cognitive tests (Liu, Duan *et al*. 2018).

(T2DM) is a progressive disease characterized by a significant decline in beta cell function, ultimately resulting in insufficient insulin secretion to meet metabolic

demands .In the early stages, increased demand due to insulin resistance induces a state of beta cell compensation mediated by increases in beta cell mass and insulin secretion to maintain euglycemia (Liu, Prentice *et al.* 2016).

(T2DM) is an expanding global health problem, closely linked to the epidemic of obesity. Individuals with T2DM are at high risk for both micro-vascular complications (including retinopathy, nephropathy and neuropathy) and macro-vascular complications (such as cardiovascular comorbidities), owing to hyperglycemia and individual components of the insulin resistance (metabolic) syndrome. Environmental factors (for example, obesity, an unhealthy diet and physical inactivity) and genetic factors contribute to the multiple pathophysiological disturbances that are responsible for impaired glucose homeostasis in T2DM (DeFronzo, Ferrannini *et al*. 2015).

Obese people with (T2DM) usually lose less weight than people without diabetes, partly because they have already lost what they can manage, and partly because many anti-diabetic drugs are obesogenic (Leslie, Taylor *et al*. 2016).

1.2.2.2.1-Diagnosis of Type 2 Diabetic Mellitus

Because the ratio of detected to undetected cases may vary over time and between places, epidemiological research aimed at defining the true prevalence of type 2 diabetes has relied on special studies in which the presence and absence of disease are defined by the oral glucose tolerance test (OGTT). The WHO recommends use of the 75 g OGTT, with diabetes defined by fasting glucose(7.0 mmol/L) or more and/or 2-hour post-challenge glucose (11.1 mmol/L) or more. the American Diabetes Association, the WHO and other authoritative bodies have approved the use of HbA1c for the diagnosis of diabetes with a cut-off of 48 mmol/mol (>6.5%) in a standardized laboratory (Forouhi and Wareham 2014).

Table (1-1): Criteria for the diagnosis of diabetes (ADA 2018).

FPG 126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.*
On or more
2h FPG 200 mg/dL (11.1 mmol/L) during OGTT. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75-g anhydrous glucose dissolved in water.*
On or more
A1C 6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
On or more
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose 200 mg/dL (11.1 mmol/L).
*In the absence of unequivocal hyperglycemia, results should be confirmed by repeat testing.

1.2.2.2.2-Complications of type 2 Diabetes Mellitus

The acute and chronic complications of diabetes mellitus are major causes of hospital admissions. Asian patients had more evidence of macro and micro vascular disease at diagnosis of diabetes. Several studies showed that the prevalence of micro-vascular and macro-vascular complications were more in Asians when compared to Europeans.

A) **Micro-vascular complications:** Micro-vascular complications include retinopathy, nephropathy, and neuropathy.

B) **Macro-vascular complications:** Macro-vascular complications include coronary artery disease (CAD), Peripheral vascular disease (PVD), and cerebrovascular Accident (CVA) (Yadav, Tiwari *et al*. 2008).

1.2.2.2.3-Risk factors of type 2 Diabetes Mellitus

Almost all populations share same risk factors for the development of (T2DM). These risk factors are broadly categorized as non-modifiable risk factors and modifiable risk factors. Age, gender and heredity are non-modifiable risk factors. Modifiable risk factors include obesity, diet, physical inactivity, smoking, alcohol consumption, psychological stress and depression. Association of genetic variation with diabetes has been well established and various susceptible genes that contribute to the development of diabetes have been identified. Gene - environment interaction is found to modulate the risk of T2DM (Nazu 2017).

1.2.2.2.4-Obesity and Diabetes Mellitus

Obesity is a complex and multifactorial disease resulting from the interactions among genetics, metabolic, behavioral, sociocultural and environmental factors (Goni, Garcia-Granero *et al*. 2018).

Obesity and (T2DM) are the leading worldwide risk factors for mortality. The inextricably interlinked pathological progression from excessive weight gain, obesity, and hyperglycemia to T2DM, usually commencing from obesity, typically originates from overconsumption of sugar and high-fat diets (Hossain, Yamaguchi *et al*. 2015).

Obesity may precede the onset of (T2DM) and is associated with an increased risk of developing T2DM. Furthermore, obesity has been linked to metabolic dysfunction and may further exacerbate T2DM -related metabolic abnormalities. Obesity-related metabolic dysfunction is also independently associated with brain alterations (Yoon, Cho *et al*. 2017).

Obesity and T2DM are also independent risk factors for several disorders, including arterial hypertension, dyslipidemia, and macroangiopathy.

These disorders have mechanisms closely related to insulin resistance, such as visceral obesity, ectopic lipid deposition and an altered profile in circulating levels of adipokines (de Araujo, Salmon *et al.* 2017).

1.3-Body Mass Index (BMI)

1.3.1-Definition

Body mass index (BMI), defined as weight divided by height squared, is the most commonly used measure of adiposity, with individuals exceeding a certain BMI threshold classed as obese, BMI, such as obesity, is positively correlated with metabolic abnormalities, many common diseases and all-cause mortality (Young, Wauthier *et al*. 2016).

The (BMI) is the metric currently in use for defining anthropometric height/weight characteristics in adults and for classifying (categorizing) them into groups (Nuttall 2015).

Diabetes and high (BMI), defined as a BMI greater than or equal to 25 kg/m², are leading causes of mortality and morbidity globally, high BMI is an important risk factor for diabetes (Pearson-Stuttard, Zhou *et al*. 2018).

1.3.2-Classification

Baseline BMI, computed as the weight in kilograms divided by the square of the height in metres, was categorised at baseline into six categories according to the World Health Organization classification: underweight (<18.5), normal weight (≥18.5 to <25), overweight (≥25 to <30), and obesity grade 1 (≥30 to <35), grade 2 (≥35 to <40), and grade 3 (≥40 kg/m^2) (Mohammedi, Chalmers *et al*. 2018).

Table (1-2): World Health Organization body mass index (BMI) classifications (Hanson, Rutten *et al*. 2014)

Classification	BMI (kg/m^2)
Severe Thinness	<16.0
Moderate Thinness	16.0-16.9
Mild Thinness	17.0-18.49
Normal	18.5-24.9
Overweight	≥25.0
Pre-Obese	25.0-29.9
Obese Class I	30.0-34.9
Obese Class II	35.0-39.9
Obese Class III	≥40

1.4-Lipid Profile

The term 'lipid profile' describes the varying levels of lipids in the blood, the most commonly reported ones being low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides (Mann, Beedie *et al*. 2014).

Lipids are a heterogeneous group of molecules that share the common properties of being predominately hydrophobic and soluble in organic solvents. Lipids range in structure from simple short hydrocarbon chains to more complex molecules, including triacylglycerols (TAGs), phospholipids (PLs) and sterols and their esters (Burdge and Calder 2015).

dipeptidyl peptidase-4 (DPP-4) inhibitors reduced the serum levels of cholesterol and triglycerides in mice and humans. GLP-1 also decreased the lipid absorption *in vivo*. Therefore, incretins may improve the endothelial function by correcting the lipid metabolism (Oyama, Higashi *et al*. 2014).

Because lipids, such as cholesterol and triglycerides, are insoluble in water these lipids must be transported in association with proteins in the circulation.

Large quantities of fatty acids from meals must be transported as triglycerides to avoid toxicity. These lipoproteins play a key role in the absorption and transport of dietary lipids by the small intestine, in the transport of lipids from the liver to peripheral tissues, and the transport of lipids from peripheral tissues to the liver and intestine (reverse cholesterol transport) (Feingold and Grunfeld 2015).

Lipids are an established potent stimulus for the secretion of gut peptides including incretins, glucose-dependent insulinotropic peptide (GIP) (Matikainen, Bjornson *et al.* 2016).

The abnormal lipid profile that often accompanies T2DM is well-known to be associated with an increased risk of atherosclerotic vascular disease, This “diabetic dyslipidaemia” is typically characterised by elevated serum triglycerides (TGs) and low high-density lipoprotein cholesterol (HDL) concentrations, together with raised apolipoprotein B and the prevalence of smaller, denser low-density lipoprotein (LDL) cholesterol particles (Schofield, Liu *et al*. 2016).

The lipid profile of T2DM patients generally presents increased VLDL, reduced HDL cholesterol, and an increased number of small dense LDL particles. However, the LDL cholesterol concentration is less often increased. VLDL is increased because of increased production by the liver (Apro 2015).

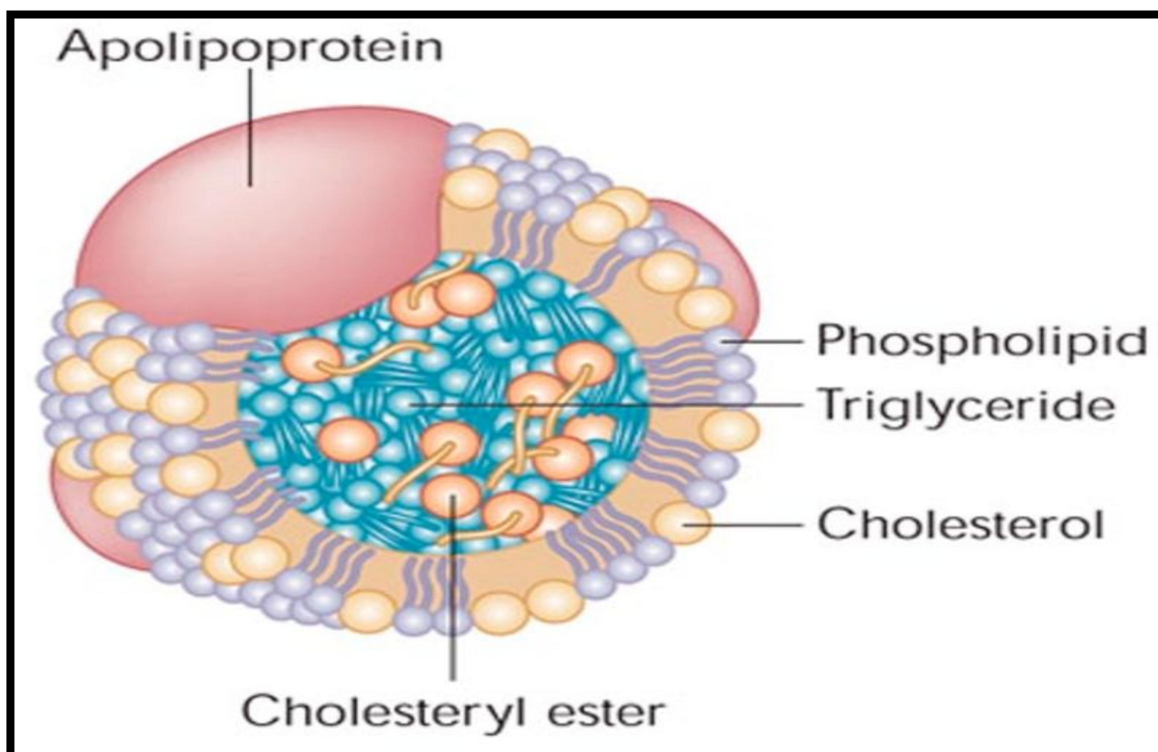


Figure (1-1): Model of lipoprotein structure (Ahnstrom 2017).

1.4.1-Cholesterol

The total body content of cholesterol is approximately 100 g, of which approximately 90% are found at the cellular levels and 10% in circulation, Cholesterol is synthesised virtually in all nucleated cells, Cholesterol synthesis starts, similarly to *de novo* lipogenesis, by the transfer of acetyl CoA from mitochondria to cytosol (Lavoie 2016).

Cholesterol is an essential structural component in cell membranes where it helps to generate semipermeable barriers between cellular compartments and to regulate membrane fluidity influencing several transmembrane signaling processes (Sjoberg 2016).

Cholesterol found in plasma membranes can be extracted by HDLs, esterified by the HDL-associated enzyme lecithin-cholesterol acyltransferase (LCAT), transferred to VLDLs and LDLs and finally re-transferred to the liver (RUSSO 2017).

Cholesterol is an essential component of the human body. Cholesterol functions in a variety of capacities including, but not limited to, stabilizing cell membranes and serving as precursor for bile acids, vitamin D, and steroid hormones. Every cell of the human body can synthesize cholesterol when needed, but cells cannot catabolize cholesterol by oxidative processes. Therefore, any excess cholesterol must be transported to the liver, secreted into bile (as cholesterol or bile acids) and eliminated from the body by the intestinal route (Jesch and Carr 2017).

1.4.2-Triacylglycerol

Triacylglycerol (TAG), also called triglycerides (TG), are one of the most important sources of energy in the body which can be utilised instantly or stored in adipose tissues to be used when energy supply is reduced. They play several significant roles such as the maintenance of the cell membrane structural integrity, cell signalling and hormonal functions. TGs are non-polar and hydrophobic, and are composed of three individual fatty acids (FAs), each linked by an ester bond to a glycerol molecule. TG derived from the diet is called exogenous TG. (Sharaf 2016).

Triacylglycerol (triglyceride; TAG) is an inert storage and transport molecule of fatty acids, TAG is the primary energy substrate stored in adipose tissues to sustain animals during fasting. TAG is also synthesized in the liver for the assembly and secretion of VLDL to transport neutral lipids to other tissues (Yen, Nelson *et al*. 2014).

1.4.3-Lipoproteins (Lps)

Lipoproteins (Lps) carry phospholipid, free cholesterol, and apolipoproteins on the surface, and triglyceride and cholsteryl ester in their core. Cholesterol, triglyceride, phospholipids, and proteins (known as apolipoproteins) are carried in plasma on generally spherical lipoprotein particles (Schaefer, Tsunoda *et al*. 2016).

Lipoproteins are complex particles containing multiple proteins and lipids aimed at transporting fats throughout the organism. In addition to the doubtless importance of lipoproteins in fat absorption, utilization and cholesterol distribution, there is strong evidence that lipoproteins play substantial, but due to their different composition, varying roles in atherosclerosis (Hubkova, Body *et al*. 2018).

(Lps) are also central to other major disorders of lipid metabolism such as metabolic syndrome, obesity and diabetes II (Gursky 2015).

(Lps) are not classified as lipids but are a group of biochemical assemblies that contains both proteins and lipids, covalently or non-covalently bound to the proteins, which allow fats to move through the water inside and outside of the cells. The proteins serve to emulsify lipid molecules (Huang and Freter 2015).

(Lps) are complex molecular assemblies that are key participants in the intricate cascade of extracellular lipid metabolism with important consequences in the formation of atherosclerotic lesions and the development of cardiovascular disease (CVD) (Kwiterovich 2013).

1.4.3.1-Structure of Lipoproteins

The surface of Lps contain amphipathic phospholipids (PL), with free un-esterified cholesterol (FC) stabilizing their assembly into monolayers where the hydrophobic fatty acyl carbon chain of the PL molecules enclose a non-polar lipid core, consisting of mainly cholesterol esters (CE) and triglycerides (TG) (Pan and Segrest 2016).

1.4.3.2-Key Enzymes of Lipoprotein-Lipid Transport

Three enzymes have important roles in lipoprotein-lipid transport—lipoprotein lipase, hepatic lipase, and LCAT.Lipoprotein lipase is synthesized in a variety of tissues, but most is present in adipose tissue and striated muscle. Hepatic lipase participates in the lipolysis of VLDL and IDL during the later stages of the formation of LDL. LCAT is responsible for the synthesis of most cholesteryl esters in plasma lipoproteins (Havel 2017).

LPL is a multifunctional glycoprotein enzyme that plays an important role on lipid metabolism. After being secreted, it adheres to the luminal surface of endothelial cells where it hydrolyzes TG in circulating lipoproteins. This constitutes the limiting step on lipoprotein elimination, such as CMs from exogenous sources, and those endogenous sources, like VLDL, in circulation (Rojas, Prieto *et al*. 2017).

Lecithin-cholesterol acyltransferase (LCAT) is a key enzyme involved in the maintenance of cholesterol homeostasis and regulation of cholesterol transport in the blood (Takahashi, Hiromura *et al*. 2013).

1.4.3.3-Apolipoproteins in Lipoproteins

Apolipoproteins are the carrier proteins for lipoproteins and they consist of a single polypeptide chain often with relatively little tertiary structure. They are required to solubilise the non-polar lipids in the circulation and in some instances to recognise specific receptors. They are classified as Apo A1, A2, A4, A5, B48, B100, C1, C2, C3, D, E, H, J, L, M and Apo (a). Most apolipoproteins are synthesised by the liver and intestine (Saba and Oridupa 2012).

Apolipoprotein B is a structural protein that is an integral component of chylomicrons, as well as very low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and low-density lipoprotein (LDL) particles. Chylomicrons are secreted by the intestine and enable the transport of dietary triglycerides to other tissues after feeding. They contain a truncated form of ApoB, referred to as ApoB48 because it is 48% of the full-length protein. The other major class of lipoproteins is high-density lipoprotein (HDL). HDL does not contain ApoB (Haas, Attie *et al*. 2013).

Table (1-3): Classes of apolipoproteins, their molecular weight and functions (Feingold and Grunfeld 2015).

Apo lipoprotein	MW	Primary Source	Lipoprotein Association	Function
Apo A-I	28,000	Liver, Intestine	HDL, chylomicrons	Structural protein for HDL, Activates LCAT
Apo A-II	17,000	Liver	HDL, chylomicrons	Structural protein for HDL, Activates hepatic lipase
Apo A-IV	45,000	Intestine	HDL, chylomicrons	Unknown
Apo A-V	39,000	Liver	VLDL, chylomicrons, HDL	Promotes LPL mediated TG lipolysis
Apo B-48	241,000	Intestine	Chylomicrons	Structural protein for chylomicrons
Apo B-100	512,000	Liver	VLDL, IDL, LDL, Lp (a)	Structural protein, Ligand for LDL receptor
Apo C-I	6,600	Liver	Chylomicrons, VLDL, HDL	Activates LCAT
Apo C-II	8,800	Liver	Chylomicrons, VLDL, HDL	Co-factor for LPL
Apo C-II	8,800	Liver	Chylomicrons, VLDL, HDL	Inhibits LPL and uptake of lipoproteins
Apo E	34,000	Liver	Chylomicron remnants, IDL, HDL	Ligand for LDL receptor
Apo (a)	250,000-800,00	Liver	Lp (a)	Inhibits plasminogen activation

1.4.3.4-Classification of Lipoproteins

Lipoproteins form major classes differing in the particle size, density, biochemical composition and function: chylomicrons, high-density lipoprotein (HDL), low-density lipoprotein (LDL), intermediate density lipoproteins(IDL), and very low-density lipoprotein (VLDL) (Phillips 2013).

There are five major groups of lipoproteins, which are divided by their densities:

(A) chylomicrons, which carry triglyceride absorbed in the gastrointestinal tract from the intestine to the adipose tissue, liver and skeletal muscle and have the lowest density; **(B)** very low-density lipoproteins (VLDL) that carry triglyceride from the liver to adipose and other tissues; **(C)** intermediate-density lipoproteins (IDL), which are formed upon the metabolism of VLDL and can carry cholesterol from the liver throughout the body; IDLs are the intermediate between VLDL and LDL and usually are not detectable in the blood; **(D)** low-density lipoproteins (LDL), which also carry cholesterol from the liver to other tissues of the body; and (**E**) high-density lipoproteins (HDL), which collect cholesterol from the tissues of the body and return this lipid to the liver (Tsai, Rainey *et al*. 2016).

1.4.3.4.1-Chylomicron (CMs)

Chylomicrons (CMs) are lipoproteins that transport dietary lipids and fat soluble vitamins from the intestine to tissues expressing lipoprotein lipase (LPL). CMs belong to the largest type of lipoprotein particles (typical sizes from 70 to 600 nm) , they are spherical in shape and consist of triglycerides (TG) (85-92%), phospholipids (6-12%), cholesterol (1-3%) and proteins (1-2%) (Chernova, Konokhova *et al*. 2018).

The density of the lipoproteins is directly proportional to the protein content. Chylomicrons mobilize dietary lipids from the intestine to other tissues. They are the largest in size among the lipoproteins; the least dense and comprise of about 80% TG (Venugopal and Jialal 2018).

GLP-1, GLP-1R agonists and DPP-4 inhibitors have all shown varying abilities to suppress enteric cell chylomicron production, made phenotypically evident by a reduction in circulating triglycerides and apolipoprotein B-48 (apoB-48) (Ryan, Patterson *et al*. 2017).

1.4.3.4.2-Very Low Density Lipoprotein (VLDL)

The liver plays a central role in whole body metabolic homeostasis. It can obtain lipids from the circulation, synthesize them and secrete them in lipoproteins into the blood stream. Very-low-density lipoproteins (VLDL) transport triglycerides (TG) from the liver to peripheral tissues, providing an energy source (Martinez-Uña, Varela-Rey *et al.* 2015).

GLP-1 can also reduce VLDL production through direct effects on the liver and via suppression of apoB-48 and CM biogenesis in the gut via both direct and indirect effects such as reducing lymph flow, suppressing gastric emptying and inhibiting gastric lipase (Zhong, Maiseyeu *et al*. 2015).

Dyslipidaemia in obesity and diabetes are associated with alterations in hepatic lipid metabolism. At least some of these alterations can be corrected by GLP-1. GLP-1 agonists, for example reduce hepatic VLDL overproduction and de novo lipogenesis in hamsters. These effects were associated with decreased hepatic lipid accumulation, and a decrease in circulating VLDL-bound TAG and plasmatic ApoB-100 (Lutz and Osto 2016).

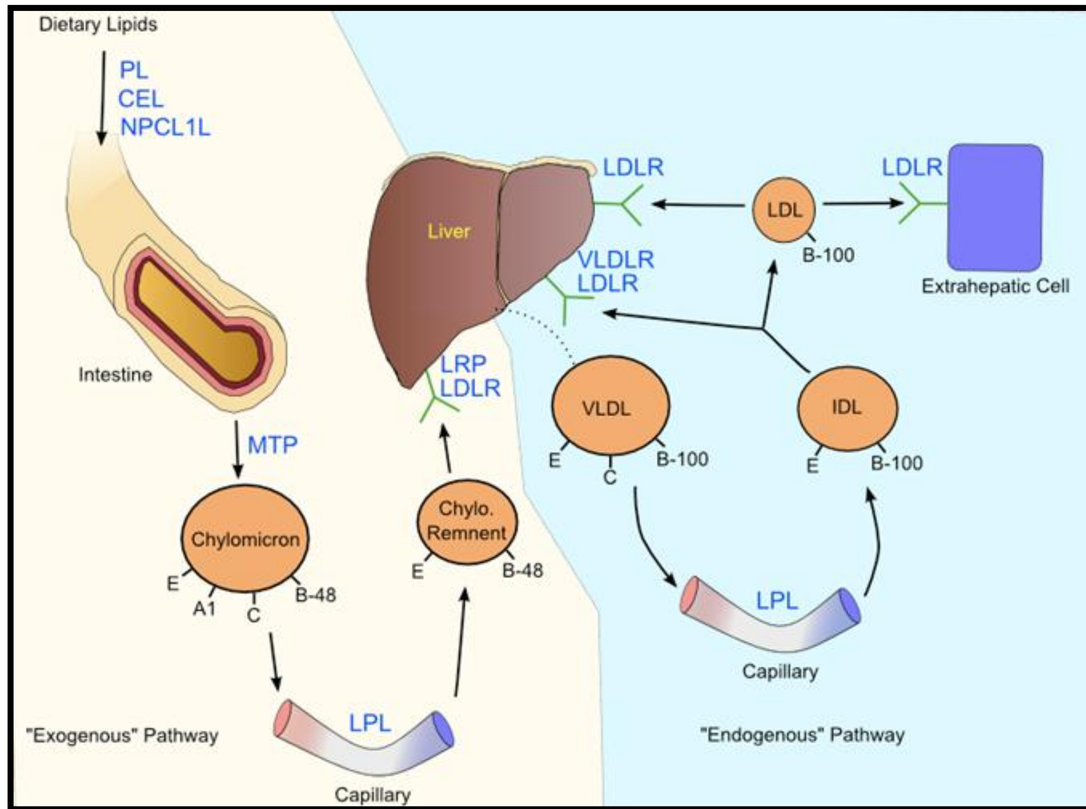


Figure (1-2): Metabolism of lipoprotein (Daniels, Killinger *et al*. 2009).

1.4.3.4.3-Intermediate Density Lipoprotein (IDL)

Intermediate density lipoprotein (IDL) consists mainly of chylomicron remnants and very low densitiy lipoprotein (VLDL) remnants that are thought to be proinflammatory lipoprotein particles (Krychtiuk, Kastl *et al.* 2016).

In metabolic experiments was discovered that intermediate density lipoproteins of density intermediate between VLDL and LDL (IDL d 1.006–1.020 g/ml) had distinct metabolic properties and hence should be viewed as a separate lipoprotein class. IDL were formed from the delipidation of VLDL and acted as a transient intermediate on the way to LDL (Meyer, Caslake *et al*. 2000).

1.4.3.4.4-Low Density Lipoprotein (LDL)

LDL is broadly defined as lipoprotein fraction with density ranging from 1.006 to 1.063 g/ml, which can be isolated by various laboratory methods. LDL particles are classified into 3 or 4 subclasses, including large (LDL I), intermediate (LDL II), small (LDL III), and, in some studies, very small (LDL IV) LDLs. LDL III and LDL IV (when discerned) are referred to as sdLDL (Ivanova, Myasoedova *et al.* 2017).

An LDL particle (20–25 nm in diameter) contains an amphiphilic phospholipid monolayer and a hydrophobic core that is rich in cholesteryl esters, Each LDL particle consists of one apolipoprotein molecule of B-100 (ApoB-100), which covers half of the particle surface via the complex amphipathic a-helix protein–lipid interaction to stabilize this remarkable nanostructure (Zhu and Xia 2017).

The terms 'cholesterol', 'LDL', and 'LDL cholesterol (LDL-C)' are frequently conflated or used interchangeably, potentially leading to confusion. Cholesterol is an essential component of cell membranes and a precursor of bile acids and steroid hormones. Importantly, cholesterol of both exogenous and endogenous origin is transported to peripheral cells largely by the apoB-containing lipoproteins in plasma. In most people, LDL particles constitute 90% of circulating apoB-containing lipoproteins in fasting blood (Ference, Ginsberg *et al*. 2017).

Elevated levels of LDL cholesterol (LDL-C) are a well-established risk factor for the development of cardiovascular disease [2]. LDL-C is composed of a spectrum of LDL particles of different densities and states of lipidation, all of which are atherogenic. Levels of the densest LDL-C particles may be disproportionately raised in patients with low HDL cholesterol (HDL-C) and high

triglycerides. Analysis of changes in LDL subtractions may provide additional information to help direct individual patient treatment (Toth, Hamon *et al*. 2016).

1.4.3.4.5-High Density Lipoprotein (HDL)

High-density lipoproteins (HDL) are endogenous nanoparticles involved in the transport and metabolism of cholesterol, phospholipids, and triglycerides. HDL is well-known as the "good" cholesterol because it not only removes excess cholesterol from atherosclerotic plaques but also has anti-inflammatory and ant oxidative properties, which protect the cardiovascular system. Circulating HDL also transports endogenous proteins, vitamins, hormones, and microRNA to various organs (Kuai, Li *et al*. 2016).

HDL is mainly secreted by the liver and small intestines. The liver, which secretes ~70-80% of the total HDL in plasma, is the main source of HDL in the circulation.HDL is heterogeneous particles regarding their size and composition. Compared with other lipoproteins, they have the highest relative density while being smallest in size. HDL has an important role in carrier in reverse cholesterol transport (RCT) and act as a carrier of cholesterol back to the liver. HDL particles are highly uniform and can be divided into several sub-types based on their composition proteins or bulk density: HDL2 and HDL3 (Zhou, Li *et al*. 2015)

The de novo synthesis of HDL involves the secretion of apoA-I by the liver and small intestine into the circulation, followed by a largely extracellular acquisition of phospholipids (PL) and cholesterol leading to the formation of nascent HDL (Oldoni, Sinke *et al*. 2014).

HDL has been demonstrated to mediate a range of beneficial actions in diabetes. An emerging body of evidence suggests a direct role for HDL in glycaemic control through its actions on pancreatic beta cells (Wong, Nicholls *et al*. 2018).

1.5-Incretin Hormones

Incretins are hormones secreted into the blood stream from the gut mucosa in response to nutrient intake. They have been characterized based on their capacity to lower blood glucose levels (Seufert 2017).

Incretin hormones are gut peptides that are secreted after nutrient intake and stimulate insulin secretion together with hyperglycaemia. GIP (glucose-dependent insulinotropic polypeptide) and GLP-1 (glucagon-like peptide-1) are the known incretin hormones from the upper (GIP, K cells) and lower (GLP-1, L cells) gut (Nauck and Meier 2018).

Both hormones are rapidly degraded by the enzyme dipeptidyl peptidase IV (DPP-IV), inhibition of which is a novel approach to enhance incretin concentrations in the treatment of type 2 diabetes (Berglund, Lyssenko *et al.* 2015).

GLP-1 is degraded by dipeptidyl peptidase-4 (DPP-4) more rapidly than GIP, and circulating levels of biologically intact GLP-1 are substantially lower than those of biologically intact GIP (Yabe, Seino *et al.* 2018).

Cleavage of the incretins by DPP-4 results in loss of these hormones' insulinotropic activities and initiates their degradation (Bohm, Wagner *et al.* 2017).

1.5.1-Types of Incretin Hormones

Incretins are naturally occurring glucoregulatory hormones released by the gut into the blood in response to nutrient ingestion. They have a short half-life because of their rapid inactivation by dipeptidyl peptidase 4 (DPP-4) enzymes. Two important incretin hormones identified to have major effects on carbohydrate metabolism are Glucose dependent insulinotropic polypeptide (GIP) and GLP-1 (glucagon-like peptide-1) (Kohli 2016).

The incretin hormones are synthesized by enteroendocrine cells (EECs) and stimulate insulin secretion from pancreatic β-cell (Cho, Fujita *et al*. 2014).

GLP-1 and GIP are produced by L-cell in the distal small intestine and K-cell in the proximal small intestine (Oh 2016).

GLP1 stimulates insulin and suppresses glucagon secretion. GIP is also insulinotropic but, in contrast, may stimulate glucagon secretion, particularly at a lower blood glucose level. Importantly, the effects of GLP-1 and GIP are glucose dependent, so that exogenous administration of GLP-1 and/or GIP, even at pharmacological doses, does not cause hypoglycaemia (Kar, Cousins *et al.* 2015).

1.5.1.1-Glucose-Dependent Insulinotropic Polypeptide (GIP)

Glucose-Dependent Insulinotropic Polypeptide is an endogenous 42-amino acid peptide hormone synthesized in and released from intestinal K-cells. GIP initially was named gastric inhibitory polypeptide based on its ability to inhibit gastric acid secretion. However, subsequent studies revealed that GIP could also stimulate insulin secretion in animals and humans (Li, Li *et al*. 2016). DPP4 cleaves GIP to release the dipeptide (Tyr-Ala) (Deacon, Nauck *et al*. 2000).GIP is a physiological substrate for DPP4 because plasma levels of intact GIP are increased in animals and humans after administration of DPP4 inhibitors, and levels of intact GIP are increased in Dpp4 deficient mice (Marguet, Baggio *et al.* 2000).

GIP is an incretin hormone, released from the gut endocrine K cells, that promotes insulin secretion from pancreatic beta cells in a glucose-dependent manner. After secretion, GIP is rapidly inactivated by dipeptidyl peptidase-4 (DPP-4) by cleavage into truncated forms that are no longer insulinotropic.The polypeptide was originally discovered as a 42 amino acid peptide (Yanagimachi, Fujita *et al.* 2016).

GIP stimulates glucagon secretion under euglycemic and hypoglycemic conditions in healthy individuals, as well as in patients with T2DM and type 1 diabetes (Christensen, Calanna *et al.* 2014). The incretin action of GIP seems to be impaired in patients with type 2 diabetes , it might be postulated that a stimulation of GLP-1 and an inhibition of GIP secretion could be a therapeutic objective in overweight patients with type 2 diabetes (Reimann and Gribble 2016).

GIP has the glucose-dependent insulinotropic effect by interacting with its receptor GIPR. It can also help increase the transcription of insulin gene and the expression of GLUT-1 (glucose transporter-1) and hexokinase-1 genes (Ma, Huang *et al.* 2018).

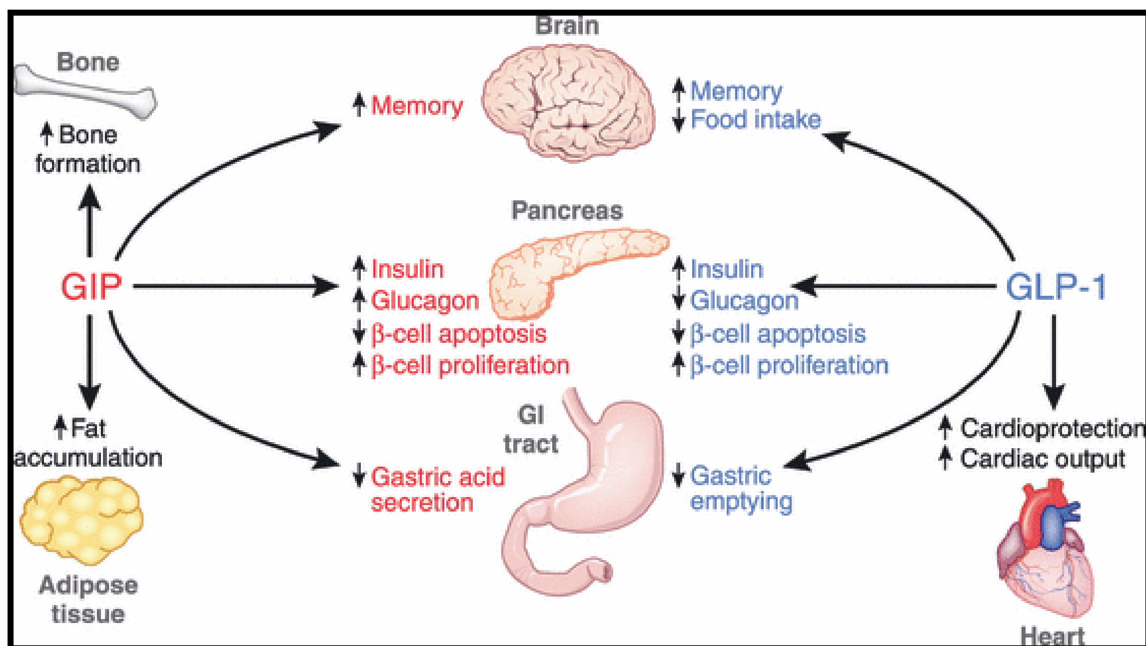


Figure (1-3):Pancreatic and exopancreatic function of glucose-dependent insulinotropic polypepide (GIP) and glucagon-like peptide (GLP-1) (Seino, Fukushima *et al.* 2010).

1.5.2-Action of Incretin Hormones in Type 2 Diabetes Mellitus

Incretin-based T2DM therapies increase circulating GLP-1 levels directly via GLP-1 analogs or indirectly through inhibition of dipeptidyl peptidase-4 (DPP-4)-inactivating enzyme. Treatment of patients with T2DM with the GLP-1 analog exenatide or with DPP-4 inhibitor preserves β-cell function and dramatically improves glucose control (Cox, Lam *et al*. 2017).

The ability of the incretin agents (glucagon-like peptide 1(GLP-1) agonists and dipeptidyl peptidase IV (DPP-4)inhibitors) to improve glycaemia with a low associated risk of hypoglycaemia, together with beneficial/neutral effects on body weight, offers a significant advantage for both patients and treating clinicians (Mudaliar and Henry 2012).

The gut hormones, (GIP) and (GLP-1) are secreted in response to nutrient intake and play an essential role for postprandial glucose regulation by potentiating glucose-stimulated insulin secretion, a phenomenon called the incretin effect (Kuhre, Albrechtsen *et al*. 2015).

The impaired incretin effect in T2DM contributes to the impaired glycaemic control. Since the incretin effect is mediated by the incretin hormones, it might be expected that by increasing levels of the intact incretin hormones, DPP-4 inhibitors should be associated with an improvement in the incretin effect (Andersen, Deacon *et al*. 2018). The incretin hormones include (GLP-1) and glucose-dependent insulinotropic polypeptide, both of which may also promote proliferation/neogenesis of beta cells and prevent their decay (apoptosis). Both hormones contribute to insulin secretion from the beginning of a meal, and their effects are progressively amplified as plasma glucose concentrations rise. (Mirosevic, Blaslov *et al*. 2017).

The incretin effect is due to the secretion of (GLP-1) and (GIP) which increases the glucose-induced insulin secretion (Larsen and Torekov 2017). In patients with type 2 diabetes, the incretin effect is impaired (Holst, Pedersen *et al.* 2017).

In addition to that, studies concerning GLP-1 secretion in patients with type 2 diabetes during an oral glucose tolerance test (OGTT) have observed both unaltered , and reduced GLP-1 responses, suggesting a varying GLP-1-secreting profile during the development and progression of type 2 diabetes or difference in the measurement technique . This is further supported by the observation that several factors (BMI, glucagon, age, and nonesterified fatty acids (NEFA)), including medications (metformin), influence the secretion of GLP-1 (Larsen and Torekov 2017).

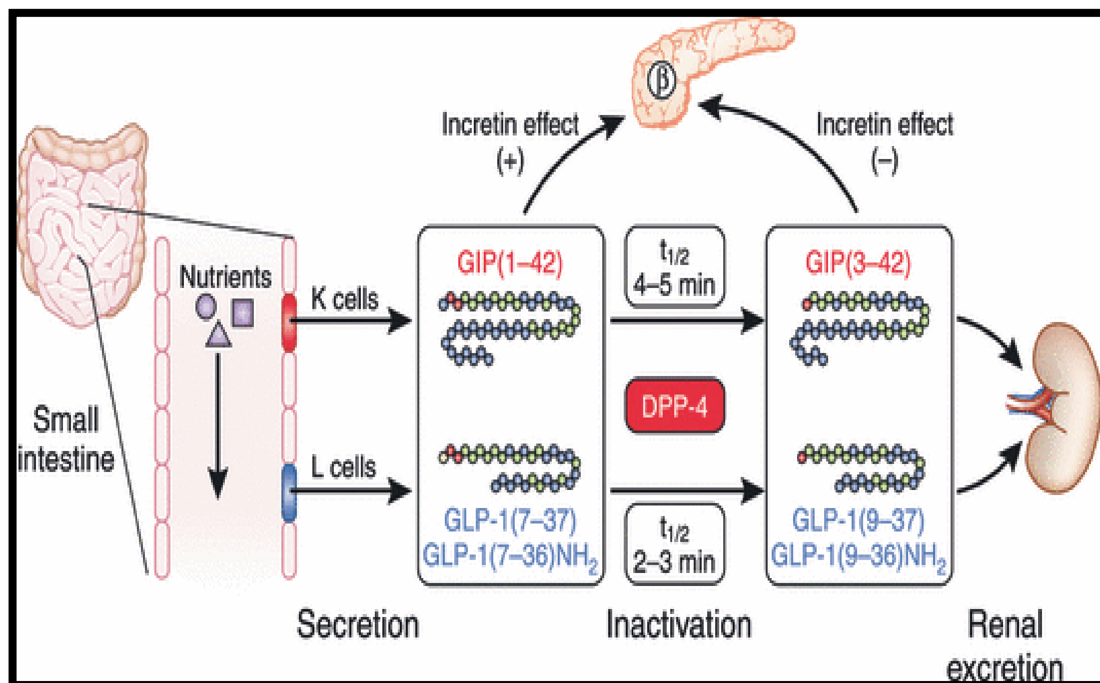


Figure (1-4): Secretion and metabolism of glucose-dependent insulinotropic polypepide (GIP) and glucagon-like peptide (GLP)-1(Seino, Fukushima *et al.* 2010).

Incretin treatments consist of either oral DPP-4 inhibitors, which decrease the clearance of secreted incretins GLP-1 and GIP, or injectable analogues of GLP-1.

Relative freedom from hypoglycaemia, an absence of weight gain, and additivity in glucose control in combination with metformin have largely driven the increasing acceptance of these drugs (Garber 2014).

In T2DM, the incretin effect is reduced, but therapeutically, incretin activity can be provided by supraphysiological dosages of GLP-1 or related agents stimulating the GLP-1 receptor (GLP-1R) . Incretin hormones may have effects beyond the stimulation of insulin secretion , and the proteolytic activity of DPP-4 is not restricted to the degradation and inactivation of the incretin hormones (Nauck 2016).

Five GLP-1 had been approved by the US Food and Drug Administration (US FDA) for the treatment of T2DM: exenatide twice a day ('exenatide BID') (Byetta®), exenatide once weekly ('exenatide QW') (Bydureon®), liraglutide once daily (Victoza®), albiglutide once weekly (Tanzeum®), and dulaglutide once weekly (Trulicity®). A sixth GLP-1 RA lixisenatide (Adlyxin™) (Alatorre, Fernández Lando *et al*. 2017).

To develop incretin-based drugs, several issues had to be resolved. First, secreted incretins undergo rapid degradation catalyzed by DPP4, which diminishes the insulinotropic effects of GIP and GLP-1. Second, it was initially reported that the insulinotropic effects of GIP are attenuated in individuals with type 2 diabetes (Seino, Kuwata *et al*. 2016).

1.6-Dipeptidyl-peptidase 4 (DPP-4)

Dipeptidyl-peptidase 4 was discovered in 1966 as a new amino peptidase with unique substrate characteristics (Hopsu and Glenner 1966).

It was later determined to be identical to the T-cell activation antigen cluster of differentiation (CD)-26, rat liver membrane glycoprotein gp110, and the mouse thymocyte-activating molecule (Vivier, Marguet *et al*. 1991).

The 70-kb human gene identified in 1992, and located on the long arm of chromosome 2 (2q24.3) and comprises 26 exons that encode a 766-amino acid protein; the classic serine protease catalytic site is encoded by two exons, exons 21 and 22, respectively. In the mouse, Dpp4 is found on chromosome 2 (2C2–2D), and interestingly, exon 21 and exon 22 are present as a single 156-bp exon (Bernard, Mattei *et al*. 1994).

(DPP4) is the target of the gliptins, a recent class of oral anti-diabetics (Zilleben, Celner *et al*. 2016).

DPP4 is a 110-KDa type 11 integral membrane glycoprotein and is expressed ubiquitously in most organs and cell types. DPP4 exists in both a soluble and membrane bound form, both of which are capable of proteolytic activity. The soluble form in the circulation is thought to arise from shedding of the membrane bound DPP4 and is the target for DPP4inhibitor as hypoglycemic agents in clinical use (Panchapakesan and Pollock 2015).

The enzyme DPP-4 is classified as a glycoprotein, composed of three distinct regions: (i) cytoplasmic-residues from 1 to 6; (ii) transmembrane-residues 7 to 29 and (iii) extracellular-residues 30 to 766. Its most important catalytic function is the degradation of incretins such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), in which metabolic regulation contributes to the control of glucose levels in the blood (Zhong, Maiseyeu *et al*. 2015).

DPP-4 enzyme rapidly deactivates GLP-1 (7–36) amide and GIP (1–42) amide into inactive forms of GLP-1 (9–36) amide and GIP (3–42) amide, respectively (Jamaluddin, Huri *et al*. 2016).

A soluble form of DPP-4 that lacks intracellular and transmembrane regions is present in body fluids such as serum/plasma, cerebrospinal fluid, synovial fluid, bile, and semen, presumably as a result of its release from many cell types, including lymphocytes, hepatocytes, and adipocytes (Kim, Yu *et al.* 2014).

Non-catalytic functions of DPP-4, such as T cell co-stimulation, depend on the interaction with another protein known as adenosine deaminase (ADA). The active site of DPP-4 is divided into sub-regions: a catalytic triad consisting of Ser630, Asp708 and His740; an oxyanion cavity containing Tyr47 and Ser631; a region with saline bridging residues such as Glu205, Glu206 and Tyr662 (Pantaleao, Philot *et al.* 2018).

(DPP-4) is a surface T cell activation antigen and has been shown to have DPP4 enzymatic activity, cleaving-off amino-terminal dipeptides with either L-proline or L-alanine at the penultimate position. It plays a major role in glucose metabolism by N-terminal truncation and inactivation of the incretins glucagon-like peptide-1 (GLP) and gastric inhibitory protein (GIP) (Klemann, Wagner *et al.* 2016).

(DPP4) is a multifunctional protein that exerts biological activity through pleiotropic actions including: protease activity , association with adenosine deaminase (ADA) , interaction with the extracellular matrix , cell surface coreceptor activity mediating viral entry , and regulation of intracellular signal transduction coupled to control of cell migration and proliferation (Mulvihill and Drucker 2014).

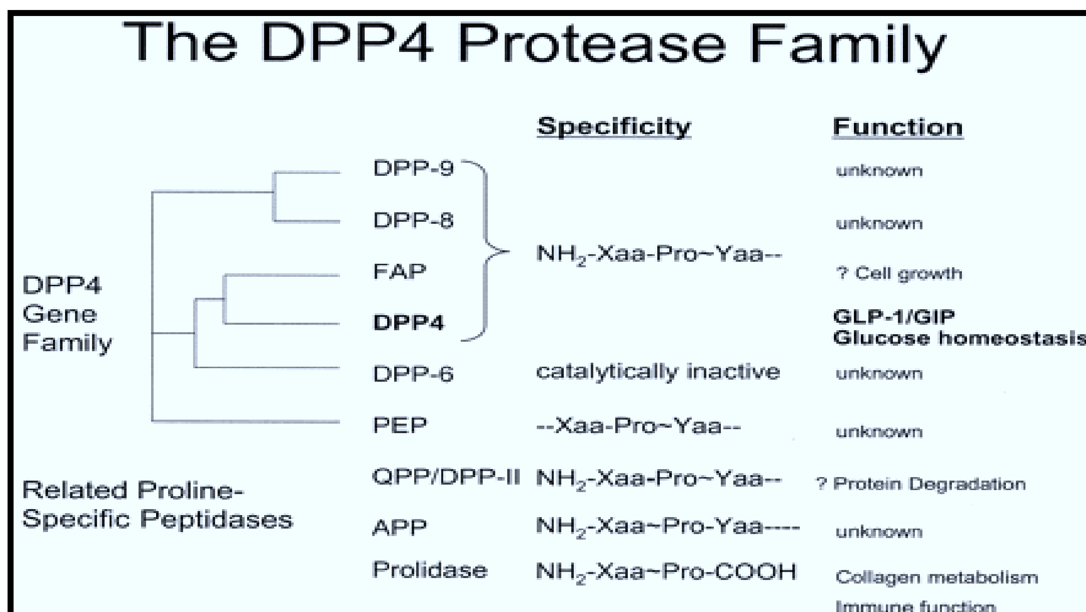


Figure (1-5): Family of DPP-4–related proteases and their substrate specificities (Drucker 2007).

1.6.1-The Regulation of Dipeptidyl-peptidase DPP-4

DPP-4 expression is influenced by hypoxia, and hypoxia inducible factor-1 α (HIF-1α) is a strong inducer of DPP-4 gene and protein. Besides HIF-1 α, hepatocyte nuclear factor-1 α (HNF-1 α), interferons, retinoic acid, and various cytokines have been shown to activate DPP-4 .The promoter of human DPP-4 gene contains putative binding sites for Sp1, AP-1/2, epidermal growth factor receptor-transcription factor site, HNF-1, signal transducer and activator of transcription 1 α, and nuclear factor- α B (NF α B) (Kim, Yu *et al*. 2014).

1.6.2-Action of the Dipeptidyl-peptidase (DPP-4) Inhibitor and the Treatment of Type 2 Diabetes Mellitus

(DPP4) inhibitors, such as sitagliptin, saxagliptin and linagliptin, are oral glucose-lowering drugs that can be used as monotherapy or combination therapy with other oral hypoglycaemic agents for (T2DM) (Kim, Schneeweiss *et al.* 2015).

Of over 380 million people with diabetes worldwide, most (85-95%) have T2DM. (DPP-4) inhibitors are a relatively new class of incretin based agents for treating type 2 diabetes. Evidence from randomised controlled trials has established that DPP-4 inhibitors reduce levels of glycated haemoglobin (HbA1c), do not affect body weight, pose a low risk of hypoglycaemia (Li, Li *et al*. 2016).

(DPP-4) inhibitors are effective antihyperglycaemic agents by virtue of their ability to inhibit the breakdown of the active form of the incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) (Berger, SinhaRoy *et al.* 2018). DPP-4 inhibition is an established option for management of hyperglycaemia in T2DM. DPP-4 inhibition has a low risk for hypoglycaemia and weight gain and has been shown to be safe from a cardiovascular point of view (Farngren, Persson *et al*. 2018).

DPP-4 inhibitors are not considered as initial therapy for the majority of patients with T2DM. Initial therapy in most patients with T2DM should begin with diet, weight reduction, exercise, and metformin (in the absence of contraindications). DPP-4 inhibitors can be considered as monotherapy in patients who are intolerant of or have contraindications to metformin, sulfonylureas, or thiazolidinediones (Dungan and DeSantis 2017).

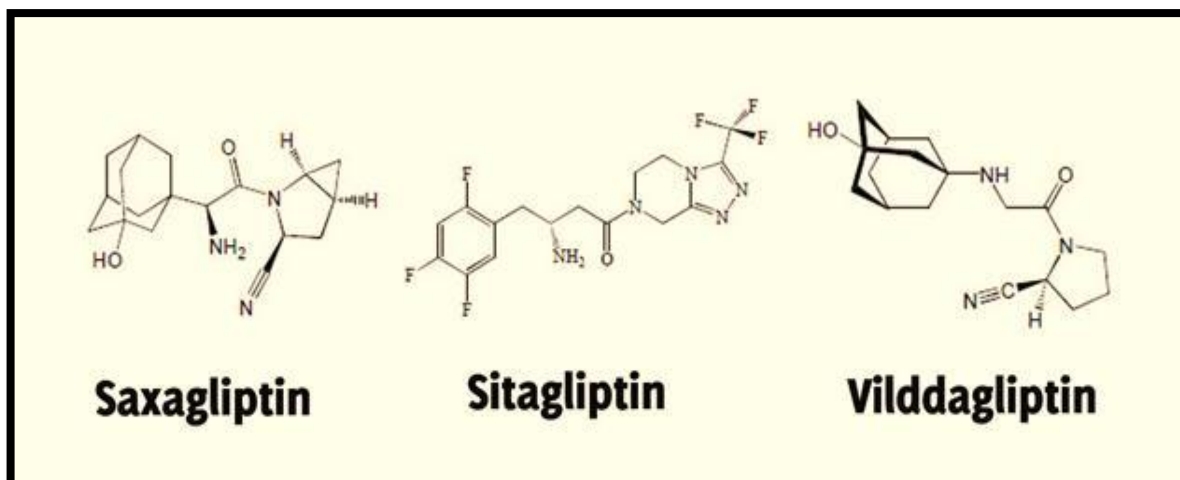


Figure (1-6): Chemical Structures of Selected DPP-4 Inhibitors (Lotfy, Singh *et al*. 2011)

The inhibition strategy of the (DPP-4) enzyme is currently employed in the treatment of T2DM. However, some substances available on the market can cause anemia, neuropathic risk, pancreatitis and nausea (Pantaleao, Philot *et al*. 2018).

DPP-4 inhibitors are a successful class of anti-diabetic agents (at least eleven different inhibitors are approved in various parts of the world), which improve glycaemic control with similar efficacy to other oral agents, but without many of the adverse effects of other therapies (Deacon and Lebovitz 2016).

They were developed in order to limit the rapid degradation of the endogenous incretin hormone, (GLP-1) and, thereby, to enhance its anti-hyperglycaemic actions. GLP-1 is a gut hormone, released in response to digestion and absorption of food in the small intestine, which is responsible for an important part of postprandial insulin secretion (Andersen, Deacon *et al.* 2018).

The classical mechanism for DPP-4 inhibition is that due to prevention of inactivation of GLP-1 in the peripheral circulation, the increased circulating intact GLP-1 results in stimulated insulin secretion and inhibited glucagon secretion, resulting in increased glucose utilization and diminished hepatic glucose production, which, through reduction in postprandial and fasting glucose, reduce HbA1C, This Perspectives in Diabetes summarizes these nonclassical effects to illustrate the mechanistic complexity of this strategy to lower glucose in T2DM (Omar and Ahren 2014).

DPP-4 inhibitors is is a new class of anti-diabetes which can prevent the rapid degradation of (GIP) and (GLP-1) through inhibition of DPP-4 (Fadini, Bonora *et al*. 2016).

The use of DPP-4 inhibition in the treatment of T2DM has increased considerably since their introduction a decade ago (Christensen, Rungby *et al.* 2016).

These second to third line treatments have been shown to have favourable effects compared with other antidiabetic drugs, such as lowering the risk of hypoglycaemia and having neutral effects on body weight and cardiovascular outcomes (Son and Kim 2015).

The first DPP-4 inhibitor to reach the market was sitagliptin, followed by vildagliptin and more recently by saxagliptin, alogliptin, and linagliptin (Plosker 2014).

DPP-4 inhibitors have different pharmacokinetic and pharmodynamic profiles, they are remarkably similar with regard to their antihyperglycaemic properties with a very safe profile (neutral concerning weight, without causing hypoglycaemia). These agents are all low-molecular-weight compounds, although they differ widely in terms of their chemical structure (Godinho, Mega *et al*. 2015).

Ordered by time-to-market, are authorized on the market, These drugs block the degradation of incretins (GLP-1 and GIP) by DPP-4, and potentiate insulin secretion following food intake (Yang, Huang *et al*. 2017).

DPP-4 inhibitors prevent the breakdown of GLP-1 and GIP, two incretins pivotal for glucose regulation. The four DPP-4 inhibitors currently approved for the treatment of T2DM in the United States include sitagliptin, saxagliptin, linagliptin, and alogliptin (Bittle 2017).

Treatment should target multiple defects in T2DM and follow a patient-centered approach that considers factors beyond glycemic control, including cardiovascular risk reduction. Therapy choices are guided by glycemic efficacy, safety profiles, particularly effects on weight and hypoglycemia risk, tolerability, patient comorbidities, route of administration, patient preference, and cost. Balancing management of hyperglycemia with the risk of hypoglycemia and

consideration of the effects of pharmacotherapy on weight figure prominently in US-based T2DM recommendations (Thrasher 2017).

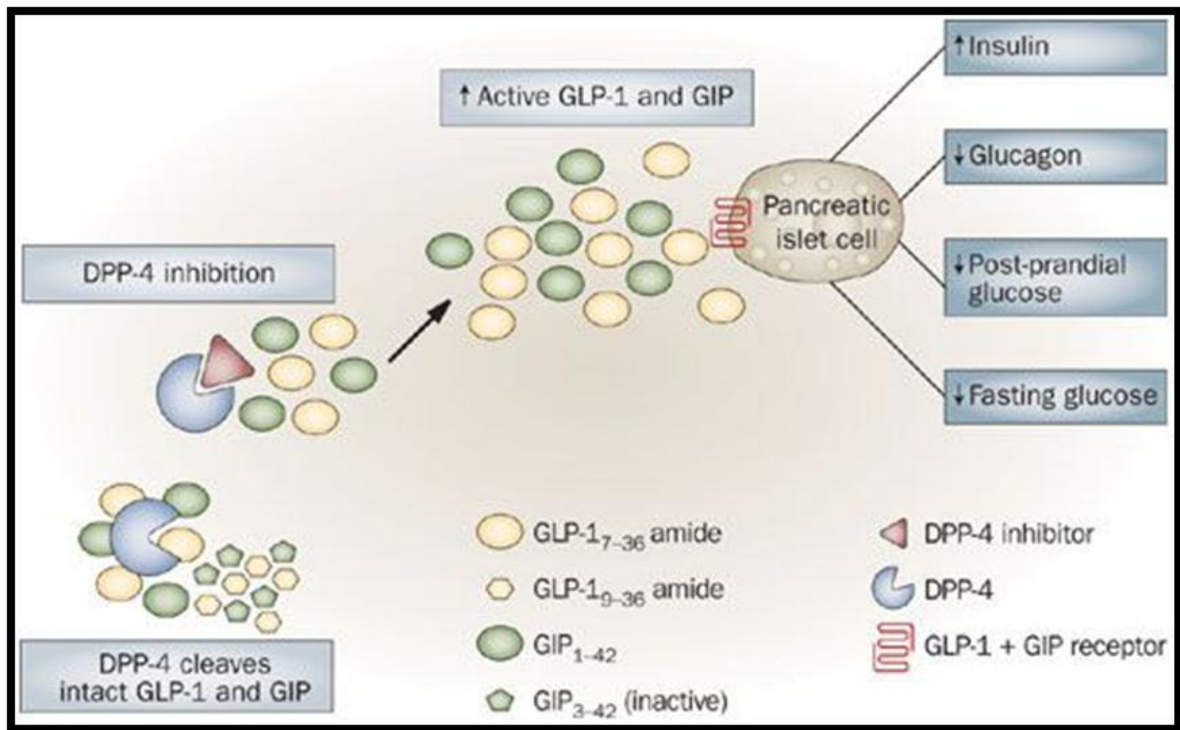


Figure (1-7):Incretin Based Therapies For Type 2 Diabetes Mellitus (Lovshin and Drucker 2009)

1.7-Genetic of type2 Diabetic Mellitus

T2DM is a disease characterized by insulin resistance and impaired pancreatic beta-cell function that affects >170 million people worldwide. With first degree relatives having ~3.5 times as much risk as compared to individuals in the general middle aged population, hereditary factors, together with lifestyle and behavioral factors, play an important role in determining T2DM risk (Scott, Mohlke *et al.* 2007).

T2DM is a complex disease that is caused by a complex interplay between genetic, epigenetic and environmental factors. While the major environmental factors, diet and activity level, are well known, identification of the genetic factors has been a challenge (Rios, Francini *et al.* 2015).

Many genetic variants contribute to the risk of T2DM. According to the 'common disease, common variant' hypothesis, a large number of common variants (with frequency >5%) with small effect sizes and low penetrance could cause the disease, whereas according to the 'common disease, rare variant' hypothesis, the disease is caused by multiple rare variants s (frequency <0.5%)with large effect sizes and high penetrance (Stancakova and Laakso 2016).

Hyperglycemic conditions in late stages of diabetic individuals include well known micro-vascular complications (nephropathy, neuropathy, retinopathy) and macro-vascular complications (atherosclerosis and cardiovascular) (Rani, Mittal *et al*. 2017).

The genetics of T2DM are not completely known. They are complex, and current evidence suggests that multiple genes in pancreatic beta cell failure and insulin resistance are involved. Specifically identified genetic variants account for about 10% of the heritable component of most cases of T2DM (Michael Jay Katz 2017).

1.7.1-Polymorphism of DPP 4 Gene and type2 Diabetes Mellitus

A DNA polymorphism is a difference in the nucleotide sequence between individuals of the same species. These differences can be single base pair changes, deletions, insertions, or even changes in the number of copies of a given DNA sequence.

The SNPs (single nucleotide polymorphisms) are the most common type of DNA polymorphism in humans.

A polymorphic variant of a gene may lead to the abnormal expression or to the production of an abnormal form of the gene; this may cause or be associated with disease (Kwok 2003, Cardiol 2014).

SNP is the simplest form of polymorphism found in human genome; on which one nucleotide substituted by another one, insertion or deletion. The SNP is estimated to occur in every 500-1000 base pairs in the human genome, and may occur both in coding and noncoding region (Shastry 2002).Since the completion of the Human Genome Project 2003 (Collins, Morgan *et al.* 2003) and the International Haplotype Map Project in 2005 (Thorisson, Smith *et al*. 2005).

SNPs are considered as being one of the most important genetic markers due to its abundance in the genome and relatively easy analysis (Shi, Wang *et al.* 2009).

Development of T2DM can be attributed to the combined effect of genetic and environmental factors. Establishment of single-nucleotide polymorphism (SNP) databases, development and improvement of cost-effective high-throughput genotyping technology, and multi-center consortium large-scale genome-wide association studies (GWAS) is an effective method to investigate genetic susceptibility to T2DM (Xiao, Zeng *et al*. 2016).

T2DM is a multifactorial disease caused by a complex interplay of multiple genetic variants and many environmental factors. genome-wide association (GWA) studies, the number of replicated common genetic variants associated with T2DM has rapidly increased (1–7) (Van Hoek, Dehgan *et al*. 2008).

Ninety-nine percent of human DNA sequences are identical. Polymorphisms are the basis of genetic heterogeneity among individuals. SNPs are alterations in DNA at the single base level that are the most frequent variations in human genome. Many techniques for genotyping had been designed based on polymerization such as allele-specific primers and tetra-primer amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) (Ugozzoli and Wallace, 1991; Ye *et al*., 2001).

Introduced tetra-primer amplification refractory mutation system PCR (tetra-primer ARMS PCR) as a simple, effective, and economical SNP genotyping method, which uses four primers in one PCR, followed by gel electrophoresis (Mesrian Tanha, Mojtabavi Naeini *et al*. 2015).

PCR is the most common technique used for low- and medium-throughput SNP genotyping (Chuang, Yang *et al.* 2008).

Several PCR methods are available and selecting the most suitable one for each research is a critical step for successful study. Type of polymorphism, accuracy of genotyping, number of samples, and available PCR equipments are factors to be taken into account when making that choice (Hamajima, Saito *et al.* 2002).

The (tetra-primer ARMS PCR or T-ARMS PCR), also known as PCR-confronting two-pair primers (PCR-CPP) or Biallelic-ARMS, which was derived from general PCR, and specially utilized for detection of known SNPs, offers the advantages of fast and cost-effective detection utilizing a single PCR reaction to detect both alleles, with no need for a restriction enzyme (Guan, Shi *et al*. 2014).

1.7.2-Gene Map of DPP 4

Human DPP 4 gene is located on the long arm of chromosome 2 and region 2q24.2 and comprises 26 exons that encode a 766-amino acid protein; the classic serine protease catalytic site is encoded by two exons, exons 21 and 22, respectively (Mulvihill and Drucker 2014) and (Drucker 2003).

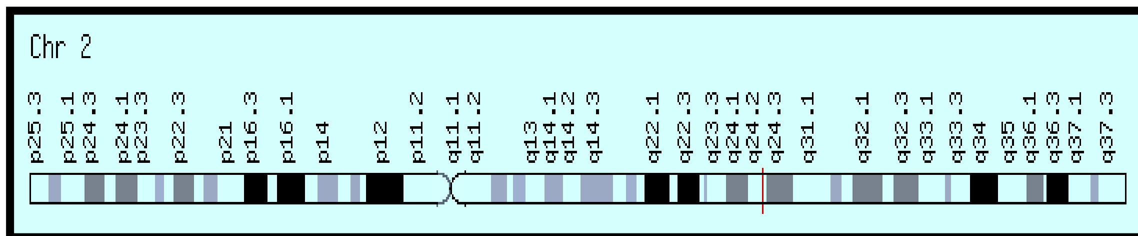


Figure (1-8): Gene map of DPP 4 (Chen, Kelly *et al*. 2003)

The protein encoded by the DPP4 gene is an enzyme expressed on the surface of most cell types and is associated with immune regulation, signal transduction, and apoptosis. It is a type II transmembrane glycoprotein, but a soluble form, which lacks the intracellular and transmembrane part, is present in blood plasma and various body fluids. DPP-4 is a serine exopeptidase that cleaves X-proline or X-alanine dipeptidesfrom the N-terminus of polypeptides. Peptide bonds involving the cyclic amino acid proline cannot be cleaved by the majority of proteases and an N-terminal X-proline "shields" various biopeptides (Vanhoof et al. 1995).

Chapter Two

Materials
&
Methods

2-Materials and Methods

2.1-Subject

A case-control study was carried on One hundred and twenty patients with age range (40-59years) who were admitted to Al-Hassan diabetic and endocrinology care center in Imam Hussein medical city/Holy Kerbala governorate /Iraq, and registered in the center as type 2 diabetes mellitus patients from January to April 2018.

The subjects of the study are divided into two groups:

a) **Patients group**: One hundred and twenty candidates (**63** male and **57** female).

b) **Control group**: One hundred and twenty participants (**65** male and **55**female).

Table (2-1): Control vs. Diabetic group

Group	Subjects	Age (year)	Age (year) (Mean±SD)	BMI (Kg/m^2) (Mean±SD)
Control	120	40-59	49.26± 4.75	28.95± 4.02
Diabetic	120	40-59	49.16± 4.66	28.69± 3.47

All subjects' history, blood tests investigations and body mass index have been collected as data for this study. Fasting blood samples have been collected during the period from January to April 2018

2.1.1-Patients Group

Patients were chosen random to have essential diabetic of various duration based on physicians' diagnosis prior to the current visit to the Al-Hassan diabetic and endocrinology care center in Imam Hussein medical city in holy Kerbala governorate where this research been conducted. Some of them were taking medications such as (*Glucophage* (metformin)) and others were not. But all patients have no obvious symptom and signs of renal or liver problems.

2.1.2-Control Group

One hundred and twenty candidates of this group have neither symptoms nor signs of Diabetic so they apparently healthy.

2.1.3-Inclusion Criteria

1- No family history of any disease.

2- Matched to patients with regard to age, gender.

2.1.4-Exclusion Criteria

Any subjects with a history of coronary arteries disease, hypertension, and renal disease were excluded from the study.

2.1.5-Specimen Collection

Almost (5 mls) of venous blood was drawn from each candidate (healthy and diabetic patients) using disposable syringes in sitting position after being fasting for at least 12 hours. (2 mls) of this sample were collected in Ethyline Diamine Tetra Acetic Acid (**EDTA)** containing tube for HbA1c, DNA extraction and PCR. The remaining was transferred into a clean plain tube(gel disposable tube), and left at room temperature for nearly thirty minutes for clotting, then centrifuged for

approximately 10-15 minutes. Serum was divided into parts for Incretin hormones (GIP), fasting serum glucose, and lipid profile.

2.2-Body Mass Index (BMI)

Body Mass Index was calculated by dividing the body weight in kilogram (kg) by the square of height in meter (m) according to the following equation

BMI = Weight (kg)/Square Height (m^2) (Park et al. 2003)

Therefore its unit is (kg/ m^2)

2.3-Materials

2.3.1-Apparatus & Equipments

All the instruments and tools which are used in this study are shown in the table below:

Table (2-2): List of Apparatus & Equipments used

NO.	Apparatus & Equipments	Company/Country
1	Balance	Precis / Switzerland
2	Clean View – UV Cabinet	Cleaver / USA
3	Electrophoresis apparatus	Consort / Belgium
4	Electrophoresis power supply	Consort / Belgium
5	Electrophoresis tank	Consort / Belgium
6	High speed Centrifuge	Mikro 200R Hettich / Germany
7	Hot Plate Magnetic Stirrer	LabTech DAIHAN / Korea
8	HumaReader HS ELISA	(Human) / Germany
9	Micro-Centrifuge	BIONEER / Korea
10	Micropipettes	Eppendorf / Germany
11	Nano drop UV- Spectrophotometer	Quawell Q5000 / USA
12	Shaking Water bath	Grant / England
13	Thermo cycler PCR instrument	Cleaver / USA
14	UV-VIS Spectrophotometer	UV-VIS /JAPAN
15	Vortex – mixture	(Karlkole) / Germany

2.3.2-Chemicals and Kits

The chemicals with their suppliers which are used in this study are shown in the table below:

Table (2-3): Chemicals and Kits with their suppliers

NO.	Chemicals	Company / Country
1	Ethidium Bromide	Sigma / USA
2	Agarose gel	Analytical Grade/ USA
3	DNA ladder (100bp)	Bioneer/ KOREA
4	TBE (Tries Borate EDTA) buffer solution	Bio basic/ Korea
5	Loading dye	Bonier/ KOREA
6	Nuclease Free Water	Promega/USA
7	PCR Premix	Bonier/ KOREA
8	Relia prep DNA purification Kit	Promega/USA
9	Glucose kit	BAIOLABO / France
10	HDL-cholesterol kit	Linear / Spain
11	Hemoglobin(HbA1c)Kit	Stan bio lab. / USA
12	Human GIP / ELISA Kit	YH Bio search Lab./ China
13	Total cholesterol MR kit	Linear / Spain
14	Triglyceride kit	BAIOLABO / France

2.3.3-Specific primers of DPP4 Gene rs1861978

The Arms-tetra primers for detection of SNP rs1861978 current study are shown in the table below:

Table (2-4): Specific primers of DPP4 Gene rs1861978

Primer	Sequence (5´ –3´)	Size(bp)	Allele	Company Country
Inner forward	CGTGAAAGCCGCAAGAGTTT	185	T	BIONEER/ Korea
Outer forward	CCTCCCCACCCTCCAACT	495		
Inner Reverse	GGTCTTCAGTGTTTAGGCTGC	350	G	
Outer reverse	TGGAGCTCAGGTCAGGCT	495		

The Arms-tetra primers of SNP rs1861978 current study are design by Dr. Hassan Mahmood Abo Al-maali / College of Pharmacy- University of Kerbala.

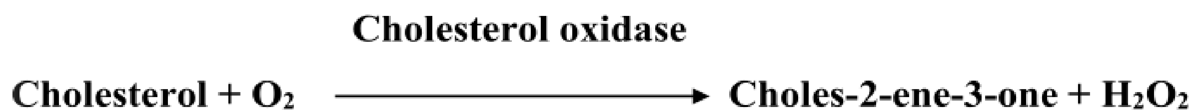
2.4-Methods

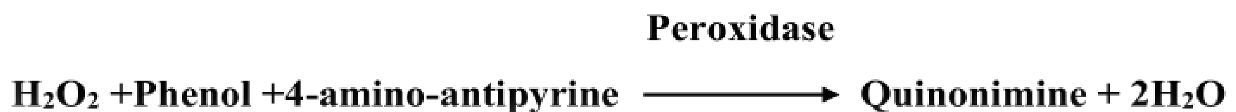
2.4.1-Serum Lipid Profile Assay

2.4.1.1-Measurement of Serum Total Cholesterol

Principle

Serum total cholesterol (TC) is determined utilizing a readymade laboratory kit for this purpose. The principle of determination is based on the enzymatic hydrolysis according to the following reaction (Allain, Poon *et al*. 1974).





The quantity of the former red dye quinonimine is proportional to the cholesterol concentration. The absorbance of quinonimine was read at 500 nm spectrophotometrically.

Reference range: (150-220mg/dl).

Reagents

Reagents	composition	Concentration
Reagents 1 (Buffer)	Phosphate buffer	100 mmol/L
	Chloro-4-phenol	5.0 mmol/L
	Sodium chloride	2.3 mmol/L
	Triton x100	1.5 mmol/L
Reagents 1 (Enzymes)	Cholesterol oxidase	100 IU/L
	Cholesterol esterase	170 IU/L
	peroxidase	1200 IU/L
	PAP	0.25 mmol/L
	PEG 6000	167 µmol/L
Reagents 1 (Standard)	Cholesterol 200mg/dL	5.17 mmol/L

Procedure

The content of vial reagent 2 (enzymes) was added to vial reagent 1 (buffer), mix gently until complete dissolution (approximately 2 minutes) to prepare work reagent. The procedure was carries out as in the following:

Reagents	Blank	Standard	Sample
Reagent	1.0 mL	1.0 mL	1.0 mL
Demineralized Water	10 µL	-	-
Standard	-	10 µL	-
Sample	-	-	10 µL

The tubes were mixed, and then let stands for 5 minutes at 37 ˚C. Record absorbance at 500 nm (480-520) against blank. The color was stable for 1 hour.

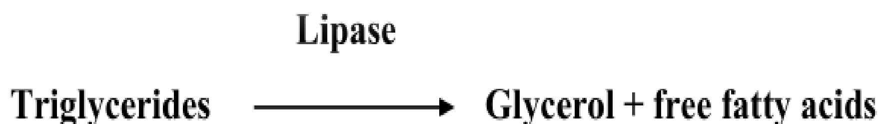
Calculation

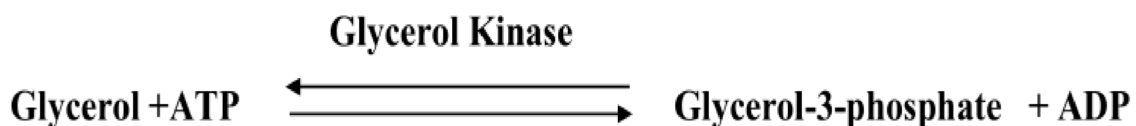
$$\text{Cholesterol (mg/dL)} = \frac{\text{Sample absorbance (at 500 nm)}}{\text{Standard absorbance}} \times 200$$

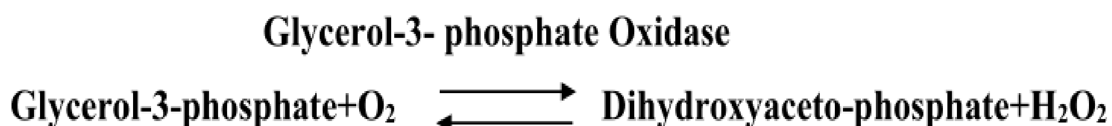
2.4.1.2-Measurement of Serum Triglyceride

principle

Triglycerides are enzymatically hydrolyzed to glycerol and fatty acids according to the following equations (Fossati and Prencipe 1982).







The absorbance was read at 500 nm spectrophotometric ally.

Reference range is: (60-160 mg/dl).

Reagents

Reagents	composition	Concentration
Reagents 1 (Buffer)	PIPES magnesium	100 mmol/L
	chloride	9.8 mmol/L
	Chloro-4-phenol preservative	3.5 mmol/L
Reagents 1 (Enzymes)	Lipase	1000 IU/L
	Peroxidase	1700 IU/L
	Glycerol-3-p-oxidase	3000 IU/L
	Glycerol kinase	660 IU/L
	PAP	0.5 mmol/L
	ATP	1.3 mmol/L
Reagents 1 (Standard)	Glycerol equivalent to triglycerides	2.28 mmol/L

Reagents preparation

The content of reagent 2 was added promptly (enzymes) into reagent 1 (buffer) and mixed gently until complete dissolution (approximately 2 minutes)

Procedure

Three sets of tubes used include blank, standard and sample. The volumes added to each tube are as follows:

Reagents	Blank	Standard	Sample
Reagent	1.0 mL	1.0 mL	1.0 mL
Demineralized Water	10 μL	-	-
Standard	-	10 μL	-
Sample	-	-	10 μL

The tubes were mixed, and then let stands for 5 minutes at 37 ˚C. or 10 minutes at room temperature .record absorbance at 500 nm (480-520) against blank. The color was stable for 1 hour.

Calculation

$$TG\ concentration\ (mg/dL) = \frac{Sample\ absorbance\ (at\ 500\ nm)}{Standard\ absorbance} x200$$

2.4.1.3-Measurement of High Density Lipoprotein Cholesterol (HDL-C)

Principle

Low density lipoprotein (LDL-C), Very Low Density Lipoprotein (VLDL-C), and chylomicrons fraction are precipitated quantitatively by the addition of phosphotungstic acid which contains magnesium chloride at pH 6.2. After centrifugation, the supernatant contains cholesterol concentration in the HDL fraction which is determined by using cholesterol kit(Warnick, Nauck *et al.* 2001).

Reference range is: (more than 40 mg/dl) for male & female.

Reagents

Reagents	composition	Concentration
Reagents 1	Phosphotungstic acid (PTA)	13.9 mmol/L
	Magnesium chloride	570 mmol/L
Reagents 1	HDL-cholesterol	2.58 mmol/L

Procedure

Three sets of tubes used include blank, standard and sample. The volumes added to each tube are as follows:

Reagents	Blank	Standard	Sample
Reagent	1 mL	1 mL	1 mL
Demineralized Water	25 µL	-	-
Standard	-	25 µL	-
Sample	-	-	25 µL

The tubes were mixed, and then let stands for 5 minutes at 37 Cº record absorbance at 500 nm against blank.

Calculation

$$\text{HDL-C (mg/dL)} = \frac{\text{Sample absorbance (at 500 nm)}}{\text{Standard absorbance}} x St.conc.x1.1$$

Where: St.conc. is standard concentration which is 100mg/dL

2.4.1.4-Determination of Low Density Lipoprotein Cholesterol

Principle

Low density lipoprotein - cholesterol (LDL–C) can be measured mathematically from the total cholesterol, triglycerides; and the HDL-C concentration using Friedwald's formula.

This formula is applicable to TG conc. <5mmole/l (400 mg/dl) (Friedewald, Levy *et al*. 1972)

LDL-C, mmol / l = Total cholesterol – (TG / 2.2) – HDL-C

LDL-C, mg / dl = Total cholesterol – (TG / 5) – HDL-C

When TG less than 5 mmol/l (400mg/dl)

Reference range is: (less than 180 mg/dl) or (3.37- 4.12 mmol/l) for male & female

2.4.1.5-Determination of Very Low Density Lipoprotein Cholesterol

Serum Very Low Density Lipoprotein Cholesterol (VLDL-C) can be determined by following equation (Friedewald, Levy *et al*. 1972)

Serum VLDL-C = TG / 2.2 when TG concentration is given in mmol/l

Serum VLDL-C = TG / 5 when TG concentration is given in mg/dl

The reference ranges: > 30 mg/dl or > 0.77 mmol/l for male & female

2.4.2-Determination of Hemoglobin HbA1c

Principle

In the method presented, a preparation of hemolyzed whole blood is mixed with a weakly binding cation-exchange resin. The non-glycosylated hemoglobin (HbA) binds to the resin, leaving free to be removed by means of the resin separator in the supernate .the present of HbA1 free to be removed by means of a resin separator in the supernate.The present of HbA1 is determined by measuring the absorbance values at 415 nm of the HbA1fraction and the total Hb fraction,calculation the ratio of absorbance (R) ,and comparing this ratio to that of a glycohemoglobin standard carried through the same procedure (Trivelli, Ranney *et al*. 1971).

Reagents

- Glycohemoglobin ion – exchange resin.
- Glycohemoglobin lysing reagent.
- Glycohemoglobin standard (lyophilized)

Procedure

- Hemolysate preparation

1- A volume of 0.5 ml of lysing reagent was added into appropriately labeled tube.
2- A volume of 0.1 ml of blood sample was added into above mentioned tube and mixed well then left for 5 minutes.

- Glycohemoglobin separation

1- A volume 0.1ml was pipetted on the prepare hemolysate into appropriately labeled resin tube.
2- The resin position was separated in the tubes approx. 1cm above ion exchange resin.
3- The tubes were mixed for 5 minutes on a hematology mixer.
4- After the incubation, the resin separation has to be into the tube until the ion exchange resin is being firmly packed in bottom of 13 mm tube.
5- The supernatant was added to be poured into a cuvette .The absorbance was read at 415 nm against water (=∆A HbA1)

- Total Heomglobin

1- A volume 5ml distilled water was dispensed to each tube.
2- A volume 0.02ml was pipetted of hemolysated to each tube, and mixed well.

3- The absorbance was read at 415 nm against distilled water.

Calculation

For each standard and unknown, the ratio (R) of the Glycohemoglobin absorbance to the hemoglobin absorbance calculated as follows:

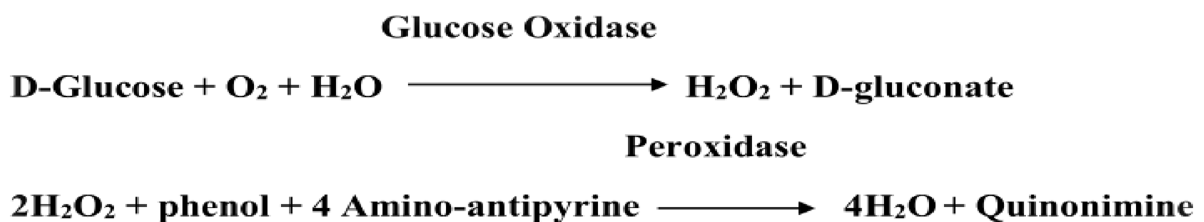
$$R = \frac{A\text{- Glycosylated}}{A\text{-Total}}$$

$$Glycohemoglobin = \frac{R(Test)}{R(standard)} x7.6$$

2.4.3-Determination of Fasting Serum Glucose Concentration

Principle

Glucose is oxidized by glucose oxidase to gluconate and hydrogen peroxide according to the following equation (Passey, Gillum *et al.* 1977)



Reagents

Reagents	composition	Concentration
Reagent 1 Buffer solution	Tris buffer PH 7	100 mmol/L
	Phenol	0.3 mmol/L
Reagent 2	Glucose oxidase	10000 U/L
	Peroxidase	1000 U/L
	4-Amino-antipyrine	6.2 mmol/L
Reagent 3 Standard	Standard glucose	100 mg/dL 5.56 mmol/L

Procedure

Reagent	Blank	Standard	Sample
Working Solution	1.0 mL	1.0 mL	1.0 mL
Standard	-	10 µL	-
Sample or Unknown	-	-	10 µL

Tubes were mixed and incubated for 10 minutes at 37 ˚C. Or 30 minutes at 25 ˚C. Then they were measured at wavelength of (505 nm) at room temperature.

Calculation

$$\text{Glucose concentration (mg/dl)} = \frac{A_{sample}}{A_{standard}} \times \text{Conc. of standard glucose}$$

Concentration of standard glucose solution = 100mg/dl

2.4.4-Measurement of serum Incretin Hormones

2.4.4.1-Measurement Glucose Dependent Insulin Releasing Polypeptide (GIP)

Principle

To assay Human Glucose dependent insulin releasing polypeptide (GIP) uses enzyme-linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology. Add (GIP) to wells that are pre-coated with (GIP) monoclonal antibody and then incubate. After incubation, add anti GIP antibodies labeled with biotin to unite with streptavidin-HRP, which forms the immune complex. Remove unbound enzymes after incubation and washing, then add substrate A and B. The solution will turn blue and change to yellow with the effect of acid. The shades of solution and the concentration of Human (GIP) are positively correlated (Vos, Krajnc *et al*. 1982).

Configuration	
Standard solution(6400pg/ml)	0.5ml×1 ebndrof
Streptavidin-HRP	6ml×1 red
Stop Solution	6ml×1 yellow
chromogenic reagent A	6ml×1 green
chromogenic reagent B	6ml×1 blue
Anti GIP antibodies labeled with biotin	1ml×1 violet
Standard dilution	3ml×1
Washing concentrate	(20ml×30)×1 big

Procedure

a) Dilution of standard solutions: (one standard original concentration), the independently dilute in small tubes was done as followed the chart below:

3200pg/ml	Standard No.5	120µl Original Standard + 120µl Standard diluents
1600pg/ml	**Standard No.4**	120µl Standard No.5 + 120µl Standard diluents
800pg/ml	**Standard No.3**	120µl Standard No.4 + 120µl Standard diluent
400pg/ml	**Standard No.2**	120µl Standard No.3 + 120µl Standard diluent
200pg/ml	**Standard No.1**	120µl Standard No.2 + 120µl Standard diluent

b) The number of stripes needed was determined by that of samples to be tested added by the standards. It was recommended that each standard solution and each blank well be arranged with multiple wells as much as possible.

c) Sample injection: **1)** Blank well: Do not add sample, anti GIP antibody labeled with biotin and streptavidin-HRP; add chromogen reagent A & B and stop solution, each other step operation is the same. **2)** Standard solution well: 50µl was Added standard and streptomycin-HRP 50µl (biotin antibodies has united in advance in the standard so no biotin antibodies are added). **3)** Sample well to be tested: 40µl was Added sample and then 10µl GIP antibodies, 50µl streptavidin-HRP. Then covered it with seal plate membrane. Shacked gently to mix. Incubate at 37 ˚C for 60 minutes.

d) Preparation of washing solution: Diluted the washing concentration (30X) with distilled water for later use.

e) Washing: carefully removed the seal plate membrane, drain liquid and shake off the remainder. Fill each well with washing solution, let stand for 30 seconds, then drain. Repeat this procedure five times then blot the plate.

f) Color development: 50µl chromogen reagent A was added to each well, and then 50µl chromogen reagent B was added to each well. Shacked gently to mixed. Incubate for 10 minutes at 37 ºC away from light for color development.

g) Stop: 50µl Stop Solution was Added to each well to stop the reaction (color changes from blue to yellow immediately at that moment).

h) Assay: Take blank well as zero, the absorbance (OD) of each well one by one was measured under 450 nm wavelength, which should be conducted within10 minutes after having added stop solution.

i) According to standards concentrations and corresponding OD values, the linear regression equation of the standard curve was calculated. Then according to the OD value of samples, the concentration of the corresponding sample was calculated. Statistical software could also be employed.

Calculation

Concentration of standards the abscissa and OD value the ordinate were made. the standard curve was drawn on the graph paper. According to the OD value of the sample, locate its corresponding concentration (which is the concentration of the sample); or calculate the linear regression equation of the standard curve according to the standard concentration and the OD value. Then substitute with the OD value of the sample to calculate its concentration

Assay range：20pg/ml→6000pg/ml. for male & female

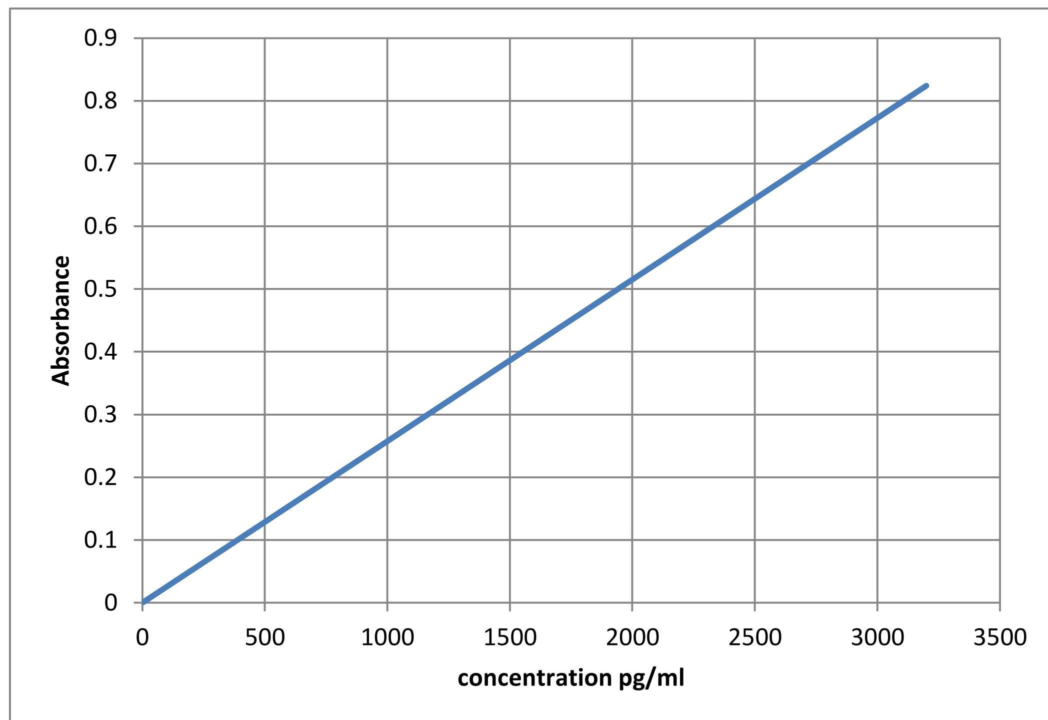


Figure (2-1): standard curve of GIP

2.5-Molecular Analysis

2.5.1-DNA extraction from blood

The ReliaPrep Blood g DNA Miniprep System has been used for the Purification of the genomic DNA from blood samples as the following:

a) 20µL of Proteinase K (PK) was dispensed into a 1.5ml micro centrifuge tube.

b) 200µL of blood was added into the (PK) tubes capped mixed.

c) 200µL of cell lysis buffer (CLB) was added to the tube, capped and mixed by vortex for at least 10 seconds.

d) The contents of the tube were added to the ReliaPrep binding column capped and placed it in a micro centrifuge.

e) Centrifuge for 1 minute at maximum speed. The binding column was checked to make sure the lysate has completely passed through the membrane. If lysate is still visible on top of the membrane, then the column was centrifuged for another minute.

f) The collection tube containing was removed and the liquid discard as hazardous waste.

g) The binding column was placed into a fresh collection tube. 500μL of column wash solution (CWS) was added to the column, and centrifuged for 3 minutes at maximum speed.

h) Step (g) was repeated twice for a total of three washes.

i) The column was placed in a clean 1.5mL micro-centrifuge tube.

j) 50–200μL of Nuclease-Free Water was added to the column and Centrifuged for 1 minute at maximum speed.

k) Then the concentration obtained DNA and purity was measured by NanoDrop technique.

2.5.2-Estimation of DNA Concentration and Purity

The concentration of DNA was measured by Nano-drop system. According to the Nano-drop manual, 1μl of each DNA sample was used, and DNA purity was measured depending on the ratio of sample absorbance at wave lengths 260 and 280 nm as shown in Figure (2-2) (Green 2012)

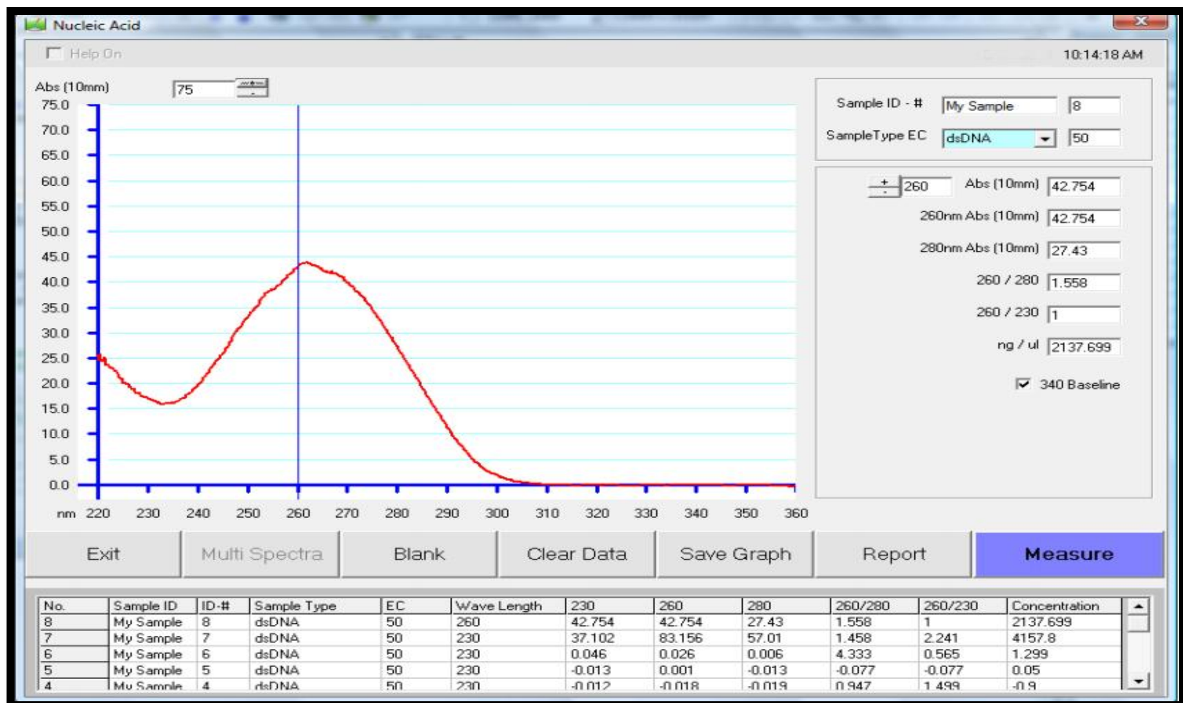


Figure (2-2): DNA Concentration and Purity

The most common technique to determine DNA yield and purity is measurement of absorbance. Although it could be argued that fluorescence measurement is easier, absorbance measurement is simple, and requires commonly available laboratory equipment. All that is needed for the absorbance method is a spectrophotometer equipped with a UV lamp, UV-transparent cuvettes (depending on the instrument) and a solution of purified DNA. Absorbance readings are performed at 260nm (A_{260}) where DNA absorbs light most strongly, and the number generated allows one to estimate the concentration of the solution. To ensure the numbers are useful, the A_{260} reading should be within the instrument's linear range (generally 0.1–1.0).

Concentration (µg/ml) = (A_{260} reading – A_{320} reading) × dilution factor × 50µg/ml

DNA concentration is estimated by measuring the absorbance at 260nm, adjusting the A_{260} measurement for turbidity (measured by absorbance at 320nm), multiplying by the dilution factor, and using the relationship that an A_{260} of 1.0 = 50µg/ml pure DNA.

To evaluate DNA purity, measure absorbance from 230nm to 320nm to detect other possible contaminants. The most common purity calculation is the ratio of the absorbance at 260nm divided by the reading at 280nm. Good-quality DNA will have an A_{260}/A_{280} ratio of 1.7–2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants are present. The ratio can be calculated after correcting for turbidity (absorbance at 320nm).

DNA purity (A_{260}/A_{280}) = (A_{260} reading – A_{320} reading) ÷ (A_{280} reading – A_{320} reading)

2.5.3-DNA Electrophoresis

Electrophoresis through agarose is a standard method used to separate, identify and purify DNA fragments. The technique is simple, rapid to perform, and capable of resolving fragments of DNA that cannot be separated adequately by other procedures (Querci, Jermini *et al.* 2006).

The agarose gel electrophoresis was done according to Harisha method (Harisha 2008), and ethidum bromide staining was added (2-3 µl) according to Robinson and lafleche method (Robinson 2000) ,ethidium bromide can bind with DNA. Ethidium is capable of forming close van der Walls contacts with the base pairs and that's why it binds to the hydrophobic interior of the DNA molecule. Molecules thatbind in this manner are called intercalating agents because they intercalate into the compact array of stacked bases, 2% agarose was prepared using this same protocol.

2.5.4-Agarose Gel Electrophoresis

The Agarose Gel Electrophoresis was done after the following steps;

a) Agarose gel was prepared by dissolving 1.5 g of agarose powder in 100 ml of 10% Tris Borate EDTA (TBE) buffer solution and boiling by electric heater (Green 2012).

b) The solution was cooled to 50˚C.

c) Two microliters of ethidium bromide solution was added.

d) The comb was fixed at one end of the tray for making wells used for loading the PCR products samples.

e) The Agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min.

f) The comb was removed gently from the tray.

g) The tray was fixed in electrophoresis chamber. The chamber was filled with TBE buffer 1%.

h) Ten microliters of each ARMS-PCR products sample were loaded into the wells in agarose gel.

i) The voltage of the electrophoresis apparatus was fixed at 100 volt to ensure an electrical field adjusted with (5-8) $v.cm^{-1}$ for 16 cm distance between cathode and anode.

j) Ultraviolet trans-illuminator was used for bands detection.

k) The gel was photographed using digital camera.

2.5.5-Preparation of Primers Solution

The lyophilized primer was dissolved using deionizer distilled water (DDH_2O) to obtain 100 pmol/µl in the master tube, then 10 pmol/µl was prepared as a working solution by transferring 10 µl from the master to another tube and the volume was completed to 100 µl by adding (DDH_2O).

2.5.6-Amplification of DNA

In order to amplify the target gene, ARMS-PCR was used with a specific primer. The ARMS-PCR reactions were performed in 25 µl volumes in PCR tubes under sterile conditions, all the volume of the reaction mixture was completed to 25 µl with using DDH_2O and the master mix which contained optimum concentrations of reaction requirements ($MgCl_2$ 1.5 mM, Taq polymerase 1 U, each dNTPs 200 µM) has been used. All amplification experiments included a negative control blank which contained all ARMS-PCR material with the exception of template DNA. Mixture of reaction is listed in table (2-5).

Table (2-5): Components of master mix for detection of DPP4 gene

No.	Material	Volume(µl)
1	**Master Mix**	12.5
2	**Inner Forward**	1.5
3	**Outer Forward**	1.5
4	**Inner Reverse**	2.5
5	**Outer Reverse**	2.5
6	**Template DNA**	4.5
Total		**25 µl**

Amplification Reaction Mixture for DPP4 rs1861978 primers were used in single PCR reaction. The ARMS-PCR mixture is prepared by mixing 1.5µl from each primer (p1, p2, p3, p4) of 8 pmol/µl primers with 4.5 µl of extracted DNA were mixed in total volume of 25 µl. Then the mixture was added to ARMS-PCR premix formula. The reaction mixture has been incubated and the following program is used to amplify the mixture.

Table (2-6) ARMS-PCR program for detection of DPP4 gene

No.	Stage	Cycle	Step	Temp.	Time
1	**Initial Denaturation**	1	1	95°C	5 minutes
2	**Denaturation**	45	1	95°C	30 seconds
3	**Annealing**		2	55°C	45 seconds
4	**Extension**		3	72°C	45 seconds
5	**Final Extension**	1	1	72°C	5 minutes
6	**Hold Phase**			4°C	

ARMS-PCR program for detection of DPP4 gene in current study design by Dr. Hassan Mahmood Abo Al-maali / College of Pharmacy- University of Kerbala

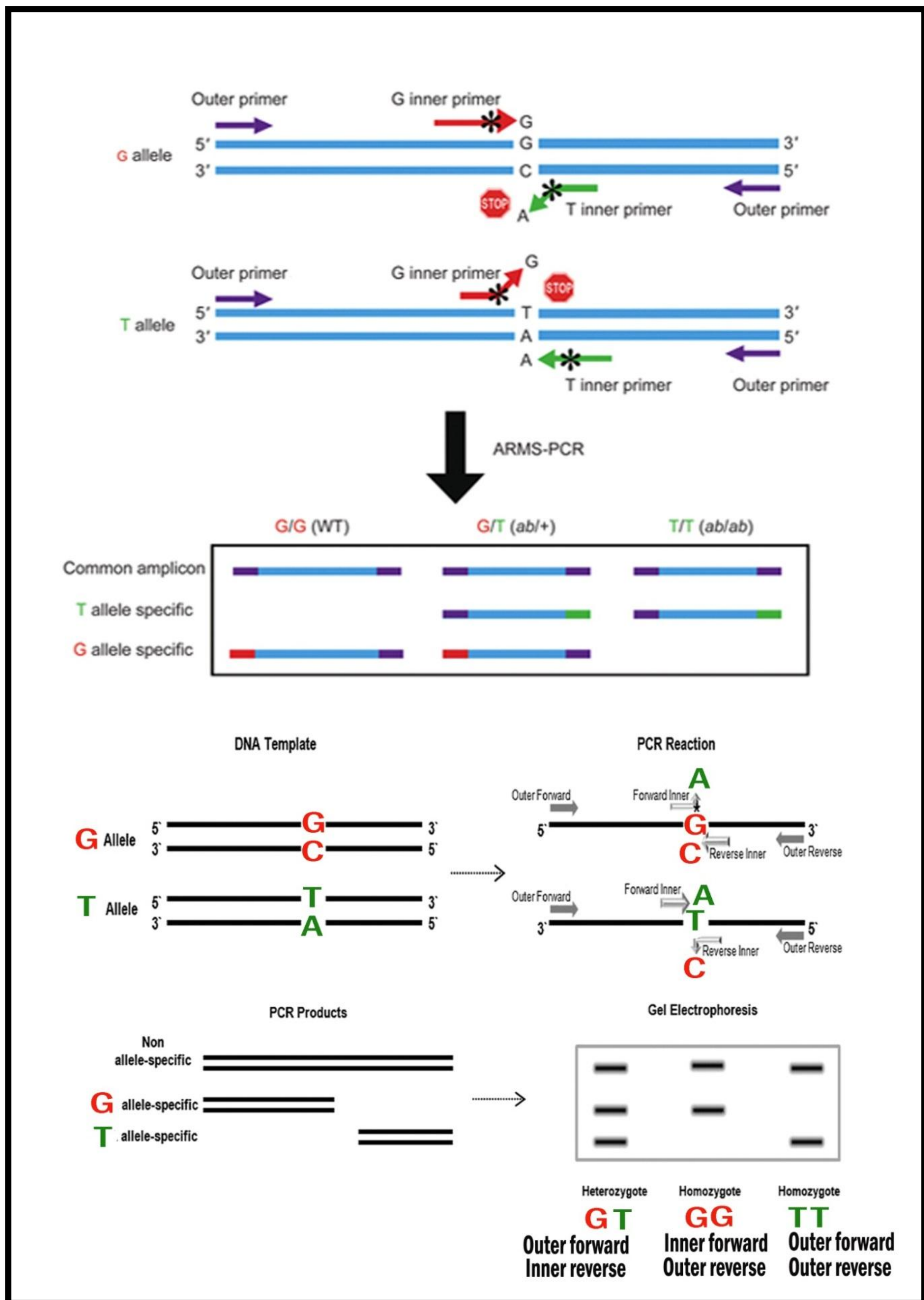


Figure (2-3): Schematic summary of ARMS-PCR primer design and DNA gel patterns of the different genotypes. Different colors indicate different primers participating in the PCR reaction

2.6-Limitation of study

1- Number of cases.

2- Time limit.

3- Cost of kits.

2.7-Statistical Analysis

The data of the study were saved in Microsoft Excel Spread sheet and analyzed on the computer using the Statistical Package for the Social Sciences (SPSS) (Version 25) and Microsoft Excel program (Version 2010). All values were expressed as mean± standard deviation (M±SD).

Statistical analyses were performed using student t-test to estimate the difference between the groups, taking ($P<0.05$) as the lowest limit of significance. Analysis of variance (ANOVA) test was used to compare between different subgroups.

The Pearson's correlation coefficient (r) is used to describe the association between the different parameters (Swinscow and Campbell 2002).

Chapter Three

Results

&

Discussion

3-Results and Discussion

3.1-Gender Distribution

Two hundred and forty Iraqi subjects have been included in this study; **128**(53.3%) males and **112**(46.7%) females. Gender distribution of the investigated groups was shown in table (3-1).

Table (3-1): Distribution of Diabetes and Control According to Gender.

Gender	Diabetic	Control	Total
	n.(%)	n.(%)	n.(%)
Male	63(52.5)	65(54.2)	128(53.3)
Female	57(47.5)	55(45.8)	112(46.7)
Total	*120(100)*	*120(100)*	*240(100)*

One hundred and twenty subjects were Diabetic patients **63**(52.5%) male and **57**(47.5%) female and male: female ratio was 1.1:1 and One hundred and twenty healthy subjects **65**(54.2%) male and **55**(45.8%), female and male: female ratio was 1.18:1.

Table (3-2) shown that Age and BMI was no significant p.vale (0.870), (0.595), and that there was a significantly higher levels of FBS , HbA1C, TG, VLDL-C , LDL-C , TC and GIP .

Table (3-2): Association between Diabetic and Control groups

Parameters	Diabetic(n. =120)	Control(n. =120)	P. Value
	Mean ± SD	Mean ± SD	
Age (years)	**49.26± 4.75**	**49.16± 4.66**	**0.870**
BMI (kg/m2)	**28.95± 4.02**	**28.69± 3.47**	**0.595**
FBS (mg/dl)	**235.70± 105.59**	**126.89± 58.69**	**<0.001**
HbA1c %	**8.15± 2.14**	**5.72± 1.01**	**<0.001**
Cholesterol (mg/dl) **(200-239)**	**203.37± 53.45**	**160.85± 41.28**	**<0.001**
TG(mg/dl) **<180**	**230.55± 96.07**	**171.34± 77.57**	**<0.001**
HDL-C(mg/dl) **(30-60)**	**36.72± 10.67**	**45.88± 11.97**	**<0.001**
LDL-C(mg/dl) **(100-190)**	**120.53± 48.06**	**80.70± 43.40**	**<0.001**
VLDL-C (mg/dl) **(2-30)**	**46.11± 19.21**	**34.26± 15.51**	**<0.001**
GIP (pg/ml)	**3384.09± 1521.07**	**2330.19± 1443.94**	**<0.001**

Student t-test between Diabetic patients and control group, p<0.05, p<0.001, SD=standard deviation, TG=triglycerides, TC=total cholesterol, HDL-C=high density lipoprotein- cholesterol, LDL-C=low density lipoprotein-cholesterol, VLDL-C=very low density lipoprotein cholesterol, GIP= Glucose-Dependent Insulinotropic Polypeptide, FBS= Fasting Serum Glucose, BMI=body mass index.

Table (3-2) show the results of some studies parameters (Age, BMI, FBS, HbA1C, TG, VLDL-C, LDL-C, TC and GIP) the results revealed that P. Value (0.870, 0.595, <0.001, <0.001, <0.001, <0.001, <0.001, <0.001, <0.001, <0.001)

According to the gender, the results in Table (3-3) showed that there was a significantly higher levels of FBS , HbA1C, TG, VLDL-C , LDL-C , TC and GIP levels, while serum HDL-C recorded a significant decrease in diabetic male group in comparison with diabetic female group (P <0.001). As well as there was a significantly higher levels of FBS , HbA1C, TG, VLDL-C , LDL-C , TC and GIP levels, while serum HDL-C recorded a significant decrease in diabetic male group in comparison with healthy male group (p<0.001).

Our results similar to other studies which show that Diabetic patients with poor and worse glycemic control had significantly higher levels of FBS, TG , TC, LDL, TG (Prabhavathi, Kunikullaya *et al.* 2014).

These results of this study agreed with the result recorded by Anna (Nordstrom, Hadrevi *et al*. 2016).

who reported that the prevalence of T2DM in 4% of women and 8% of men, another study has found a higher prevalence of T2DM in men than in women (Soriguer, Goday *et al*. 2012).

Epidemiological studies have shown that the prevalence of T2DM is generally higher in males than in females (Toshikuni, Tsuchishima *et al*. 2017).

In most countries, even though females have lower mortality rates than males, they experience poorer health (Malmusi, Artazcoz *et al*. 2011) . Diabetes tends to affect males more than females since more males are diagnosed with T2DM (Canada 2011).

Another study has showed that the prevalence of T2DM was 1.2 times higher in men than in women (Noh, Han *et al*. 2017).

Table (3-3): Comparison between Diabetic group and Control group in the measured Parameters according to Gender using ANOVA test.

Parameters	Diabetic n.=120		Control n.=120	
	Male n.=63 Mean±SD	Female n.=57 Mean±SD	Male n.=65 Mean±SD	Female n.=55 Mean±SD
Age (years)	48.69±4.61	49.89±4.87	48.80±4.61	49.60±4.64
BMI (kg/m2)	29.11± 3.82	28.77± 4.28	28.82± 3.51	28.55± 3.46
FBS (mg/dl)	245.37± 124.06[b*]	225.02± 80.21[b*]	109.65± 48.72	147.28± 63.24
HbA1c %	9.16± 2.22 [a,b*]	7.04± 1.38 [a,b*]	5.74± 1.09	5.82± .92
TC (mg/dl)	216.70± 49.35[a,b*]	188.63± 54.35 [a,b*]	157.82± 41.51	164.43± 41.08
HDL-C (mg/dl)	37.73± 6.88[b*]	35.60± 13.69[b*]	46.56± 11.24	45.08± 12.85
VLDL-C (mg/dl)	56.61± 14.92 [a,b*]	34.50± 16.63 [a*]	31.90± 13.72	37.06± 17.10
TG (mg/dl)	283.06± 74.63[ab*]	172.51± 83.15[a*]	159.53± 68.61	185.30± 85.52
LDL-C (mg/dl)	122.36± 46.37 [b*]	118.52± 50.20 [b*]	79.35± 44.29	82.29± 42.68
GIP (pg/ml)	3449.3± 1431.1 [b*]	3311.9± 1624.4 [b*]	2313.92± 1476.10	2349.44± 1418.31

P value derived from ANOVA test,*Significant: $p<0.05$, ** highly significant: $p<0.001$, No significant: $p>0.05$, n.=number, SD=standard deviation, BMI=body mass index, TG=triglycerides, TC=total cholesterol, HDL-C=high density lipoprotein-cholesterol, LDL-C=low density lipoprotein-cholesterol, VLDL-C=very low density lipoprotein cholesterol, GIP= Glucose-Dependent Insulinotropic Polypeptide, FBS= Fasting Serum Glucose. [a] ANOVA test= male vs. female Diabetic patients, [b] ANOVA test= male or female Diabetic vs. control.

Previous studies have shown that biologically there is no difference between men and women in the prevalence and characteristics of T2DM. The difference is likely to be much wider in developing countries where the position of women in the society is still poor. In this context the gender differences in the care of T2DM becomes very important (Shrestha, Kosalram *et al*. 2013).

Another factor that may increase a tendency in many populations to T2DM in men compared to women is a much higher prevalence of tobacco smoking in men (Ng, Freeman *et al*. 2014).

The overall prevalence of T2DM was lower in women compared to men. Although some references did not mention sex as an independent risk factor for T2DM (Powers 2015). Onat et al. and Meisinger et al. studies have shown that the male excess in the incidence and prevalence of T2DM, which is found in some populations, has been attributed to sex-related differences in insulin sensitivity, consequences of obesity and regional body fat deposition and other contributing factors such as hypertension, smoking and alcohol intake (Naseribafrouei, Eliassen *et al*. 2018)

3.2-Age distribution

The results of the present study showed that the mean age of Diabetic patients were Mean±SD (49.26± 4.75) and the normal control group Mean±SD (49.16± 4.66) (p=0.870), as shown in table (3-2) .There was also, no significant difference between age and other parameters respectively as shown in table (3-10).

Age distribution of the investigated groups was shown in table (3-4).

Table (3-4): Distribution of Diabetic and healthy Control according to Age.

Group	Diabetic n.=120	Control n.=120	Total
	n.(%)	n.(%)	n.(%)
(40 – 49) years	63(52.5)	74(61.7)	137(57.0)
(50 – 59)years	57(47.5)	46(38.3)	103(43.0)
Total	*120(100)*	*120(100)*	*240(100)*

There is evidence that diabetes complications, hospitalization and mortality are more prevalent among older people with diabetes (Ki, Baek *et al*. 2014).

A study by Shaw has showed that between 2010 and 2030, a 69% increase in T2DM is expected among adults aged 20-79 years in low-income countries compared with a 20% increase in the same age group in high income countries (Shaw, Sicree *et al.* 2010).

The results of this study were in agreement with Samira Alsenany study who found an increased prevalence of T2DM in people aged 58.5 years or older. Similarly, another study noted that the incidence of diabetes increases with age (Alsenany and Al Saif 2015).

The results of present study were Similar to the finding that were observed in Iran and in Iraq, where diabetes mellitus was more common among older people, with 10.9% of those aged >60 years having the disease (Cockram 2000),(Mansour, Al-Maliky *et al*. 2014).

The prevalence of T2DM increases with age such that in developing countries, most diabetics are in the age bracket of 45 to 65 years, while in developed countries the largest number is found in those aged 65 years and above (Yeasmin, Nahar *et al*. 2015).

Papadopoulos et al. study has showed that the prevalence of diabetes increases with age in older people and many studies have investigated the relationship between diabetes and quality of life in older people. Rubin et al. study show that the patients were classified into three groups according to age and assessed; all sub-parameters except physical functioning and general health parameters did not differ significantly between the three groups. Although there are studies in the literature indicating that the quality of life is related to age, and the quality of life in young people is better, there were also studies indicating that there was not relationship between quality of life and age (Altınok, Marakoglu *et al*. 2016).

3.3-Smoking

The results revealed that **92** (38.3%) subjects were smokers and **148** (61.7 %) were non-smokers, Figure (3-1). There was also, significant correlation between smoker with VLDL-C and LDL-C (r=-0.244, P=0.007), (r=-0.244, P=0.007) respectively as shown in table (3-10).

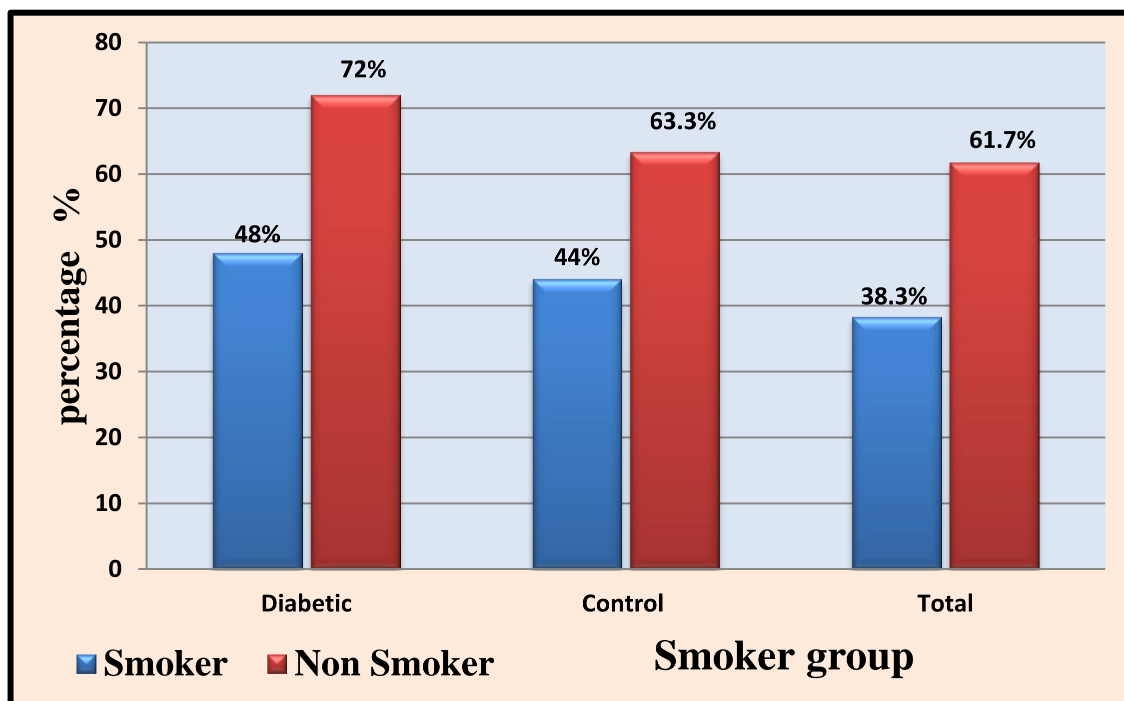


Figure (3-1): Distribution of Diabetic and control according to smoking.

According to smoking the results in Table (3-5) showed that there was a significantly higher levels of FBS , HbA1C, TG, VLDL-C , LDL-C , TC and GIP, while serum HDL-C recorded a significant decrease in diabetic smoking group in comparison with diabetic non-smoking group (P <0.001). As well as there were significantly higher levels of FBS , HbA1C, TG, VLDL-C , LDL-C , TC and GIP levels, while serum HDL-C recorded a significant decrease in diabetic smoking group in comparison with healthy smoking group (p<0.001).

In Japan, systematic review study found that increased risk of T2DM in smokers compared with nonsmokers (Akter, Goto *et al*. 2017).

another meta-analyses based on worldwide data reported that passive smoking is associated with a 21–28% higher risk of T2DM (Pan, Wang *et al*. 2015).

A study observed a stronger association of amount smoked with risk of diabetes in China (Liu, Bragg *et al*. 2018). A systematic review and meta-analysis of prospective cohort studies assessed the relationship between active smoking and risk of mortality and cardiovascular events among patients with diabetes (Sliwinska-Mosson and Milnerowicz 2017). Smoking is associated with a 40 to 50% increase in the risk of type 2 diabetes. While smoking in the Caribbean is on average much higher in men than in women, its prevalence in men is typically between 10 and 20%, lower than most world regions (Sobers-Grannum, Murphy *et al*. 2015).

The total serum cholesterol, LDL, VLDL and Triglyceride values were higher in smokers as compared to Non-smokers, .On the other hand, LDL & VLDL levels were also significantly increased in smokers than non-smokers and are in agreement with results of Kesaneimi and Grundy (Verma, Kumar *et al*. 2018).

Table (3-5): Comparison between Diabetic group and Control group in the measured parameters according to smoker & non-smoker using ANOVA test.

Parameters	Diabetic n.=120		Control n.=120	
	Smoker n.=48 Mean±SD	Non-smoker n.=72 Mean±SD	Smoker n.=44 Mean±SD	Non-smoker n.=76 Mean±SD
Age (years)	49.35±4.91	49.21±4.68	49.00±5.00	49.26±4.49
BMI (kg/m2)	29.32±3.77	28.70±4.20	28.88±3.54	28.58±3.45
FBS (mg/dl)	252.24±103.02 ^{b*}	224.68±106.57 ^{b*}	117.88±47.33	132.12±64.09
HbA1c %	8.83±2.44 ^{a,b*}	7.69±1.79 ^{a,b*}	5.84±1.01	5.74±1.02
TC (mg/dl)	218.08± 53.05 ^{a,b*}	193.56± 51.79 ^{a*}	191.38± 49.16	143.18± 21.32
HDL -C(mg/dl)	34.67± 7.73 ^{b*}	38.09± 12.11	41.25± 12.20	48.57± 11.06
VLDL-C (mg/dl)	51.48± 20.67 ^{a,b*}	42.52± 17.41^{a*}	38.96± 15.71	31.55± 14.83
TG (mg/dl)	257.4± 103.3 ^{a,b*}	212.62± 87.08 ^{a*}	194.81± 78.56	157.75± 74.15
LDL-C (mg/dl)	131.92± 48.60 ^{a,b*}	112.94± 46.49 ^{a*}	111.17± 52.70	63.06± 23.17
GIP (pg/ml)	3346.4±1563.3 ^{b*}	3409.1±1502.7 ^{b*}	1926.8±1272.0	2563.7±1493.2

P value derived from ANOVA test,*Significant: $p<0.05$, ** highly significant: $p<0.001$, No significant: $p>0.05$, n.=number, SD=standard deviation, BMI=body mass index, TG=triglycerides, TC=total cholesterol, HDL-C=high density lipoprotein-cholesterol, LDL-C=low density lipoprotein-cholesterol, VLDL-C=very low density lipoprotein cholesterol, GIP= Glucose-Dependent Insulinotropic Polypeptide, FBS= Fasting Serum Glucose. [a] ANOVA test= Smoker vs. Non-smoker Diabetic patients, [b] ANOVA test= Smoker or Non-smoker Diabetic vs. control.

3.4-Body Mass Index

The results obtained have shown that BMI has no significant association between diabetic patients and control groups (p=0.595), as shown in Table (3-2). Moreover, there are significant correlations between BMI and total cholesterol TC as shown in table (3-10).

Table (3-6):Incidence rate of BMI in Diabetic Patients and Control individuals

Classification	BMI (kg/m^2)	Diabetic n.=120		Control n.=120	
		Male n.(%)	Female n.(%)	Male n.(%)	Female n.(%)
Normal	(18.5-24.9)	**7(11.1)**	**6(10.5)**	**6(9.2)**	**8(14.5)**
Preobese	(25.0-29.9)	**30(47.6)**	**28(49.1)**	**33(50.7)**	**28(50.9)**
Obese class I	(30.0-34.9)	**21(33.3)**	**17(29.8)**	**22(33.8)**	**15(27.2)**
Obese class II	(35.0-39.9)	**5(7.9)**	**6(10.5)**	**4(6.1)**	**4(7.3)**
Total		**63(100)**	**57(100)**	**65(100)**	**55(100)**

According to the obesity, the results in Table (3-7) showed that there was a significantly higher levels of BMI, FBS , HbA1C, TG, VLDL-C , LDL-C , TC and GIP levels, while serum HDL-C recorded a significant decrease in diabetic obese group in comparison with diabetic normal group (P <0.001). As well as there was a significantly higher levels of BMI, FBS , HbA1C, TG, VLDL-C , LDL-C , TC and GIP levels, while serum HDL-C recorded a significant decrease in diabetic obese group in comparison with healthy obese group (p<0.001).

There were several studies which evaluate the relationship between BMI and DM world-wide. This study found that there was no association between BMI in both genders with Diabetic group and the control group. several studies have shown that it is a superior predictor for diabetes development compared with BMI (Schulze, Heidemann *et al.* 2006). In another Western study, all-cause mortality increased with increasing BMI in adults without DM but decreased with increasing BMI among adults with DM (Jackson, Yeh *et al.* 2014).

This study found that was no significant relation between obesity and overweight with sex, age and the area of residence. There was a significant relation between BMI with total cholesterol (Azita, Asghar *et al*. 2009).

Table (3-7): Comparison between Diabetic group and Control group in the measured parameters according to normal & Obese using ANOVA test.

Parameters	Diabetic n.=120		Control n.=120	
	Obese n.=107 Mean±SD	Normal n.=13 Mean±SD	Obese n.=106 Mean±SD	Normal n.=14 Mean±SD
Age (years)	49.27± 4.86	49.23± 4.02	49.33± 4.72	47.93± 4.18
BMI (kg/m2)	29.75± 3.47 [a*]	22.38± 1.50 [a,b*]	29.38± 3.09	23.48± 0.63
FBS (mg/dl)	238.42± 107.97 [b*]	213.35± 83.70 [b*]	124.38± 56.35	145.94± 73.80
HbA1c %	8.23± 2.16 [b*]	7.46± 1.94 [b*]	5.77± 1.02	5.79± 1.05
TC (mg/dl)	207.92± 53.70 [a,b*]	165.93± 34.03 [a*]	160.23± 40.59	165.61± 47.57
HDL-C (mg/dl)	36.48± 10.53 [b*]	38.63± 12.10 [b*]	45.23± 12.07	50.82± 10.34
VLDL-C(mg/dl)	46.62± 18.90 [b*]	41.90± 21.94	35.30± 15.60	26.44± 12.67
TG (mg/dl)	233.10± 94.54 [b*]	209.52± 109.71	176.51± 78.04	132.20± 63.37
LDL-C (mg/dl)	124.80± 48.09 [a,b*]	85.39± 31.21 [a*]	79.69± 42.67	88.34± 49.67
GIP (pg/ml)	3403.9± 1518.6 [b*]	3220.3± 1593.2	2386.8± 1433.2	1901.5± 1506.9

P value derived from ANOVA test,*Significant: $p<0.05$, ** highly significant: $p<0.001$, No significant: $p>0.05$, n.=number, SD=standard deviation, BMI=body mass index, TG=triglycerides, TC=total cholesterol, HDL-C=high density lipoprotein-cholesterol, LDL-C=low density lipoprotein-cholesterol, VLDL-C=very low density lipoprotein cholesterol, GIP= Glucose-Dependent Insulinotropic Polypeptide, FBS= Fasting Serum Glucose. [a] ANOVA test= Obese vs. Normal Diabetic patients, [b] ANOVA test= Obese or Normal Diabetic vs. control.

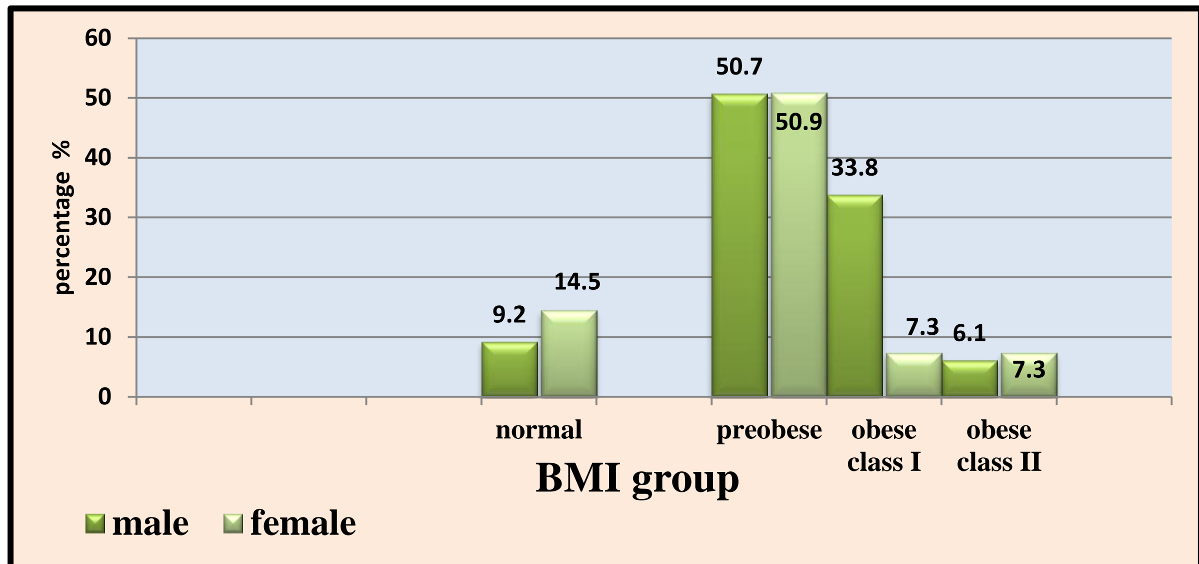


Figure (3-2): Distribution of Diabetic according to BMI.

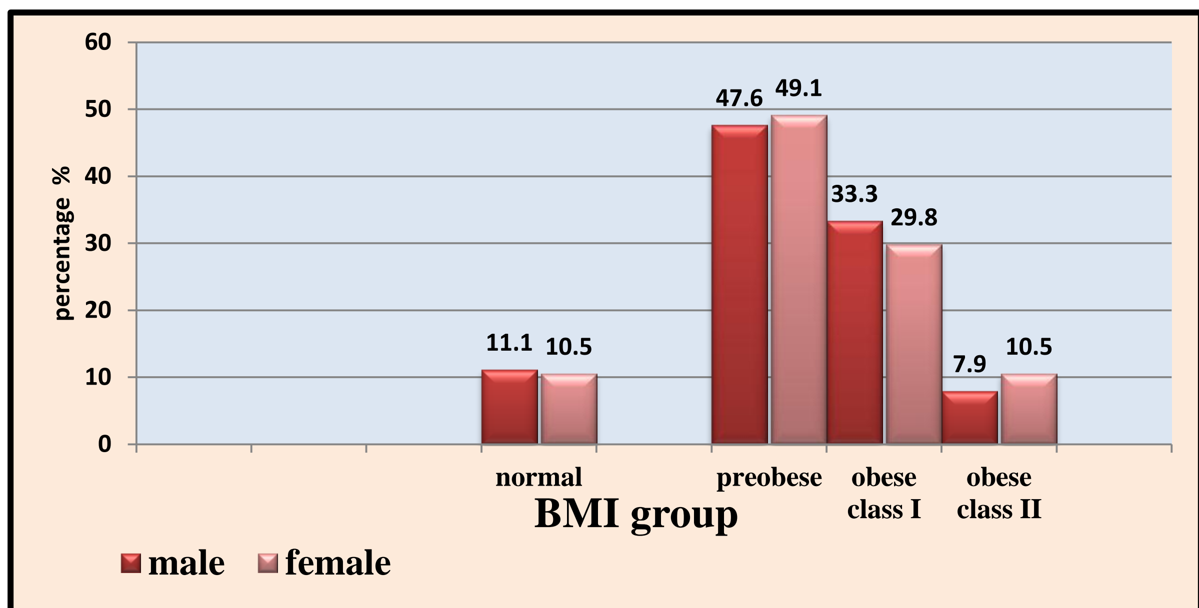


Figure (3-3): Distribution of Control according to BMI.

3.5-Fasting Serum Glucose FSG

The results showed that serum glucose is significantly high in Diabetic patients compared with control group (p=<0.001), table (3-2).

There was also, a correlation between serum glucose with HbA1c, TC-C, TG, VLDL-C and GIP (r =0.255, p=0.01), (r =0.286, p=0.02), (r =0.286, p=0.02), and (r =0.229, p=0.012) respectively as shown in table (3-10).

Table(3- 8):Levels rate of FSG in Diabetic patients and Control individuals

Classification	Fasting Serum Glucose	Diabetic		Control	
		Male n.(%)	Female n.(%)	Male n.(%)	Female n.(%)
Normal	**80 - 100**	1(1.5)	0 (0)	37 (56.9)	15 (27.2)
Impaired Glucose	**101 - 125**	8 (12.6)	1 (1.7)	17 (26.1)	13 (23.6)
Diabetic	**>126**	54 (85.7)	56 (98.2)	11 (16.9)	27 (49.0)
Total		63 (100)	57 (100)	65 100	55 (100)

Dhanya et al, showed that the mean serum fasting glucose levels in diabetic group were 136.30 mg/dL and the mean serum glucose in controls was 97.78 mg/dL (Dhanya and Hegde 2016). results of this Study were in agreement that have shown that high blood glucose level as high FBS and high HbA1c and dyslipidemia as high TC-C, TG and LDL-C and low HDL are the main pathogenesis for micro and macro vascular complication of DB type 2;as it was revealed in the meta-analysis study by Sarwar N et al, high blood glucose level was linked with cardiovascular complications of DM (Zarrintan, Mobasseri *et al.* 2015). On the other hand, a study by Shrestha L et al, has stated that FBG correlated significantly with HbA1c values (Shrestha, Jha *et al.* 2012).In a study by H. Surekha Rani Et.al., it is observed that FBS, Chol, VLDL, LDLs, TGs were high and the levels of HDLs were low compared to controls (Rani, Madhavi *et al.* 2005).Results of this study demonstrated a significant positive correlation of blood sugar with, cholesterol, TG and VLDL-C. Similarly, cholesterol showed positive correlation with TG, LDL-C and VLDL-C. Significant negative correlation of TG

with HDL-C in the present study was in agreement with the earlier study of Mahato et al. (VinodMahato, Gyawali et al. 2011).

Results showed a correlation between serum glucose with GIP ($r = 0.229$, $p = 0.012$) as in the rats, GIP secretion was stimulated by glucose, galactose, and sucrose but not by fructose. However, the ability to obtain multiple blood samples over the course of 2 hours in human subjects revealed differences in the time course of GIP secretion. The rise in plasma GIP levels in response to sucrose was significantly delayed relative to the rise in response to glucose and galactose (Morgan 1979).

3.6-Hemoglobin HbA1c

The results showed that HbA1c has a significant association between Diabetic patients and control groups ($p = < 0.001$), table (3-2).

Moreover, there is a correlation between HbA1c with serum glucose, total cholesterol, TG, VLDL-C and GIP ($r = 0.515$, $p = 0.00$), ($r = 0.384$, $p = 0.00$), ($r = 0.803$, $p = 0.00$), ($r = 0.803$, $p = 0.00$), and ($r = 0.229$, $p = 0.012$) respectively as shown in table (3-10).

Table(3-9):Levels rate of HbA1c in Diabetic patients and Control individuals

Classification	Fasting Serum Glucose	Diabetic		control	
		Male n.(%)	Female n.(%)	Male n.(%)	Female n.(%)
Normal	**<6**	0 (0)	0 (0)	30 (46.1)	17 (30.9)
Pre-Diabetic	**6**	6 (9.5)	26 (45.6)	14 (21.5)	25 (45.4)
Diabetic	**14>6**	57 (90.4)	31 (54.3)	21 (32.3)	13 (23.6)
Total		63 (100)	57 (100)	65 (100)	55 (100)

According to the Diabetes Complications and Control Trial, HbA1c is the gold standard of glycemic control (Ketema and Kibret 2015).

As indicated in the present results, the mean HbA1c levels in Diabetic patients (cases) were significantly higher than that in controls. Similar results were obtained by Nomani, et al. who found that type 2 diabetic patients had higher HbA1C levels than non-diabetics (Nomani, Nabi *et al*. 2016). Large study of more than 13000 Chinese, Malays, and Indian adults, HbA1c provides good discrimination between individuals with and without moderate retinopathy. Our data suggest the HbA1c cutoff of 6.5% is reasonable for the diagnosis of diabetes in all three ethnic groups in Asia (Sabanayagam, Khoo *et al*. 2015). The case for HbA1c as a diagnostic test for diabetes has therefore been submitted to a very rigorous examination based upon the principles of evidence based medicine (Florkowski 2013).Various studies have described this before, that the correlation between HbA1c and HDL-C was negative, however there was a positive, significant correlation between HbA1c and TC, LDL-C, and TGs (VinodMahato, Gyawali *et al.* 2011).

Correlation of HBA1c with TC and TG, but not with HDL-C and LDL-C was shown by previous studies (Mullugeta, Chawla *et al.* 2012), (Zadhoush, Sadeghi *et al.* 2015) whereas on the contrary, Babikr et al. Reported association of HBA1c with HDL-C and LDL-C, but not with TC and TG (Klisic, Kavaric *et al*. 2017).

The present study found significantly increased levels of TG and VLDL and decreased HDL levels similarly observed by Elizabeth et.al, observed that LDL and HDL cholesterol were significantly associated with HbA1c. HDL cholesterol was inversely associated with HbA1c whereas LDL cholesterol was positively associated with HbA1c in diagnosed diabetics (Rani, Madhavi *et al*. 2005).

Various studies have described this before that the correlation between HbA1c and HDL-C was negative, however there was a positive, significant correlation between HbA1c and TC, LDL-C, and TGs (Hussain, Ali *et al*. 2017).

HbA1c greater than 7.0% exhibited a significant increase in TC, LDL-C, TG, and compared with those with HbA1c up to 7.0%. Improving glycemic control may substantially reduce the risk of cardiovascular events. It has been projected that a decrease in the HbA1c value by 0.2% could lower the mortality by 10% (Kishore, Kim *et al*. 2012).

3.7-Glucose dependent insulinotropic polypeptide (GIP)

To the best of our knowledge, this was the first study that measured incretin levels in Kerbala. The results show that Glucose dependent insulinotropic polypeptide (GIP) levels decreased in control group compared with Diabetic group.

The results obtained show that GIP has significant association between Diabetic patients and control groups (p=<0.001), table (3-2).

There is, also, significant correlation between GIP with TC and LDL-C respectively as shown in table (3-10), and figures (3-4), (3-5).

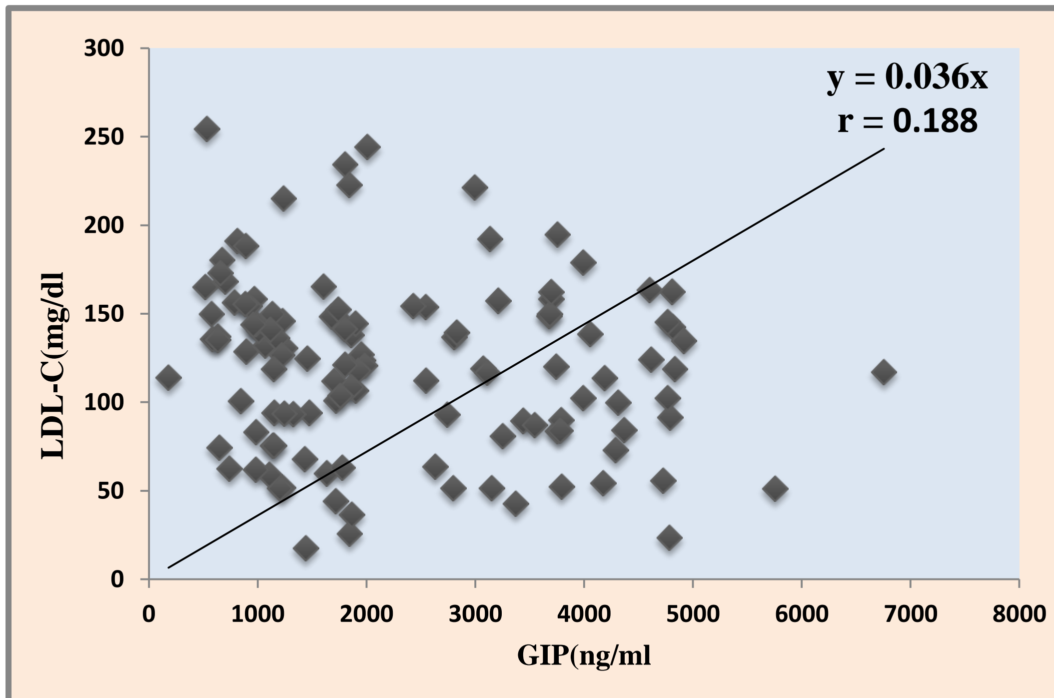


Figure (3-4): Correlation between serum GIP and serum LDL-C.

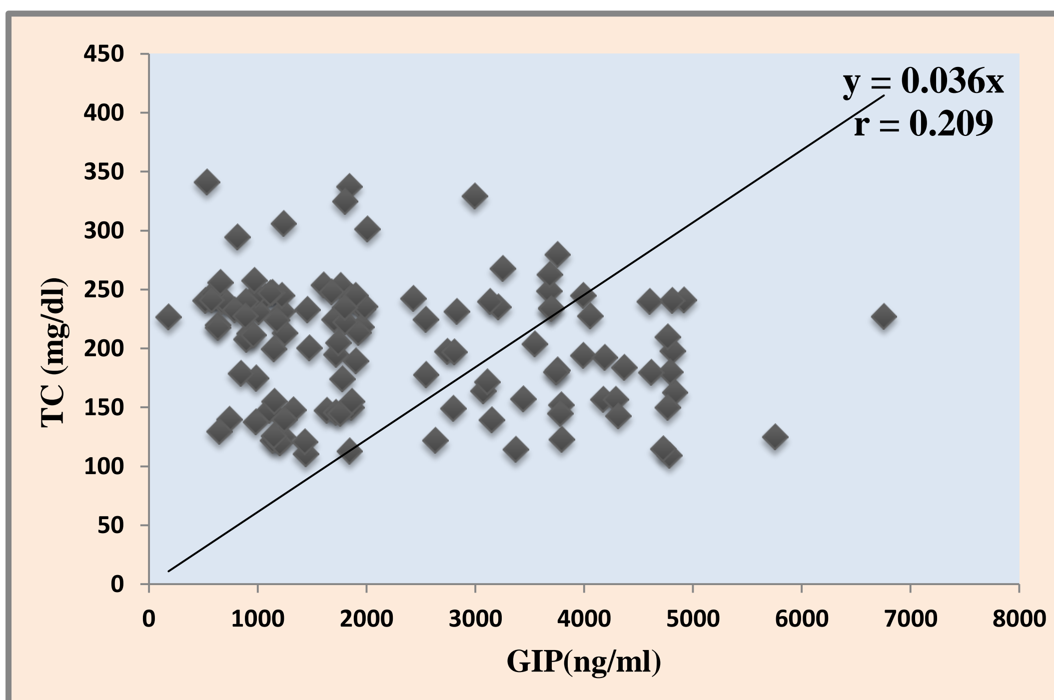


Figure (3-5): Correlation between serum GIP and serum TC.

The current study was the first extensive examination of human GIP including patients with T2DM and matched healthy controls. Most of the studies excluded based on these considerations showed increased GIP secretion in diabetic patients and have generated the common notion that GIP secretion is higher in patients with diabetes than in matched non-diabetic subjects (Calanna, Christensen *et al*. 2013).

The present study, GIP levels were increased significantly in subjects who have diabetes. This result was also in line with previous report showing that GIP levels were significantly elevated in newly-diagnosed diabetes group when compared with the normal group (Chia, Odetunde *et al*. 2014).

Zhong et al. study showed that the GIP and glucagon levels decreased after a mixed meal in patients with new diagnosed T2DM by treatment with single dose acarbose but Miyawaki K, et al. study has shown that the secretion of GIP increases significantly after the excessive ingestion of nutrients, indicating that GIP plays an important role in the development of obesity and insulin resistance induced by a high-calorie diet (Chen, Fu *et al*. 2016).

Serum GIP was positively correlated with serum levels of LDL cholesterol(Yamaoka *et al*. 2010). since the other authors assessing the incretins concentration in patients with diabetes mellitus reported that GIP level can be equal, or elevated in comparison to healthy people (Ross, Brown *et al.* 1977).

The lack of GIP effect in T2DM has given rise to try and link polymorphisms in the GIP receptor with the T2DM phenotype. Two earlier studies from Europe and Japan have failed to establish an association between T2DM and GIP receptor polymorphisms (Kubota *et al*. 1996, Almind *et al*. 1998). Noteworthy, Meier et al reported that plasma GIP concentrations were elevated in some T2DM patients (Meier *et al*. 2001).

Table (3-10): Correlations Between the Parameters in Diabetic Patients

	age	smoker	BMI	Glucose	HbA1c	TC	HDL	VLDL	TG	LDL	GIP
age	1	0.101	0.075	-0.115	0.079	0.110	0.005	0.085	0.085	0.088	0.013
smoker	-	1	0.067	-0.046	-.073	.043	.173	-0.061	-0.061	0.034	1
BMI	-	-	1	-.138	.031	.189*	-.023	.101	.101	.176	-0.002
Glucose	-	-	-	1	.515**	.258**	-.077	.286**	.286**	.189*	0.098
HbA1c	-	-	-	-	1	.611**	-.023	.803**	.803**	.364**	0.078
TC	-	-	-	-	-	1	-.008	.525**	.525**	.904**	0.209*
HDL-C	-	-	-	-	-	-	1	.092	.092	-.268**	0.173
VLDL-C	-	-	-	-	-	-	-	1	1.000**	.163	0.101
TG	-	-	-	-	-	-	-	-	-	.163	0.101
LDL-C	-	-	-	-	-	-	-	-	-	-	0.188*
GIP	-	-	-	-	-	-	-	-	-	-	1

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

BMI (Kg/m^2); Glucose (mg/dl), Cholesterol (mg/dl), S.TG (mg/dl), S.HDL (mg/dl), S.LDL (mg/dl), and VLDL (mg/dl); GIP (ng/ml).

3.8-Lipid Profile

The study results show that total cholesterol TC ,HDL-C,VLDL-C,TG and LDL-C are significantly elevated in Diabetic patients (p=<0.001), compared with normal control group as shown in table (3-2).

Firstly, serum total cholesterol shows significant correlation with VLDL-C, TG, LDL-C and GIP (r=0. 525, p=0.00), (r=0. 525, p=0.00), (r=0. 904, p=0.00) and (r=0.209, p=0.00) respectively.

While there is non-significant negative correlation with serum HDL-C (r =-.008, p>0.05) respectively was found.

Secondly, serum TG shows significant positive correlation with serum total cholesterol and serum VLDL-C (r =0. 525, p=0.00) and (r =1.00, p=0.00) respectively non-significant correlation with serum HDL-C (r=0.092, p>0.05) as shown in table (3-10).

Over 70% of patients with T2DM had one or more types of dyslipidemia. Similarly, these results reveal high prevalence of hypercholesterolemia, hypertriglyceridemia and high LDL-C levels, which are well known risk factors for cardiovascular diseases among patients. In diabetes many factors may affect blood lipid levels, because of interrelationship between carbohydrates and lipid metabolism. Therefore, any disorder in carbohydrate metabolism leads to disorder in lipid metabolism and vice versa (Dixit, Dey *et al*. 2014).

Sendhav et al. in their study documented that lipid profile showed a significant rise (p <0.01) of triglycerides, total cholesterol, low-density lipoprotein-cholesterol (LDL-C), and very low-density lipoprotein-cholesterol (VLDL-C) along with increase in fasting blood glucose among diabetics in comparison with controls (p < 0.01) (Sendhav SS 2017) . in Shaik et al. study, was able to document a significant increase in TGs, LDL-C, TC and drop in HDL-C among diabetics when compared to normal individuals (Hussain 2018).The present study showed

that the level of dyslipidemia parameters are very high in type 2 diabetic patients, 61.4% with a high level of LDL, 52.2% with a low level of HDL, 51.2% with a high level of TG, and 68.1% with a high level of non-HDL (Safo 2018).

Shyamala K. et al. show that the mean value of TC, VLDL-C and LDL-C were higher in overall T2DM patients than the normal range and HDL-C was lower in T2DM patients (Venkatesh and Sudheer 2018). The present study analysed the effects of lipid on the diabetic, control and found that the subjects with Diabetic showed a marked increase in the prevalence of hypercholesterolemia, hypertriglyceridemia, and obesity. These results could be interpreted by a research result that found a significant correlation between these parameters and they have also found lipid abnormalities during diabetes induced dyslipidemia are hypercholesterolemia, hypertriglyceridemia and elevated LDL cholesterol (Ozder 2014). Other study in Saudi Arabia and Korea, consistent to this study, reported that there was a positive correlation between BMI with TC, respectively (Al-Ajlan 2011).

Patients with T2DM compared to healthy ones have different cholesterol metabolism because they synthesis higher level of cholesterol. Also in other studies, by Comparing diabetic people with other individuals show the metabolism of cholesterol and LDL in these patients is more than healthy people (Fadaei, Asadi *et al*. 2017).

This study has shown that serum TG shows significant positive correlation with serum total cholesterol and serum VLDL-C. Similar results of significance with serum cholesterol and serum triglycerides with panic disorder and major depression disorder as reported by Hamidreza et al in a descriptive analytic study conducted 100 major depressive patients in Iranian population (ROUHAFZA, SADEGHI *et al*. 2005).

3.9-Molecular analysis

3.9.1-Estimation of DNA concentration

Table (3-11): DNA concentration and purity

DNA sample	Mean +SD
DNA conc. (µg/ml)	67.83+32.41
DNA purity	1.63+0.51

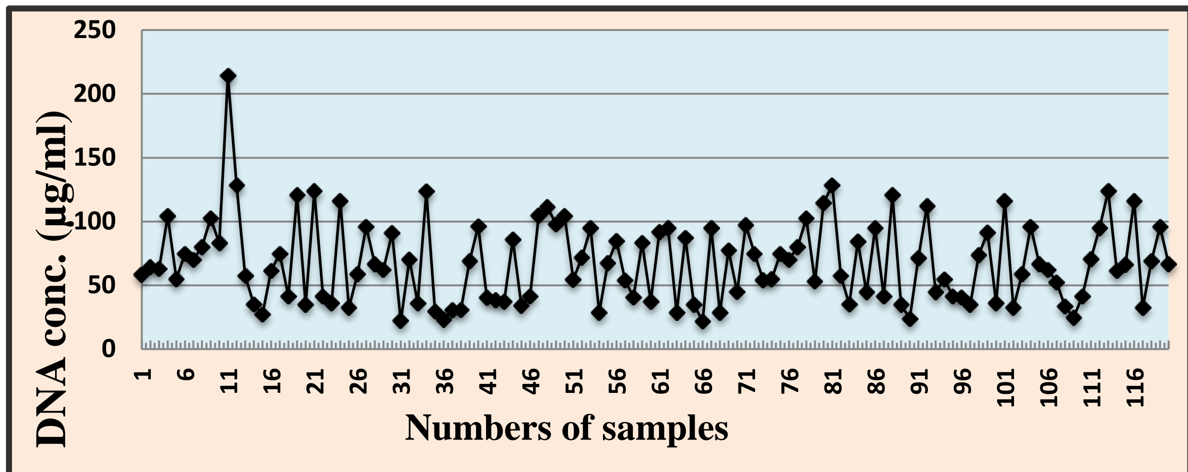


Figure (3-6) Concentration of DNA extracted from study individuals.

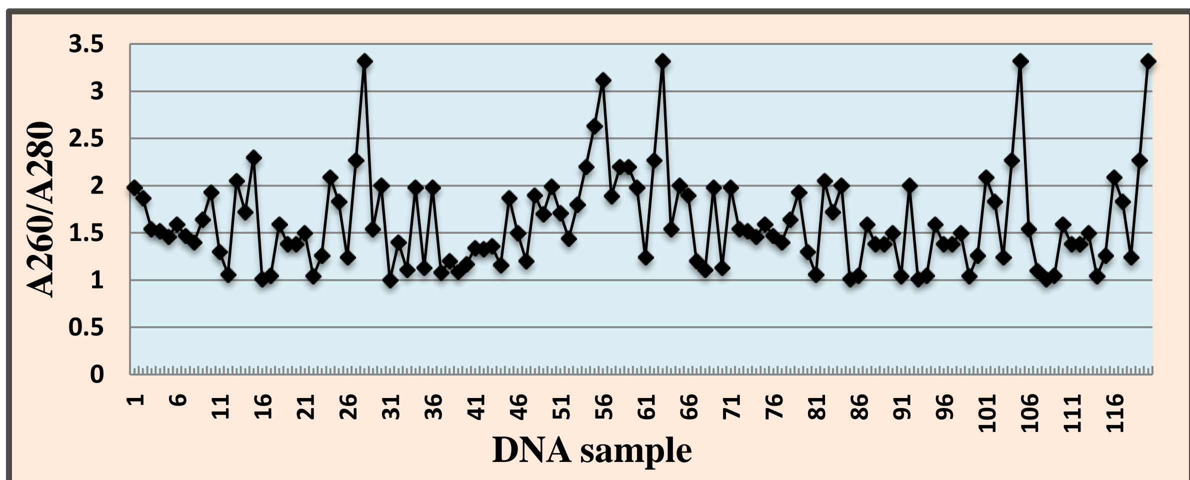
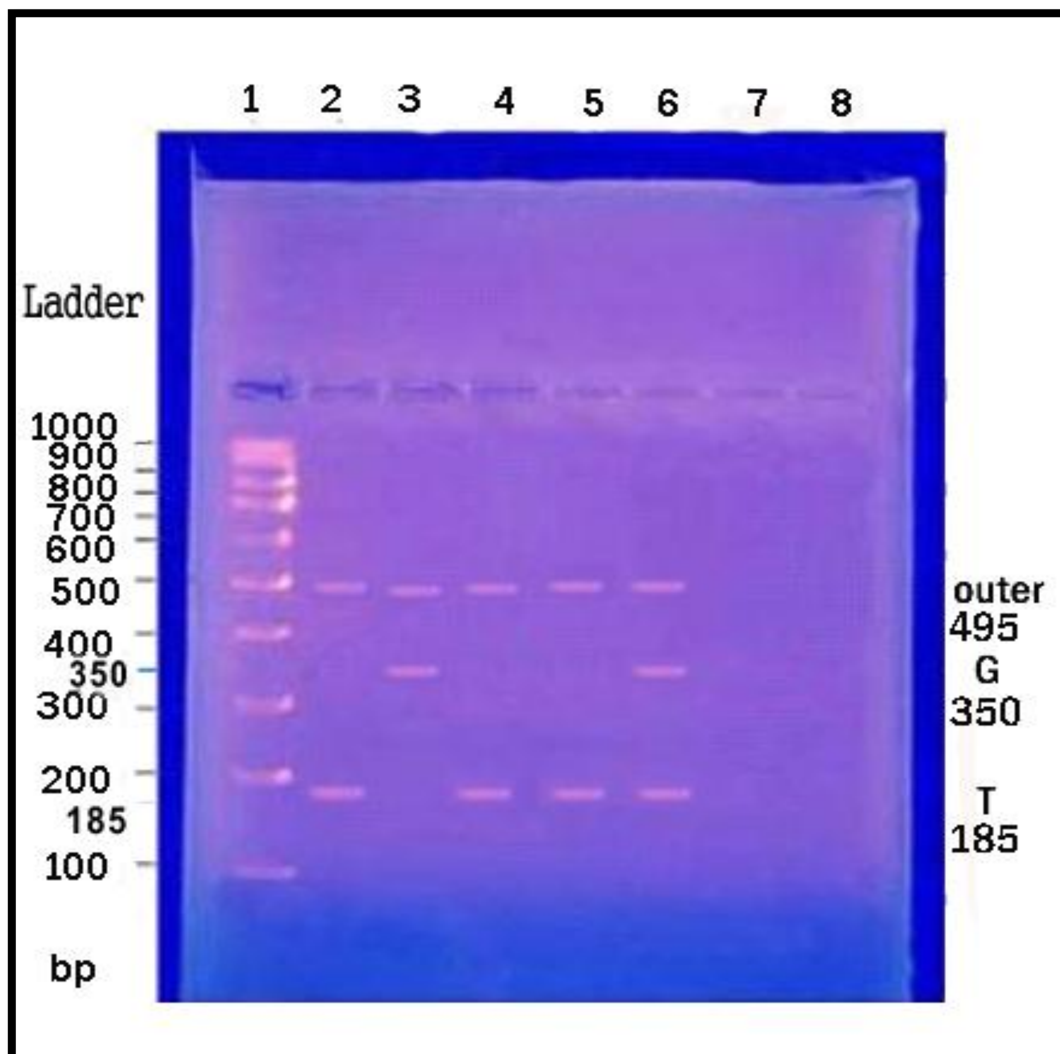


Figure (3-7) Purity of DNA extracted from study subjects.

3.9.2-Results of amplification reactions

3.9.2.1-rs1861978 mutation

Amplification the ARMS-PCR product of DPP4 gene polymorphism rs1861978 from the figure (3-8) we see the outer band in 495bp and the G allele in 350bp and T allele in 185bp, Three genotypes of rs1861978were generated: Lane 3 is the GG genotype (homozygous wild type), lane 6 is the G/T genotype (heterozygous) and lanes 2, 4, 5 are the T/T genotype (homozygous).



Lane 1 is size marker, lanes 2, 4, 5 are the T/T genotype (homozygous), Lane 3 is the GG genotype (homozygous wild type) and lane 6 is the G/T genotype (heterozygous).

Figure (3-8) ARMS-PCR product of DPP4 gene.

Table (3-12): DPP4 rs1861978 Genotype in T2DM and Control

Genotype	T2DM N (%)	Control N (%)
G/G	11 (9.1)	6 (5)
G/T	50 (41.6)	36(30)
T/T	59 (49.1)	78 (65)
Total	120 (100)	120(100)

Table (3-12) shown that the Genotypes G/G, G/T, T/T are 11,50,59 in T2DM and are 3,36,78 in control this results were compare with the finding of Radwan H. Ahmed et al. that result were revealed 4,55,255 in T2DM and are 3,30,131 in control.

3.9.3-DPP4 Gene and Diabetic

3.9.3.1-DPP4 Genotype and other parameters

According to the Genotype, the results from Table (3-13) showed that there was a significantly higher levels of FBS , HbA1C, VLDL-C , LDL-C and TC, while serum HDL-C recorded a significant decrease in diabetic G/G group in comparison with healthy G/G group (P <0.001). As well as there was no significantly different levels of FBS , HbA1C, VLDL-C ,TG , HDL-C, LDL-C and TC levels, in diabetic G/G group in comparison with diabetic G/T group (p>0.05). As well as there was no significantly different levels of FBS, HbA1C, VLDL-C, TG, HDL-C, LDL-C and TC, in diabetic G/G group in comparison with diabetic T/T group (p>0.05).

Result of this study has shown significant associations between DPP4 SNP and Diabetes. Different as study that selected seven SNPs in the DPP4 gene (rs3788979, rs1558957, rs1861978, rs4664443, rs12617656, rs17574, rs7608798)

based on previous indications that were studied with risk factors for T2DM,and found no association was found between T2DM and other SNPs rs1558957, rs1861978, rs2160927, rs17574, rs7608798, rs1014444 (Ahmed, Huri *et al*. 2016). The Hap Map project also showed that the allelic distribution of many DPP4 SNPs in Japanese and Chinese was the opposite of that in Europeans(Buchanan, Torstenson *et al*. 2012).

Table (3-13): Relation between DPP4 genotype and other Parameters

Parameter	Diabetic (n.=120) mean ± SD			Control (n.=120) mean ± SD		
	G/G (n.=11)	G/T (n.= 50)	T/T (n.= 59)	G/G (n.= 6)	G/T (n.= 36)	T/T (n.= 78)
Age (years)	48.81±4.33	49.26±4.93	49.35±4.75	49.16±4.53	48.88±4.77	49.29±4.67
BMI (kg/m^2)	29.06±3.90	29.67±4.30	28.32±3.77	28.18±4.90	29.27±3.81	28.46±3.20
FBS (mg/dl)	262.1±120.48 [c*]	247.7±108.11	220.53±100.05	102.1±11.86	104.88±29.88	138.96±66.99
HbA1c %	8.27±1.95 [c*]	8.46±2.10	7.86±2.20	5.83±0.98	5.75±1.11	5.78±0.99
TC (mg/dl)	203.51±61.90 [c*]	205.68±49.50	201.39±55.90	129.7±15.49	173.19±48.83	157.56±37.11
HDL-C (mg/dl)	34.40±6.88 [c*]	36.41±10.12	37.42±11.74	48.16±14.11	42.36±11.45	47.34±11.87
LDL-C (mg/dl)	126.28±56.27 [c*]	121.41±47.87	118.74±47.42	51.87±12.78	95.57±52.04	76.06±38.47
VLDL-C (mg/dl)	42.84±16.24 [c*]	47.86±20.80	45.24±18.47	29.74±14.93	35.26±15.59	34.16±15.65
TG (mg/dl)	214.20±81.19	239.3±104.01	226.19±92.33	148.7±74.65	176.31±77.93	170.80±78.27
GIP (pg/ml)	4571.3±842.5 [a,b,c*]	3589.3±1607.8 [a,b,c*]	2988.8±1406.4 [a,b,c*]	1949.4±734.9	2376.9±1413.5	2337.9±1505.5

P value derived from ANOVA test,*Significant: $p<0.05$, ** highly significant: $p<0.001$, No significant: $p>0.05$, n.=number, SD=standard deviation, BMI=body mass index, TG=triglycerides, TC=total cholesterol, HDL-C=high density lipoprotein-cholesterol, LDL-C=low density lipoprotein-cholesterol, VLDL-C=very low density lipoprotein cholesterol, GIP= Glucose-Dependent Insulinotropic Polypeptide, FBS= Fasting Serum Glucose, [a] ANOVA test= G/G vs. G/T Diabetic patients, [b] ANOVA test= G/G vs. T/T Diabetic patients, [c] ANOVA test= G/G vs. G/G Diabetic patients vs. control.

According to the genotype, the results showed that there was a significantly higher levels of FBS , HbA1C, VLDL-C , LDL-C , TC and GIP levels, while serum HDL-C recorded a significant decrease in diabetic GG genotype (homozygous wild type) group in comparison with healthy G/G genotype (homozygous wild type) group ($P < 0.001$). As well as there was no significantly levels of FBS , HbA1C, TG, VLDL-C , LDL-C , TC and GIP levels, while serum HDL-C recorded no significant decrease in diabetic GG genotype (homozygous wild type) group in comparison with healthy G/T genotype (heterozygous) group or T/T genotype (homozygous) ($p > 0.05$).

This study investigates DPP4 allelic distributions and their association with incretin hormones (GIP) in kerbala T2DM, which may have clinical significance.

Moonesinghe et al. observed that SNP rs12617656and rs1861978 (SNPs in the DPP4 gene) showed a stronger association with T2DM in Indians. Such an association might be attributed the gene-environment and gene-gene interactions that might have contributed to the differences in gene-disease associations among the different ethnic groups (Moonesinghe, Ioannidis *et al*. 2012).

Ma, Lu. et al. revealed the association of the DPP4 polymorphisms rs3788979, rs1861978 and rs12469968 with CAD risk in a Chinese Han population with type 2 diabetes. The genetic variants at DPP4 gene SNP rs3788979 have a synergetic effect with obesity to increase the risk of CAD in Chinese Han population with T2 DM (Ma, Lu *et al*. 2017).

Chapter Four

Concolusions

&

Recommendations

4-Conclusions and Recommendations

4.1-Conclusions

From all data and correlations of different variables in the present study, it could be concluded that:

1- The mean serum glucose and blood HbA1c levels in patients were significantly higher than that in controls.

2- The average levels of cholesterol, triglycerides and LDL-C were significantly found to be higher in cases compared to controls. On the other hand, HDL-C was significantly lower in patients. The Pearson correlation test revealed negative significant correlation between GIP and Total cholesterol (TC). On the other hand, there was a negative significant correlation between GIP and LDL-C level.

3- The mean level of serum GIP was higher in local diabetic patients compared to controls.

4- The t-test showed no significant in BMI of diabetic patients compared to controls.

5- This study shows that genotype of SNP rs1861978 of DPP4 is association with Diabetic patients.

6- There is significant association between DPP4gene and incretin hormones (GIP) in Diabetic, (G/G) genotype of SNP rs1861978 of DPP4siginificat with GIP.

4.2-Recommendations

This study recommends that it is necessary to:

1- Increase the sample size and use a cohort study to have more benefit.
2- Measure enzymatic activity of DPP4 and incretin hormones gene (GIP, GLP) in patients with type 2 diabetes mellitus.
3- Measure the level of another incretin hormones glucagon-like peptide (GLP).
4- Study the association between SNP of incretin hormones (GIP, GLP) and incretin hormones levels (GIP, GLP) in patients with type 2 diabetes mellitus.
5- Study the cases of patients with type 2 diabetes mellitus that take drugs (Glucophage, Thiazolidinedione) and study the effects of these drugs with incretin hormones levels (GIP, GLP).
6- Study another SNP of DPP4.or increase numbers of SNP in new study to show if any association between SNP of DPP4 and incretin hormones, in Iraqi population.
7- Further studies with functional investigations will be necessary to confirm the association between the DPP4 genetic polymorphisms and T2DM.

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الخلاصة

الخلفية

يرتبط مرض السكري من النوع الثاني بزيادة معدلات انتشار الامراض والوفيات في جميع أنحاء العالم. وهو مرض متعدد الجينات مع عواقب وخيمة.
تظهر العديد من المؤشرات ان بداية تطور هذا المرض تعتمد على عوامل بيئية. لذلك فان دراسة الطفرات الجينية المتعلقة بداء السكري النوع الثاني ستضيف الى المعرفة وتساهم في تطوير العلوم الطبية للعالم اجمع .

ويمكن ايضا ان تستخدم هذه الدراسات للطفرات للمعرفة المستقبلية لتصميم الاختبار التشخيصي للتنبأ فيما اذا كان الشخص قابل للاصابة بمرض السكري من النوع الثاني في المستقبل ام لا لان مرض داء السكري من النوع الثاني يرتبط ببعض الطفرات وكذلك يعتمد على التفاعل مع البيئة المحيطة.

نظرًا لأن العديد من الجينات المعنية بالأنظمة من النوع الثاني من داء السكري ، فقد اقترحت علاقة تعدد الأشكال النيوكليوتيدية الفردية rs1861978 في الداي ببتديل ببتيديز -4 أن ترتبط بمرض السكري.
من ناحية أخرى ، وجد أن هرمونات الإنكريتين مثل بوليببتيد GIP في الأبحاث الحديثة مرتبطة بمرض السكري من النوع الثاني في ظروف مختلفة.

الهدف من الدراسة

اجريت دراسة الحالات والشواهد لتقييم العلاقة بين هرمونات الانكرتين والطفرة الجينية rs1861978 في الداي ببتديل ببتيديز -4 لمرضى السكري النوع الثاني.وعلاقة الطفرة بالاصابة بمرض السكري.

الهدف من هذه الدراسة هو التحقق من تواجد علاقة بين تعدد الاشكال النوكليوتيدية للطفرة الجينية rs1861978 في الداي ببتديل ببتيديز -4 لمرضى السكري النوع الثاني في العراق واجراء التقييم لتاثير مستويات هرمون الانكرتين في مصل الدم للمرضى .

طرائق العمل

لقد تم اختيار مئة وعشرون حالة مرضية مصابة بمرض السكري النوع الثاني بناءا على تشخيص الاطباء الاختصاص بمركز الحسن(ع) لمرضى السكري والغدد الصماء في مدينة الامام الحسين (ع) الطبية في مدينة كربلاء المقدسة. بعض المرضى يتناول الادوية العلاجية والبعض الاخرلاياخذ اي علاج وجميعهم لم يظهر اي مشاكل صحية في الكبد او الكلى لحد تاريخ اخذ العينات.

كذلك تم اختيار مئة وعشرون فردا من الاصحاء ومن الذين ليس لديهم اعراض مرض السكري النوع الثاني حيث انهم يتمتعون بصحة جيدة.

لقد تم اخذ التاريخ المرضي لجميع الحالات وقياس ضغط الدم واجراء اختبارات الدم الحياتية ومؤشر كتلة الجسم ، وقد تم جمع بيانات هذه الدراسة وجمع عينات الدم خلال الفترة من كانون الثاني إلى نيسان 2018 . حيث تم جمع نموذج الدم من حالة الصيام لجميع المشاركين في هذه الدراسة لقياس كل من هرمونات الانكريتين ، نسبة السكر في الدم ، و نسبة الدهون.

وتم سحب 2 مل من هذه العينية ووضعاه في انابيب EDTA لاجراء فحوصات الحمض النووي وتفاعلات الكوثرة.

النتائج

اظهر مؤشر كتلة الجسم عند مرضى داء السكري النوع الثاني ارتباط وعلاقة واضحين للغاية ($p<0.01$).

كذلك لوحظ وجود ارتفاع في معدلات كل من الدهون الثلاثية، الكولسترول الكلي ، و البروتينات الدهنية واطئة الكثافة مع اختزال في معدلات البروتينات الدهنية عالية الكثافة في المرضى المصابين بارتفاع سكرالدم بالمقارنة مع الاشخاص الاصحاء .

ايضا اظهر المرضى بداء السكري النوع الثاني من العراقيين في هذه الدراسة وجود علاقة ذات دلالة احصائية بين الطفرة الجينية rs1861978 في الداي ببتديل ببتيديز -4 وداء السكري ($p < 0.001$).

الاستنتاج

اظهرت هذه الدراسة انه يوجد ارتباط بين الطفرة rs1861978 في الداي ببتديل ببتيديز -4 مع مرض داء السكري النوع الثاني .

كذلك اظهرت هذه الدارسة انه يوجد ارتباط بين مستويات هرمون GIP مع مرض داء السكري النوع الثاني.

كذلك وجدت الدراسة ارتفاع كل من هرمون الانكرتين ومستوى السكر بالدم والتراكمي في مرضى داء السكري بالمقارنة مع الاشخاص الاصحاء.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء

كلية الطب

فرع الكيمياء الحياتية

الترابط بين أنزيم الداي ببتديل ببتيديز-4 وتعدد الاشكال الجيني (rs1861978) ومستوى الانكرتين لمرضى السكري النوع الثاني في كربلاء المقدسة

رسالة مقدمة الى

مجلس كلية الطب / جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في الكيمياء السريرية

من قبل

أسعد مناف مصطفى الموسوي

بكلوريوس علوم كيمياء / جامعة بابل 2002

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