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Preptin and Irisin as Metabolic Biomarkers in Juvenile Diabetes Mellitus

A Thesis

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

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Dedication

This work is dedicated to everyone who fell martyr in the defense of our homeland.

To my mom, dad, sister, and brother and to my uncle who died in corona pandemic.

Zeina Ajam

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First of all, I 'm extremely appreciative for the Lord of Heaven for all the help and facilities that he put in my way to complete this work. I really appreciate the effort of my supervisor professor Dr. Seenaa Badr Mohammed not only for her guidance, but also for her support, encouragement, patience, and kindness.

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Summary

Type 1 diabetes is a chronic autoimmune disorder marked by insulin depletion as a result of pancreatic β-cell damage leading to hyperglycemia. It is commonly occurs in children, adolescent, and young adults. Hyperglycemia eventually develops as the insulin levels decreased, which makes the patients at the risk of oxidative stress and diabetes complications. Due to the absence of a definitive treatment, patients are dependent on everlasting insulin injections.

Preptin is a newly discovered peptide hormone derived from pro-insulin-like growth factor-II (IGF-II), synthesized mainly in pancreatic β-cells and coincides with insulin in response to increased blood glucose levels. As preptin has a role in the amplifying of insulin secretion, it might be used as a physiological amplifier for the glucose-mediated insulin secretion.

Irisin is a novel exercise-induced myokine secreted from human muscles and mediates the beneficial effects of exercise such as thermoregulation and weight loss. Since there are grateful benefits of exercise induced-irisin, particularly in glucose homeostasis, we suggest that irisin could be used as a therapeutic agonist in patients with type 1 diabetes to increase glucose utilization.

C-peptide is a linker chain cleaved from proinsulin in pancreatic β-cells. It was discovered that C-peptide has numerous beneficial effects on various cell types and tissues. C-peptide is used as a biomarker for the function of β-cells because it is secreted in similar quantities with insulin, evade first pass metabolism by the liver, and has a longer half-life than insulin.

The present study aims to investigate the levels of irisin and preptin in patients with type 1 diabetes and determine their correlation with C-peptide levels. In addition to determine the correlation of these hormones with glucose levels, to determine the possibility of their usage as a co-therapy with insulin.

Sample collection was performed from the 1st of September of 2022 until the 13th of February 2023. The practical side of the study was performed in the laboratory of Chemistry and biochemistry department at the College of Medicine/University of Babylon.

The present case-control study involved 100 participants with an age range (2-16 years), divided into two groups: 50 apparently healthy control group and 50 patients with type 1 diabetes. The random glucose levels in all participants were estimated by a spectrophotometer, while the levels of preptin, irisin, and C-peptide were measured by Enzyme-Linked Immunosorbent Assay (ELISA) technique. Hemoglobin A1C is measured using an automated analyzer.

The study showed a significant decrease in C-peptide levels in the patient group ($P \leq 0.05$). Random blood sugar was increased considerably in the patient group ($P<0.001$). Preptin and irisin levels were significantly increased in the patients group compared with the control group ($P<0.05$).

In addition, this study showed a positive correlation between preptin and irisin ($P<0.001$). Preptin and irisin also correlated positively with C-peptide ($P<0.001$). In regression analysis, irisin was a significant predictor for circulating preptin and C-peptide levels. There was also a positive significant correlation between HbA1c and RBS ($P=0.028$).

In conclusion, the positive correlation between preptin and irisin support that there is a relationship between irisin and pancreatic β-cells, and so the development of diabetes mellitus. Irisin is a strong predictor for preptin levels, and thus residual β-cells, therefore it might be used as a predictive marker with C-peptide for T1D. Through the strong association between preptin and C-peptide, preptin could be used as a physiologic amplifier for the second phase insulin secretion.

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List of abbreviations

Abbreviation	Meaning
2-h PG	2-h plasma glucose
4-AP	4-aminophenazone
ACC	Acetyl-CoA carboxylase
ADA	American diabetes association
ADAM	Disintegrin and metalloproteinase
AGEs	Advanced glycated end products
AKT	Protein kinase-B
ALP	Alkaline phosphatase
AMPK	Adenosine monophosphate-activated protein kinase
APC	Antigen presenting cells
ATF	Activating transcription factor
BAT	Brown adipose tissue
CAD	Coronary artery disease
CAM	Cellular adhesion molecule
CDC	Centres for disease control and prevention
COX2	Cyclooxygenase 2
CREB	cAMP- response element binding protein
CVD	Cardiovascular disease
DAG	diacylglycerol
DCCT	Diabetes Control and Complications Trial
DKA	Diabetic ketoacidosis
ERK	Extracellular-signal regulated kinase
FABP	Fatty acid binding protein
FAK	Focal adhesion kinase
FAS	Fatty acid synthase

FFA	Free fatty acid
FNDC5	Fibronectin type III domain-containing 5
FOXO1	Forkhead box protein O1
FPG	Fasting plasma glucose
G6PC	Glucose-6 phosphatase
GAD65	Glutamic acid decarboxylase
GDF	Growth differentiation factor
GLUT	Glucose transporter
GOD	Glucose oxidase enzyme
GPCR	G- protein coupled receptor
GSK	Glycogen synthase kinase
GWAS	Genome Wide Association Studies
HK	Hexokinase
HLA	Human leukocyte antigen
HSL	Hormone sensitive lipase
HUVEC	Human umbilical vein endothelial cell
IA-2	Islet autoantigen-2
IAA	Insulin autoantibodies
ICA	Islet Cell autoantibodies
ICAM	Intracellular adhesion molecule
IDDM2	Insulin dependent diabetes mellitus
IDF	International diabetes federation
IFN	Interferon
IGF	Insulin like growth factor
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit related protein
IL	Interleukine

INS	Insulin gene
ISPAD	International society for paediatric and adolescent diabetes
JNK	c-Jun N-terminal kinase
KC	keratinocyte chemo attractant
LADA	Latent autoimmune diabetes in adults
LDL	Low density lipoprotein
LRP5	LDL receptor related protein 5
LXRα	Liver x receptor-α
MAPK	Mitogen-activated protein kinase pathways
MCP 1	Monocyte chemoattractant protein-1
MCP-1	monocyte chemotactic protein 1
MEF2	Myocyte enhancer factor
MHC	Major histocompatibility complex
MIP 1	Macrophage inflammatory protein 1
MyD88	Myeloid differentiation primary response protein 88
NADH	Nicotinamide adenine dinucleotide + H
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-κappa B
NGSP	National Glycohemoglobin Standardization Program
NICE	National Institute for Health and Care Excellence
NK	Natural killer cells
NO	Nnitric oxide
NOX4	NADPH-oxidase4
OGTT	Oral glucose tolerance test
OGTT	Oral glucose tolerance test

PCK-1	Phosphoenolpyruvate carboxykinase 1 (also known as PEPCKC)
PEPCKC	Phosphoenolpyruvate carboxykinase
PGC-1α	Peroxisome proliferator-activated receptor-γ co-factor 1α
PI3K	phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase-C
PKC	Protein kinase C
PLC	Phospholipase -C
PPARA	Peroxisome proliferator-activated receptor alpha
PRMT3	Protein arginine methyltransferase-3
PYGM	Muscle glycogen phosphorylase
R1, R2	Reagents
RAC-1	Ras-related C3 botulinum toxin substrate 1
Rho A	Ras homolog family member A
ROCK1	Rho-kinase-1
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2
SCR	S-locus cysteine rich protein
sp7	Transcription Factor, also called Osterix
SREBP1C	Sterol regulatory element-binding protein 1C
STAT	Signal transducer and activator of transcription
T1DM	Type 1 diabetes mellitus
TFAM	Mitochondrial transcription factor
TGF-β	Transforming growth factor beta
TLR4	Toll like receptor-4

TNF	Tumour necrosis factor
UCP	Uncoupling protein
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VNTR	Variable number of tandem repeats
WAT	White adipose tissue
WHO	World Health Organization
ZEB	Zinc finger transcription factor
α4β7	Lymphocyte Peyer's patch adhesion molecules(LPAM-1)
αLβ2	Lymphocyte function-associated antigen 1(LFA-1)

Introduction and Literature Review

1. Introduction and literature review

1.1 Diabetes mellitus:

1.1.1 Definition:

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by increased blood glucose levels, which after a long time, leads to serious damage to the eyes, neurons, kidneys, heart, and blood vessels. The underlying reasons for the disease are either aberrant insulin secretion, insulin resistance, or most frequently both [1]. The traditional signs of severe hyperglycemia include polyuria, polydipsia, weight loss, polyphagia, and impaired vision. Prolonged hyperglycemia can also be associated with growth impairment and susceptibility to specific infections [2]. Diabetes' consequences affect nearly most of the body's tissues, and it is considered the leading cause of increased cardiovascular morbidity and mortality, cerebrovascular disease, renal failure, blindness, and amputations [3].

Other consequences of diabetes can affect the foot and ankles of the diabetic patients such as ulcerations, infections, or gangrene. Long-term hyperglycemia may also affect cognitive function [4]. In recent decades, an upsurge has been occurred globally in the prevalence of diabetes, and the main causes are economic expansion, global aging, fast urbanization, and dietary changes in various income-level nations [5].

1.1.2 Classification of Diabetes Mellitus (DM):

To administer successful treatment, classification should be identified properly [6], table (1-1). The main two etiopathogenetic groups of diabetes include: T1DM, in which pancreatic β-cells are destroyed by the immune system, and T2DM, a more prevalent category, can be caused by a combination of both insulin resistant and insufficient insulin secretion [7]. Another new kind of diabetes, overlaps between T1D and T2D, is termed latent autoimmune diabetes in adults (LADA) that is characterized by the existence of islet autoantibodies,

occurs in middle and later stages of life, presence of both T1D and T2D risk genes, a leaner habit, and a faster development to the need for insulin injection than individuals with T2D [8].

Additional types of diabetes that may develop from different causes include monogenic diabetes syndrome (e.g. maturity-onset diabetes), diseases of the exocrine pancreas (e.g. cystic fibrosis), and drug or chemical-induced diabetes [6]. Furthermore, gestational diabetes is another form of DM, which is a common medical condition known to affect pregnant women [9].

Table (1-1). The classification of DM [10]**:**

1-Type 1—β-cell destruction, usually leading to absolute insulin deficiency **• Autoimmune** **• Idiopathic**
2-Type 2—may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance.
3-Other specific types of diabetes **• genetic defects of β-cell function** **• Genetic defects of insulin action** **• Diseases of the endocrine pancreas** **• Endocrinopathies** **• Drug- or chemical-induced** **• Infections** **• Uncommon forms of immune-related diabetes** **• Other genetic syndromes sometimes associated with diabetes**
4-Gestational diabetes

1.1.3 Diagnosis of Diabetes Mellitus:

In order to make a diagnosis of diabetes, plasma glucose criteria such as the fasting plasma glucose (FPG) or the 2-hour plasma glucose (2-h PG) value (OGTT), or HbA1c test may be used, as shown in table (1-2).

Table (1-2). The diagnostic criteria for DM according to the American Diabetes Association (ADA) [11]

FPG ≥126 mg/dL (7.0 mmol/L). **-Fasting is defined as no caloric intake for at least 8 h.***
OR
2-h PG ≥200 mg/dL (11.1 mmol/L) during OGTT. **-The test should be performed as described by WHO, using a glucose load containing the equivalent of 75 g of anhydrous glucose dissolved in water. ***
OR
HbA1c ≥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. ***
OR
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, diagnosis requires two abnormal test results from the same sample or in two separate test samples

1.1.4 Diabetes complications:

Diabetes complications include: coronary artery disease (CAD), cardiovascular disease (CVD), stroke, renal disease, peripheral neuropathy, diabetic retinopathy, lower extremities amputation, hypertension, dyslipidaemia, hearing impairment, obstructive sleep apnoea, dementia, and increased incidences of specific cancers [12]. Complications of diabetes can be classified into acute and chronic complications.

1.1.4.1 Acute complications:

This type of complications can occur at any time including hypoglycaemia (glucose levels <70 mg/100 ml in children), hyperglycaemia and diabetic ketoacidosis (a life-threatening emergency) [13].

1.1.4.2 Long-term complications of diabetes:

Chronic diabetes complications can be subdivided into microvascular and macrovascular complications [14].

1-Microvascular complications: As glucose combustion results in the production of electrons, which are stored mainly in NADH, the more glucose levels in blood the more electrons are produced. As a consequence, prolonged hyperglycaemia leads to a redox imbalance between NADH and NAD^+ favouring NADH direction. This is certainly what happens in diabetes, and the polyol pathway plays a major role in breaking the redox imbalance [15]. Polyol pathway consists of two reactions, in which excess glucose is converted to sorbitol by aldose reductase and sorbitol is converted to fructose by sorbitol dehydrogenase. The first reaction occurs at the expense of NADPH, while the second reaction occurs at the expense of NAD^+, leading to $NADH^+$ production. Thus, the total products of the polyol pathway include sorbitol, fructose, and NADH [16].

Polyol pathway activation can results in decreased NADPH/$NADP^+$ ratio and nitric oxide production, increased protein glycation and development to non-alcoholic fatty liver disease due to the higher content of fructose, induce accumulation of sorbitol which in turn induce osmotic stress, and increase NADH/NAD^+ ratio leading to the production of reactive oxygen species (ROS) and oxidative stress. As glucose levels rise in the bloodstream, polyol pathway activation increases, and the consequences of this process are diabetic complications including retinopathy, nephropathy, and neuropathy (figure 1-1) [15].

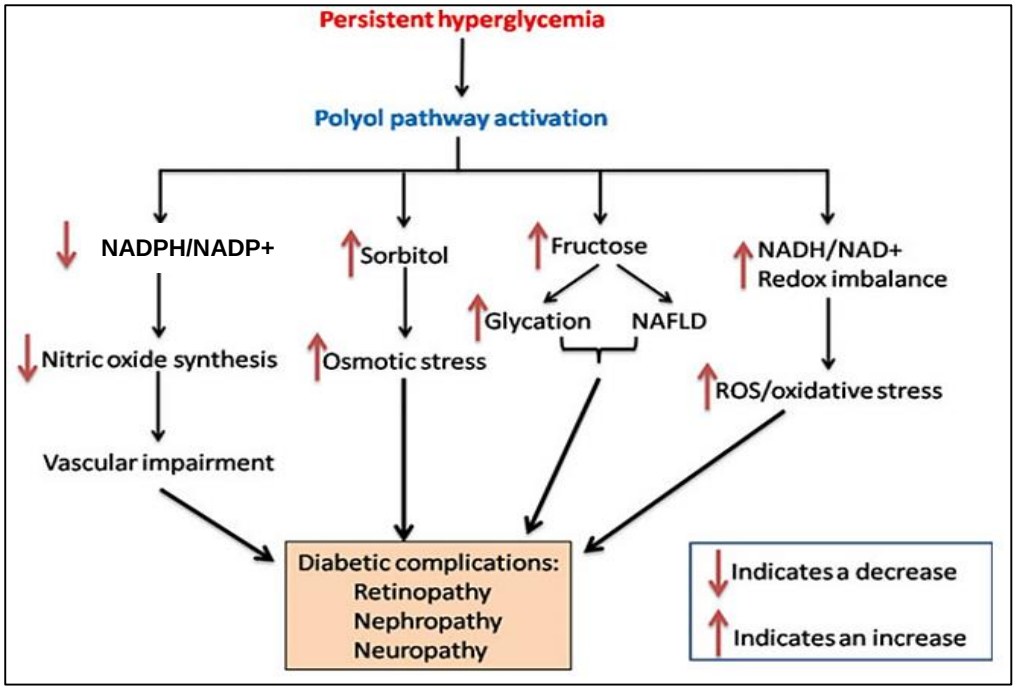

Figure 1-1. The effects of polyol pathway induced by prolonged hyperglycaemia [15].

In diabetic patients, retinopathy is the most prevalent microvascular complication, and it is considered a major cause of blindness [17]. Due to the decreased levels of sorbitol dehydrogenase in certain tissues including retina, sorbitol accumulates and results in osmotic stress which is considered the main underlying cause of developing diabetes-related retinopathy [18].

Diabetic nephropathy (DN) can lead to end-stage renal failure worldwide [19]. In the kidney, NADPH oxidase-4 (NOX4) is considered to be the key enzyme that produces ROS, which eventually leads to defective endothelial function, inflammation and apoptosis [20]. DN is recognized when there is an increased level of albumin in urine and the severity is based on the grade of albuminuria, which can be classified as microalbuminuria (when albumin excretion rate of 30 to 300 mg /24 hours) and macroalbuminuria (when albumin excretion exceeds 300 mg per /24 hours). Both types of albuminurea are linked to serious renal and cardiovascular conditions [19]. Measurement of the albumin to creatinine ratio is the most reliable, quickest, and easiest method for diagnosing microalbuminuria [21].

Diabetic neuropathy, which appears late in patients with T1D, is damage to sensory neurons that results in neuropathic pain with either central or peripheral symptoms in various patterns (e.g. numbness and pain). Dysregulation of metabolic pathways in diabetes propagates oxidative stress and AGEs, which are considered potent initiators of nerve damage and to the modification of blood arteries supplying peripheral neurons [22].

According to the International Society for Paediatric and Adolescent Diabetes (ISPAD), screening for diabetes complications in patients with T1DM should begin at puberty or from age 11 years within 2-5 years of disease duration and annually thereafter [23].

2- Macrovascular complications: patients with diabetes have an increased risk of developing cardiovascular disease and death due to heart failure. Macrovascular complications arise as a result of prolonged hyperglycaemia that leads to increased fluid load. However, these complications require many years to be obvious [24].The basic mechanism of macrovascular complications is atherosclerosis, which occurs in the arterial walls due to increased inflammation and injury to the wall in the peripheral or coronary vascular system [25].

Chronic inflammation and injury to endothelial walls results in accumulation of oxidised lipid from low density lipoprotein (LDL). Oxidation of LDL may be promoted by angiotensin II that in turn causes infiltration of monocytes. Then, monocytes differentiate into macrophages which in turn accumulate oxidised lipids to form foam cells. Once foam cells are formed, it stimulates proliferation of macrophages and T-lymphocyte attraction, followed by a proliferation of smooth muscle of arterial wall and collagen accumulation, which induced by T-lymphocytes leading to the formation of a lipid-rich atherosclerotic lesion covered by a fibrous cap. Rupture of the more advanced atherosclerotic lesions can cause platelet activation, thrombus development, and acute vascular infarction [26], [27].

1.2 Type 1 diabetes mellitus (T1DM):

Diabetes mellitus type 1 (T1DM) is a chronic autoimmune disease marked by insulin depletion as a result of pancreatic β-cell death leading to hyperglycemia. Due to the absence of a definitive treatment, patients are dependent on everlasting insulin injections [28]. T1D constitute about 5-10% of all cases of diabetes [2], mostly occurs in children and it is a multifactorial disease with a significant genetic component that, in combination with particular environmental conditions, causes the disease to first manifest [29].

1.2.1 Epidemiology of T1DM:

According to the International Diabetes Federation (IDF), about 537 million adults are living with diabetes in the world with a prediction to rise the number to 643 million by 2030. There is about 1,211,900 children and adolescent younger than 20 years have type 1 diabetes globally [30]. Standardized registry data show that the incidence of diabetes mellitus type 1 has increased over the past three decades to 3-4% [31].

The prevalence of T1D in Asia was 6.9 per 10,000 people, and the incidence was 15 per 100,000, while in Europe the prevalence is 12.2/10,000 population and the incidence is 15/100,000 population. In America, the incidence of T1D was 20/100,000 population and the prevalence was 12.2/10,000 people, and the prevalence of type 1 diabetes in Africa was 8 per 100 000 population with incidence rate 3.5 per 10,000 people. Globally, the incidence was 15/100,000 population and the prevalence is 9.5/10,000 [32].

The prevalence of type 1 diabetes in Iraqi children of Baghdad province was found 159 per 100,000, which was approximate to the prevalence in Saudi Arabia, less than that in Al-Kuwait, but higher than that in Turkey [33].

1.2.2 Pathogenesis of T1D

Autoimmune T1D (best described as type 1A diabetes) occurs due to the loss of immunologic tolerance to pancreatic beta cells. Both genetic susceptibility, numerous genetic variants and environmental factors may develop chronic autoimmunity against auto-antigens expressed on β-cells such as insulin, glutamic acid decarboxylase (GAD65), islet autoantigen-2 (IA-2), and zinc transporter (Zn-8) leading to β-cell damage and progression to T1D, as shown in figure (1-2). Recent studies suggest that the key pathogenic mechanism of the disease is the autoimmune destruction of β-cells, which involves pancreatic islet infiltration by CD4 and CD8 T-lymphocytes and macrophages leading to insulitis [34], [35].

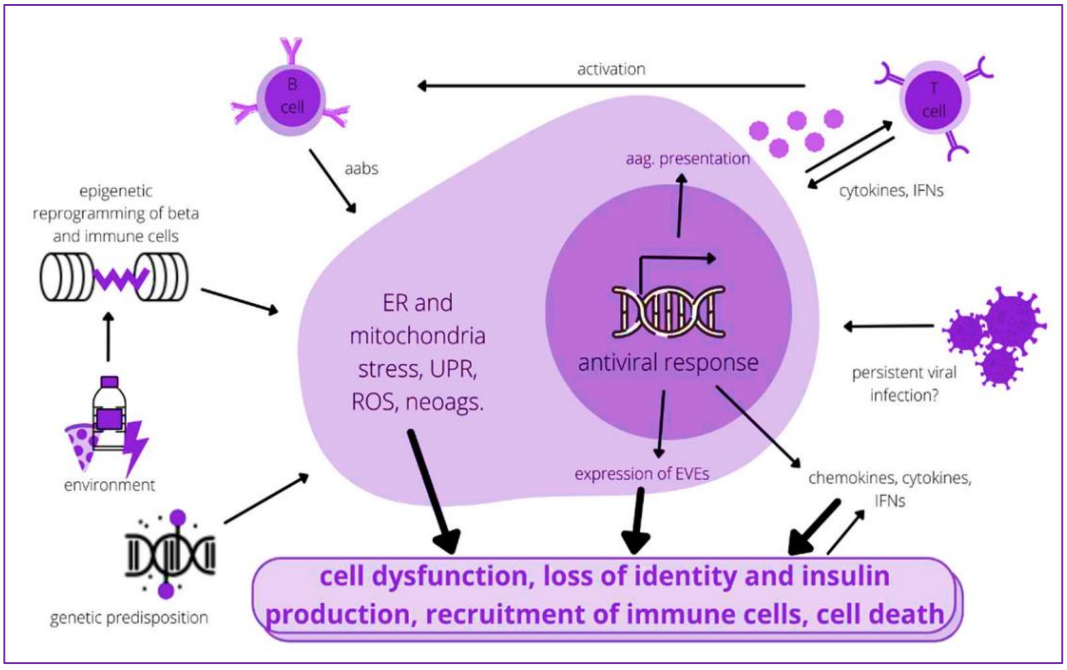

Figure 1-2. The pathogenic influences on β-cells [36]**.** Aab-autoantigens, ROS-reactive oxygen species, ER-endoplasmic reticulum, IFNs-interferons, EVEs-endogenous viral elements, neoags-neoantigens, aab.-autoantibodies, UPR-unfolded protein response.

The pathogenesis of T1D is divided into three stages. In stage I two or more autoantibodies appear with normo-glycaemia and without symptoms, in stage II autoantibodies remain but with dysglycaemia and without symptoms, while stage III is characterized by the presence of autoantibodies, dysglycaemia, and clinical symptoms [37], as shown in figure (1-3).

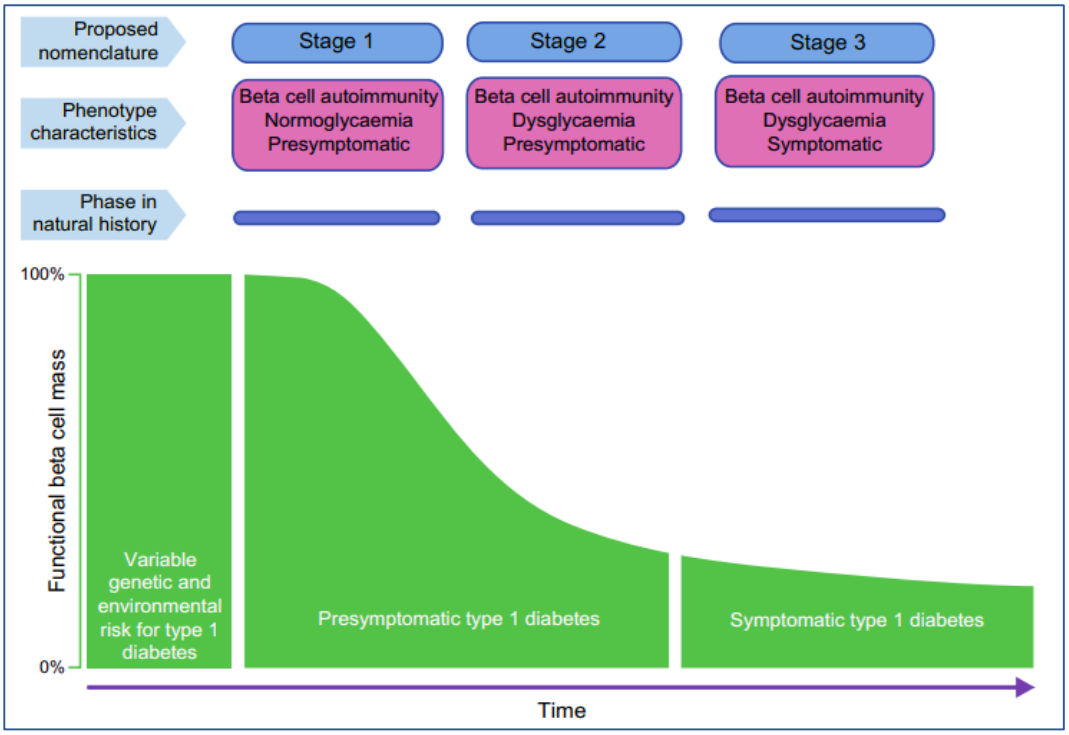

Figure 1-3. Stages of pathogenesis in type 1 diabetes [38]**.**

1.2.3 Risk factors for developing the disease

Different factors are associated with the development of T1DM, including the following:

1. Genetic factor

Type 1 diabetes is a heritable disease with identical twin concordance 30-70%, sibling risk 6-7%, and 1-9% if children have parents with diabetes [39]. Although most patients lack a family history when diagnosed, patients with first degree relatives with T1D have a higher risk of developing the disease [40]. In children with diabetic fathers, the heritability risk is 6-9% while with diabetic mothers is 2-4% and reach to 30% if the parents are both diabetics [41].

More than 50 genetic risk loci have been identified by genome-wide association studies. The main genes predisposing to T1D are within the major histocompatibility complex (MHC) region, often called HLA (human leucocyte

antigen) which is located on chromosome 6. HLA complex polymorphic alleles are responsible for 40–50% of T1DM genetic risk [42].

HLA system is a complex of genes encoding for proteins on the surface of cells that are responsible for immune system regulation. It is responsible for differentiation between self and non-self cells. Within HLA complex, loci more strongly associated with T1DM are those coding for HLA class I (A, B) which present cytosolic peptide antigens for CD8 T-lymphocytes and class II (DR, DQ) which present foreign antigens to CD4 T-lymphocytes. However, a stronger predisposition comes from HLA class II [43] that is expressed on the antigen presenting cell {APC} surfaces as in dendritic cell, macrophage, activated T and B lymphocytes and activated endothelial cells [44].

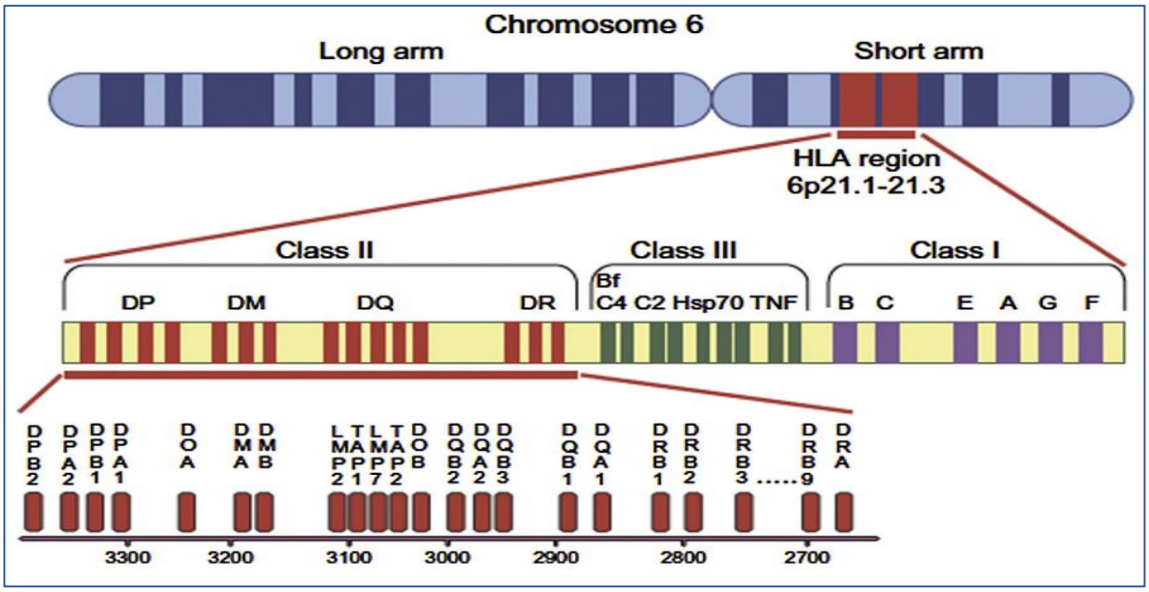

Figure 1-4.The HLA region on the short arm of chromosome 6 [45]**.**

There are also non-HLA genes have strong association with T1D was initially described as IDDM2, which is a variable number of tandem repeats (VNTR) located next to the insulin gene (INS). These insulin polymorphisms regulate insulin expression in the thymus and are likely to impact the development of immunological tolerance to insulin [46].

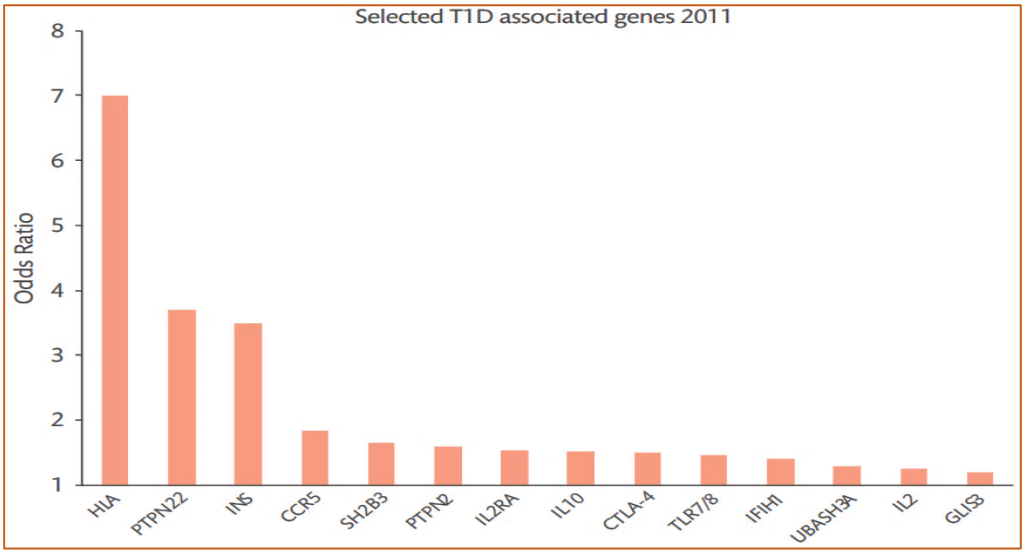

Figure 1-5. Non-HLA genes that are associated with type 1 diabetes

2. Autoimmunity:

The autoimmunity of T1D is represented by the infiltration of mononuclear cells as T and B-lymphocytes, APC, and natural killer cells (NK) in the pancreatic islets. The exact mechanism of autoimmunity is not yet completely clarified. However, a possible mechanism is that the damage of β–cell by autoimmunity in genetically predisposed individuals is stimulated when they exposed to environmental triggers, such as a virus [47].

The destroyed β–cells then presented by HLA-II (MHC-II) on APC to CD4+ T-lymphocytes which in turn facilitate the activation of CD8+ lymphocytes. Subsequently, the activated lymphocytes produce cytokines such as interlukin-2 (IL-2), tumor necrosis factor (TNF)-α, and interferon (IFN) γ. These cytokines with the reactive oxygen species cause a mitochondrial damage, thus depleting ATP production inducing necrosis and apoptosis of the islet cells [36], [48].

Such T-helper cells assist the B-lymphocytes to produce autoantibodies against peptide self-antigens in the islet cell including islet cell autoantibodies (ICA), resulting in a progressive destruction of β-cells and chronic inflammatory process called insulitis. Other antibodies directed against islet cells include

autoantibodies towards insulin (IAA), glutamic acid decarboxylase isoform (GAD-65), zinc transporter isoform-8 (ZnT-8), tyrosine phosphatase-like insulinoma associated antigen-2 (IA-2), and tetraspanin 7 [49], [50].

The process of autoimmunity appears months to years before the disease becomes obvious. The presence of one autoantibody is not necessarily to develop the disease, while two or more autoantibodies which usually appear 6-12 months following the first antibody predict the development of T1D over 10-15 years later [51], [52]. Nevertheless, the progression of the disease to the clinical onset may start before weeks in those who develop diabetes before age five [53].

3. Environmental factors

It is believed that a variety of environmental factors contribute to the onset of autoimmunity and progression to typ1 diabetes, these factors include the following:

a-Viral infections: the most possible environmental factors associated with the causation of T1D are viral infections with Epstein-Barr virus, Coxsackei, mumps, rubella, and enterovirus [54]. In two years ago, different studies have also indicated the implication of SARS-COVID-19 in the development and progression of the disease [55].

Viruses can cause T1D by one of two mechanisms, either direct cytotoxicity or the induction of an autoimmune reaction [56]. In the direct manner, the viral infection affects the secretion of pro-inflammatory cytokines (e.g. TNF-α, IFN, NO) resulting in the destruction of pancreatic β-cells [57]. In the case of viral-induced autoimmunity, the most common concept is the molecular imitation model. In which exogenous viral proteins resembles β-cells auto-antigens (e.g. GAD) which results in cross reaction of autoreactive B and T lymphocytes with viral and self-antigens, thus leading to the destruction of β-cell [58].

b- Gut microbiota: the gut microbiome has vital roles in the body, including defence against foreign pathogens, production of energy, and maintaining the integrity of intestinal epithelia. Furthermore, the gut microbiome plays a critical role in the maturation and development of the immune system especially in early life, and any imbalance in bacterial composition (gut dysbiosis) will lead to dysregulation of immune response resulting in β-cell damage and developing T1D in genetically predisposed individuals [59].

Gut dysbiosis may also result in the dismantling of intestinal tight junctions and so ''the leaky gut'' that in turn causes a passage of microbial antigens into the circulation and induction of autoreactive T-cells, promoting autoimmunity and destruction of β-cell in genetically susceptible subjects [60].

c- Dietary factors: numerous nutritional factors have been linked to an increased risk of developing T1D by affecting the immune system such as vitamin D, cow's milk, eating solid food and cereals at an early age (i.e. rice, gluten, fruit), and polyunsaturated fatty acids. However, various observational studies revealed contradictory findings regarding the relationship of these factors with the disease [61], [62].

1.2.4 Pathophysiology of T1DM

The autoimmune destruction of pancreatic β-cell results in insulin deficiency, which eventually leads to metabolic instabilities. The α-cells of pancreas are also impaired resulting increased secretion of glucagon, which is normally must be suppressed by hyperglycemia .Since there is no respond to the increased glucose levels by the cells, glucagon cannot be suppressed by hyperglycemia [63].

The elevated levels of glucagon aggravate the metabolic disruption that results from insulin deficiency, and the most noticeable disruption is diabetic ketoacidosis (DKA) which develops rapidly in the absence of insulin

administration. As well as, reduced insulin levels result in uncontrolled lipolysis, which leads to the release of free fatty acids that suppress glucose metabolism in peripheral tissues such as skeletal muscles. Impaired glucose utilization as well as decreased insulin levels affect gene-expression of enzymes required for target tissues to respond to insulin such as glucokinase in the liver and GLUT4 class of glucose transporter in adipose tissue [64], [65].

As a result of this disruption, the required enzymes for glycogenolysis and gluconeogenesis will be activated in the liver, leading to the production of excessive amounts of glucose. And since the peripheral tissues cannot utilize glucose due to the absence of insulin, glucose builds up in the circulation exceeding the renal threshold for excretion (more than 180 mg/dl). Furthermore, because glucose is an osmotic diuretic, its secretion from the kidneys is followed by a loss of water and electrolytes, activating the thirst mechanism in the patients (polydipsia) [66].

Impaired glucose metabolism as well results in increased mobilization of triglycerides leading to an excess amount of free fatty acids (FFA). The subsequent depletion of calories, electrolytes, increased dehydration, and physiological stress result in the secretion of stress hormones (i.e. cortisol, epinephrine, glucagon, and growth hormone). Low insulin and elevated counter-regulatory hormone levels in plasma are both responsible for enhanced lipolysis, impaired lipogenesis, and shunts of fatty acids to the formation of ketone bodies in the hepatic mitochondria (i.e. acetoacetate, and β-hydroxybutyrate) [67].

The accumulation of ketoacids leads to a decrease in bicarbonate concentration, and retention of these strong acids leads to the development of high anion gap metabolic acidosis, accomplished with quick heavy respiration as a compensatory mechanism to remove excess CO2 [Kussmaul Respiration]. The non-enzymatic conversion of acetoacetate results in the production of acetone, which is exhaled in the breath with a fruity odor [68]. Excess ketone bodies and

hyperglycemia causes osmotic diuresis leading to hypovolemia (depletion of extracellular volume) with contraction of arterial blood volume. Peripheral tissues then exposed to hypoperfusion due to reduced blood volume, making the tissues to be deprived of oxygen. This in turn can cause lethargy, stupor and coma in diabetic patients [69].

1.2.5 Signs and symptoms of T1DM:

The general symptoms of undiagnosed diabetes typically include: polydipsia, polyphagia, polyuria, slow healing of sores, genital itching, weight loss, blurred vision, and nausea. In children or teenagers particularly, T1D signs and symptoms can develop rapidly and worsen over weeks or even days. Signs such as increased urination, increased thirst, tiredness, and sudden weight loss tend to be the most noticeable symptoms, with diabetic ketoacidosis in more than 20% of newly diagnosed type 1 diabetic children [70], [71].

1.3 Preptin

1.3.1 Definition:

Preptin is a newly discovered peptide hormone. It was first isolated from the pancreatic beta-TC6-F7 cell lines of rats by Buchanan and colleagues in 2001 [72]. It is derived from proinsulin-like growth factor II (proIGF-II), synthesized mainly in pancreatic β-cells, coincides with insulin secretion and is involved in mineral metabolism. Increased and decreased concentrations of circulatory preptin can regulate insulin secretion, and thus it is thought to be a physiological amplifier of glucose-mediated insulin secretion [73].

1.3.2 Synthesis and structure:

Preptin is synthesized in the pancreas, salivary gland, mammary tissue, liver and kidneys [74]. Buchanan *et al.* identified a 34-amino-acid peptide (3948 Da) corresponding to Asparagine (69)-Leusin (102) of the proinsulin-like growth factor IIE-peptide (pro-IGF-IIE), which they have termed preptin. Insulin-like growth factor II (IGF-II; also known as somatomedin A) cleaved into the

following three chains: (1) insulin-like growth factor II, (2) insulin-like growth factor II Ala-25 Del, and (3) preptin, figure (1-6) [75].

The amino acid sequence of preptin has been largely preserved through the evolutionary process in both mice and humans. Human preptin is 79.41% similar with mouse preptin and 73.53% similar with rat preptin [75]. Being a derivative of pro-insulin-like growth factor II (pro IGF II), preptin is considered the latest member of insulin family [76].

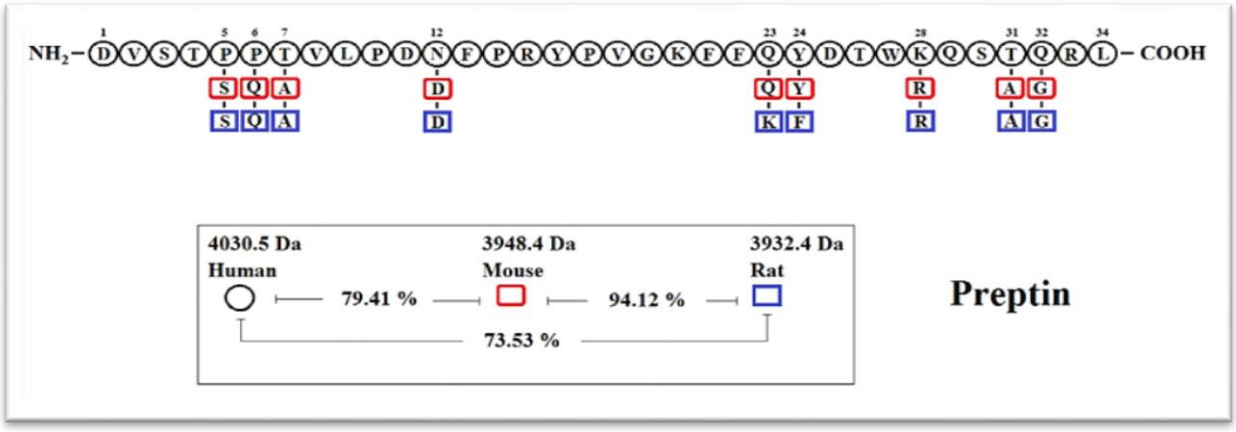

Figure 1-6. The chemical structure and amino acid sequence of preptin [75]**.** D-aspartic acid, N-asparagine, F-phenylalanine, Y-tyrosine, K-lysine, Q-glutamine, W- tryptophan, R-arginine.

Preptin is cleaved by proteases at the 21st phenylalanine amino acid fragment. The truncated preptin peptide that results from this cleavage (preptin 1–16) does not impact insulin secretion. Full length (34-amino acid) preptin, however, physiologically increases glucose-mediated insulin secretion. The half-life of preptin in circulation is shorter than 5 min and the increases or decreases in the circulatory preptin amount are associated with insulin levels in humans [77].

1.3.3 Mechanism of action:

One of the possible mechanism(s) of preptin is that stimulating insulin secretion, acts not only at the level of the amplifying pathway but also the triggering pathway. High-glucose concentrations stimulate insulin release and increase glucose sensitivity in terms of insulin secretion, electrical activity, and

Ca^{+2} signalling for changes in glucose metabolism. Preptin action as well produced in a calcium-dependent manner, as shown in figure (1-7) [78].

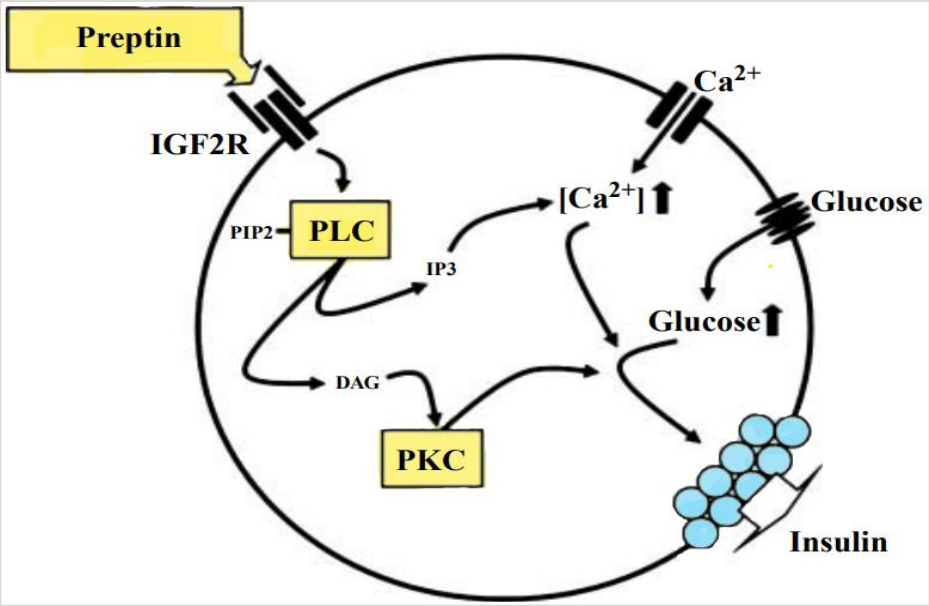

Figure 1-7. Regulation of insulin secretion by preptin [78]**.** PIP2-Phosphatidylinositol 4,5-bisphosphate, DAG-diacylglycerol, IP3-inositol trisphosphate, PKC-protein kinase-C.

As preptin is a fragment of a larger precursor (proIGF2), it is possible that preptin acts on cells through the IGF2R, which is a type-I transmembrane glycoprotein. Preptin influences the secretory process through the activation of IGF2 as a consequence of an influx of extracellular Ca^{2+}. Receptor-operated non-nutrients that are coupled to PLC may activate Ca^{2+}dependent protein kinases by the IP3-induced release of Ca^{2+} from intracellular stores and activate conventional and/or novel isoforms of PKC by the generation of DAG [79].

1.3.4 Physiologic functions of preptin:

It was found that preptin has an anabolic effect on bone, by its ability to reduce osteoblast apoptosis through the MAP-kinase pathway [80], which is synergetic with the effects of insulin hormone. Moreover, preptin has been found to elevate insulin secretion, increases cell differentiation and cell activity of osteoblasts and osteoclasts in vitro and in vivo [81], [82], figure (1-8).

Preptin has been assumed to inhibit glucose production by the liver. However, this property of preptin is still on debate and it may be a key player in the etiopathology of diabetes in the future [75].

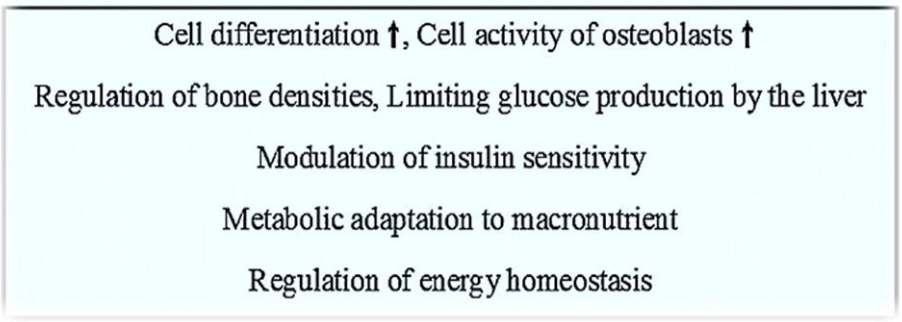

Figure 1-8. The main physiologic actions of preptin [75]

1.4 Irisin:

Irisin, a novel exercise-induced myokine, was first discovered by Pontus Bostroöm from mouse skeletal muscle in 2012 at Harvard University. In response to exercise, irisin is secreted from human muscle and mediates the beneficial effects of exercise such as thermoregulation and weight loss. Since irisin plays a key role in glucose homeostasis, maintains a balance in bone formation and resorption, promotes favourable processes in the nervous system, and increases endothelial proliferation, it has been proposed as a potentially attractive therapeutic target for diabetes and metabolic disorders [83].

1.4.1 Structure and synthesis:

Irisin is composed of 112 amino acids with an approximate molecular weight of about 12kDa, and it is produced from the proteolytic cleavage of its precursor fibronectin type III domain C (FNDC5). The expression and synthesis of FNDC5 stimulated by peroxisome proliferator-activated receptor-γ (PPAR-γ) co-factor 1α (PGC-1α), figure (1-9), a master regulator of genes involved in metabolism and energy expenditure [75], and it is overexpressed in skeletal muscle, heart, liver, and brown adipose tissue [84].

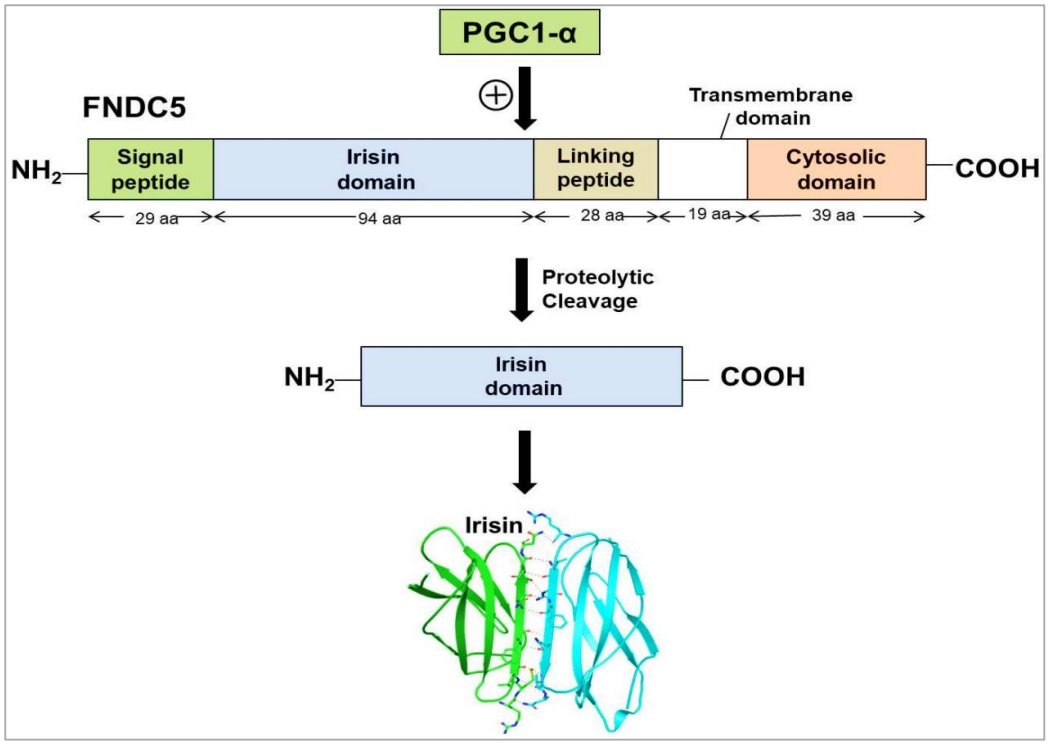

Figure 1-9. Schematic representation of FNDC5 structure and formation of irisin [85].

Exercise increases the transcriptional co-activator PGC1-α which in turn induces the expression of FNDC5 gene. FNDC5 ,which consists of 212 amino acids, is a membrane bound protein composed of signal peptide, a fibronectin III domain, a hydrophobic transmembrane domain, and a carboxy-terminal domain located in the plasma [86].

After exercise, the extracellular N-terminal part of FNDC5 (composed of most of the fibronectin III domain) is released by a proteolytic cleavage, which then glycosylated and dimerized as irisin (figure 1-10). FNDC5 is found abundantly in skeletal muscle and it is identical 100% between humans and mice. It is also released from adipose tissue, pancreas, brain, heart, Kupffer cells, stomach, tongue, sinusoidal epithelial cells, and optic nerves [87]. Circulating irisin is removed specifically from the body through the hepato-biliary system and the kidneys [88].

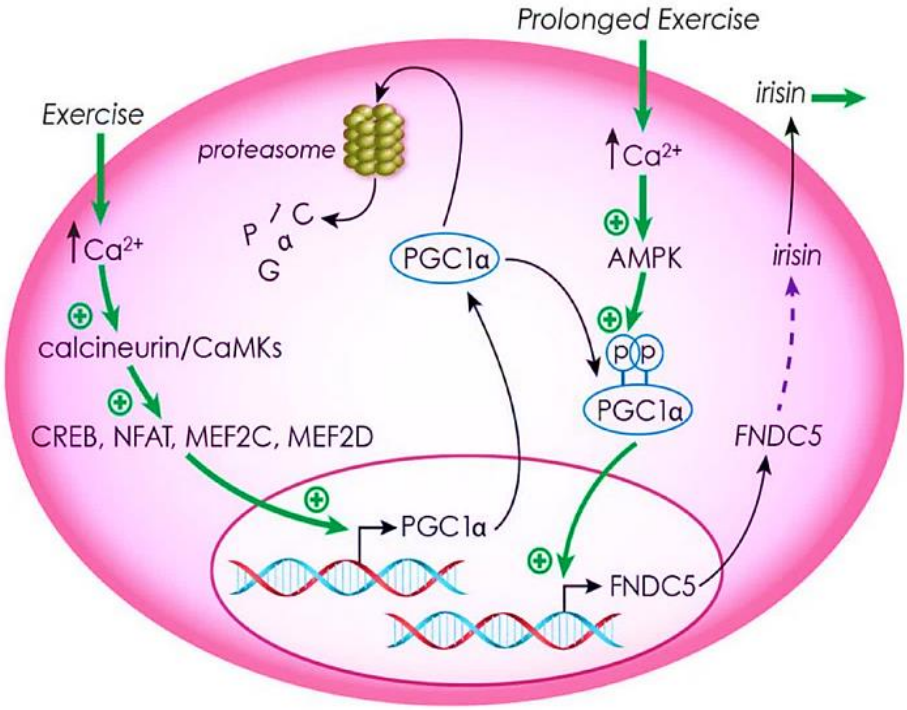

Figure 1-10. Mechanism of irisin synthesis [84]**.** MEF-Myocyte enhancer factor, CREB-cAMP response element-binding protein, NFAT-Nuclear factor of activated T-cells.

It is still unclear which exact protease enzyme cleaves FNDC5 protein to irisin; however, in recent years it has been indicated that ADAM family is responsible for the proteolytic cleavage of FNDC5. A disintegrin and metalloproteinase (ADAM) family is a well-known protease family, which proteolytically converse some surface-expressed proteins into soluble variants, and ADAM 10 is the candidate protease enzyme that cleave cell membrane protein FNDC5 into irisin [89].

1.4.2 Physiological functions of irisin

It has been found that irisin is a multifactorial hormone acts locally in both autocrine/paracrine manner. It can influences the functions of skeletal muscle, pancreas, liver, bone, and brain, enhancing insulin sensitivity, metabolism, osteogenesis, and cognition, figure (1-11). Furthermore irisin improves insulin sensitivity by promoting insulin receptors in skeletal muscle and heart, improving metabolic processes of lipid and glucose in the liver, promoting pancreatic β-cell function, and browning of white adipose tissue [90].

Recent studies have shown that irisin has anti-inflammatory, anti-metastatic, anti-cancer, anti-depression, anti-hypertension, anti-cardiac hypertrophy properties. Its anti-inflammatory properties are exhibited via the down-regulation of toll-like receptor 4 (TLR4). Furthermore, irisin is capable of reducing oxidative stress that occur in endothelial cells and macrophages [91].

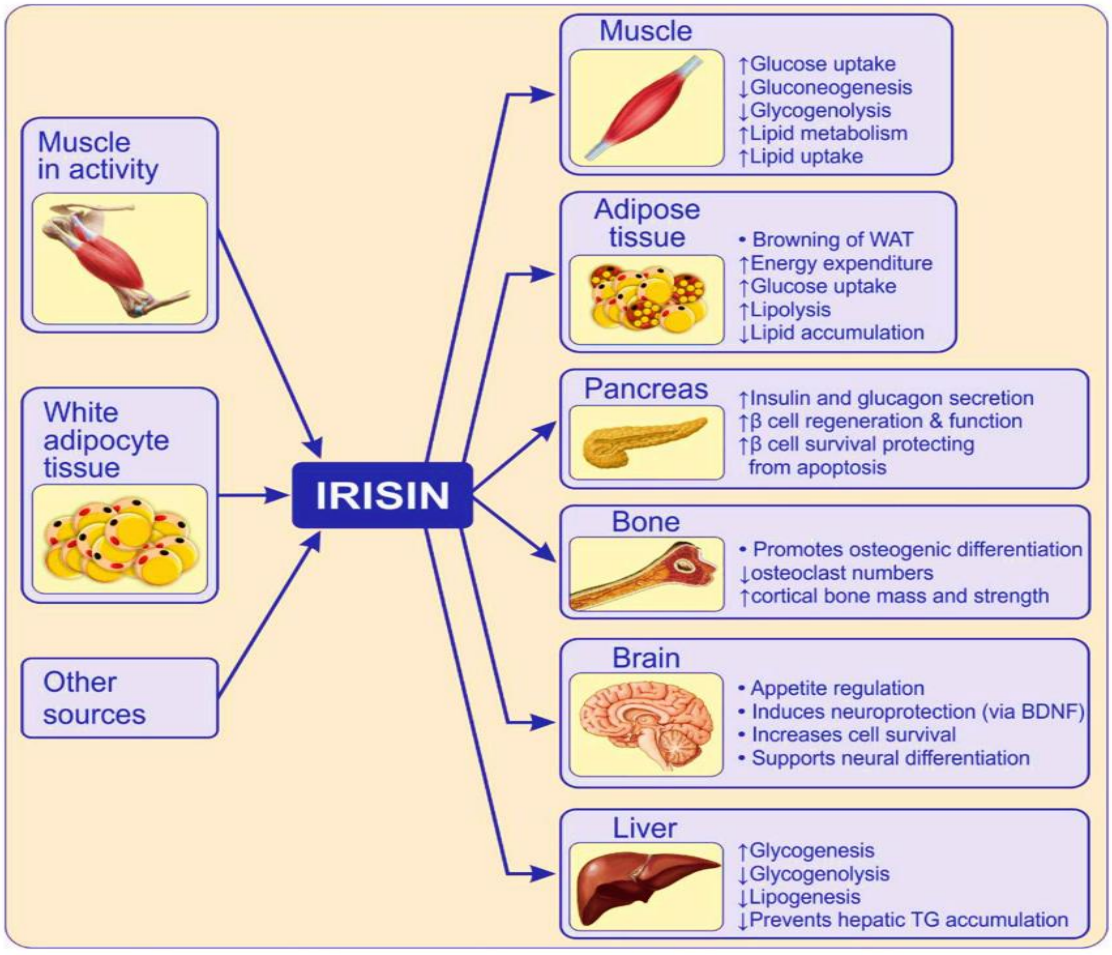

Figure 1-11. Sources of irisin and its functions [90].

1.4.3 Mechanism of action:

Recent studies indicates that irisin acts primarily through the mitogen-activated protein kinase pathway (MAPK). However, other signalling cascades mediate the functions of FNDC5/Irisin, including the AMPK, PI3/AKT, and STAT3/Snail pathways. The major functions in the body that are elicited by *fndc5/irisin* gene are mediated by p38 and ERK1/2 signalling. These signalling cascades are involved in the browning of white adipose tissue, glucose uptake by

muscle, inducing neural cell differentiation, and osteocyte proliferation [92]. MAPK signalling plays a vital role in fundamental cellular processes such as mitosis, gene expression, metabolism, motility, survival, apoptosis, and differentiation [93].

1.4.3.1 Direct effect of irisin on glucose homeostasis in adipose tissues:

After secretion from muscle, irisin stimulates browning of WAT through p38-MAPK and extracellular-signal regulated kinase (ERK) pathways which induce the expression of the UCP1 gene followed by UCP1 protein synthesis, figure (1-12). Brown adipose tissues (BAT) have higher energy expenditure and higher numbers of mitochondria due to increased oxygen consumption, and accumulate smaller lipids than do WAT. After cold exposure BAT have ten times GLUT4 expression than WAT, thus increased glucose uptake, and it seems to be related to irisin secretion from skeletal muscle which also increased after cold exposure (shivering) [92], [94].

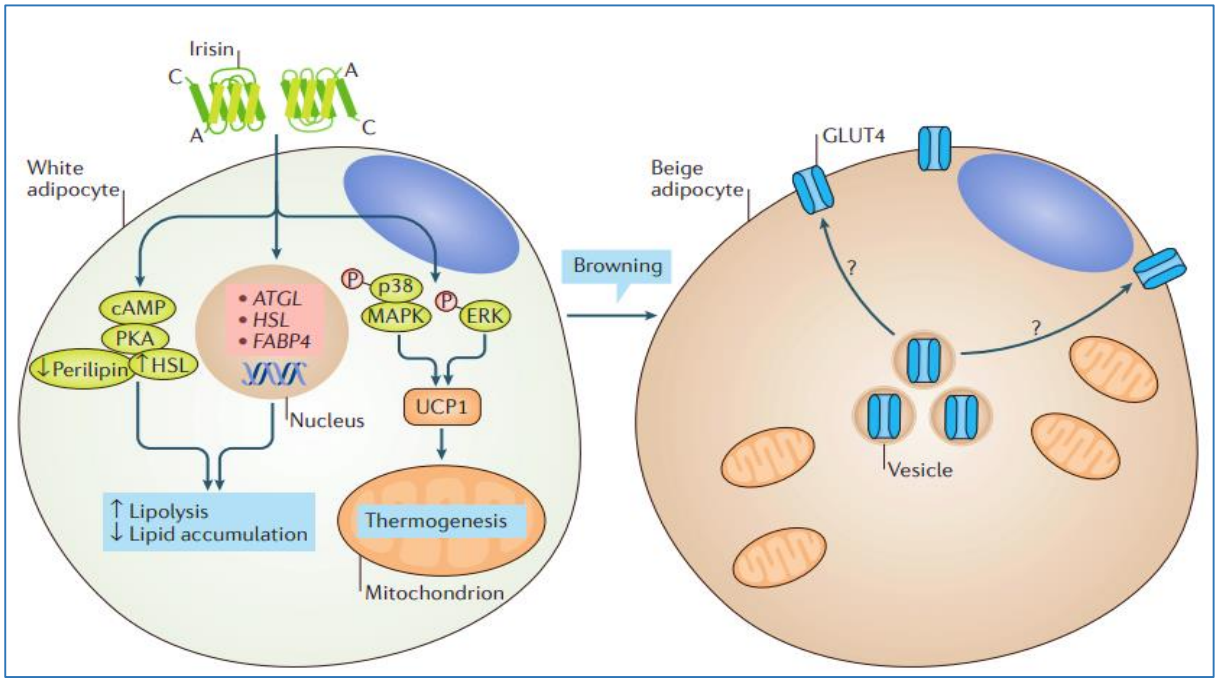

Figure 1-12. Signalling pathways of irisin in adipocytes [95]**.** ERK-extracellular signal-regulated kinase, UCP-uncoupling protein.

Furthermore, irisin induces lipolysis through the cyclic AMP(cAMP)-protein kinase A (PKA)- perilipin- hormone sensitive lipase (HSL) pathway and stimulates intracellular lipid metabolism through the up regulation of the expression of genes such as Pnpl2 (which encodes triglyceride lipase), Hsl and of proteins as fatty acids binding protein-4 (FABP4) [92], [96].

1.4.3.2 Direct effect of irisin on glucose homeostasis in muscles:

Studies in the last two decades have identified the muscle as the largest endocrine organ. The secretome of skeletal muscle consists of numerous peptides regulating multiple physiological processes in an auto-, para-, and endocrine manner, especially muscle growth, energy metabolism, immune, endothelial, and central nervous system function [97]. Myokines, such as irisin, act as cross-talk mediators between muscles and other tissues (i.e. liver, adipose tissue, pancreas, brain, bone, and intestine), participating in different metabolic processes involved in these organs [98].

Irisin can activate the AMP-activated protein kinase (AMPK) either by reducing ATP, increasing ROS, or by raising intracellular Ca+ levels. AMPK in turn stimulates the expression of PPARA, HK2, and GLUT4 genes, and inhibits the expression of PYGM and PCK1. The increased expression of GLUT4 and HK2 combined with increased GLUT4 translocation from the cytoplasm to the membrane (mainly by p38-MAPK) increases glucose uptake by myocyte. On the other hand, inhibition of PYGM and PCK1 expression reduces glycogenolysis and gluconeogenesis. The irisin–AMPK pathway also promotes fatty acid β-oxidation. Finally, irisin stimulates mitochondrial biogenesis by regulating the expression of PPARA and TFAM genes and of UCP3 protein [99], [100], (Figure 1-13).

In addition irisin has the ability to increase the expression of insulin-like growth factoe-1 (IGF1), which is a positive regulator of muscle growth [101].

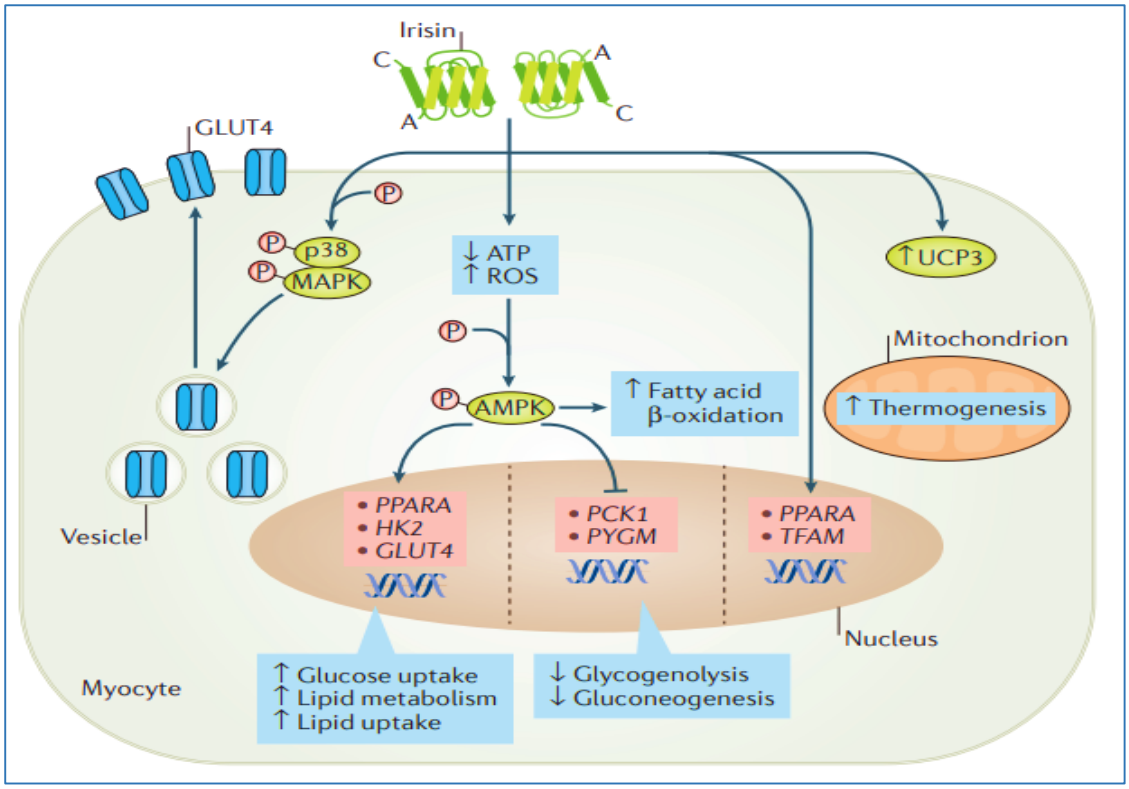

Figure 1-13. Signalling pathways of irisin in myocytes [95]**.** PCK1- Phosphoenolpyruvate carboxykinase 1, PYGM-muscle glycogen phosphorylase, TFAM, mitochondrial transcription factor.

1.4.3.3 Effect of irisin on glucose homeostasis in liver:

In hepatocytes, irisin decreases gluconeogenesis through down regulation of PEPCKC and G6PC via the phosphoinositide 3-kinase PI3K-AKT-FOXO1 and AMPK signalling pathways, and activates glycogenesis through the activation of glycogen synthase via the PI3K-AKT-glycogen synthase kinase-3 (GSK3) pathway. In addition, irisin inhibits palmitic-induced lipogenesis and lipid accumulation, as well as oxidative stress by reducing the expression of PRMT3. This leads to reduced expression of several lipogenic markers {e.g. LXRα, SREBP1C, ACC, FAS } and inflammatory markers [102], [103], figure (1-14).

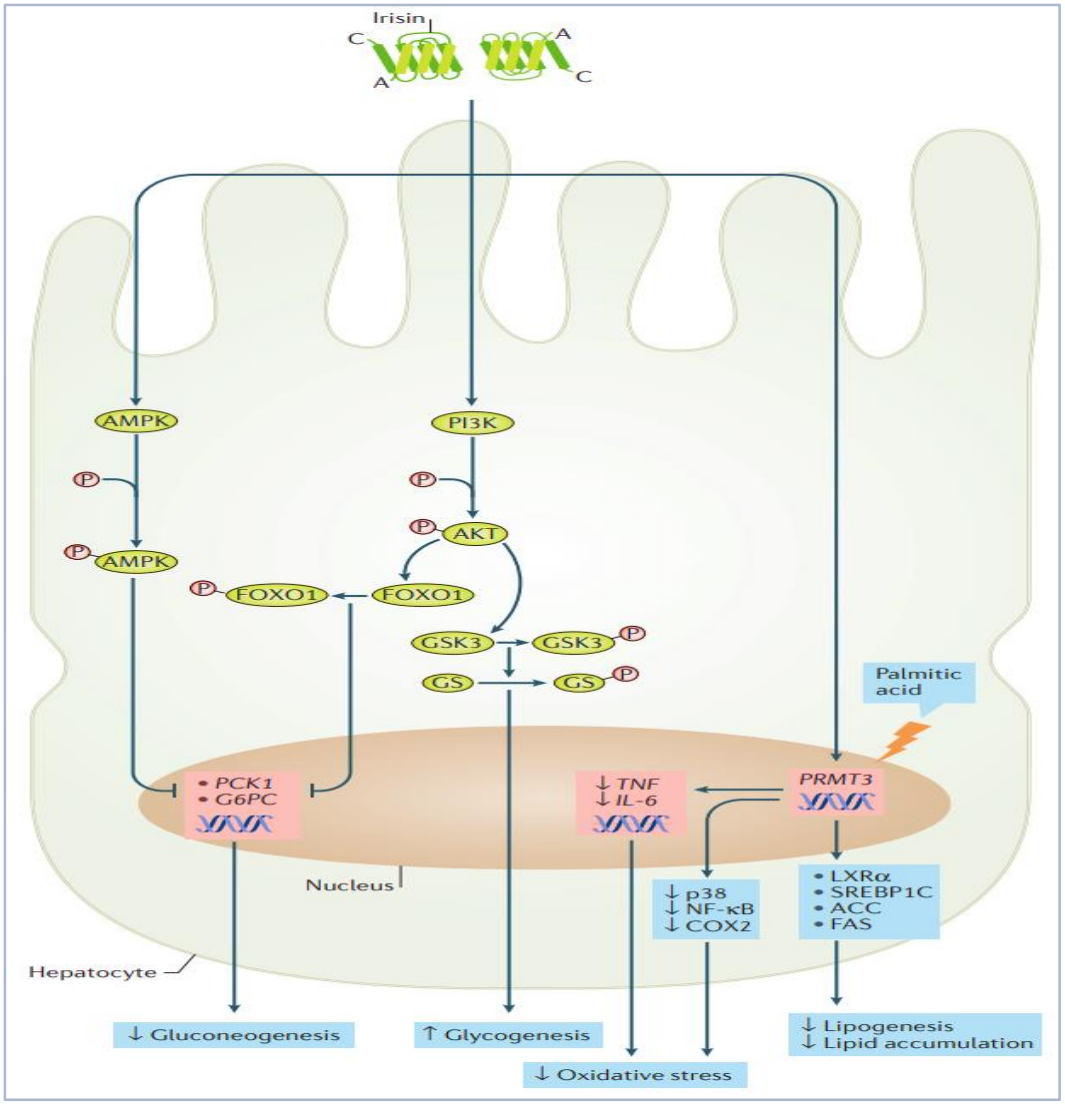

Figure 1-14. Signalling pathways of irisin in hepatocyte [95]**.** Liver x receptor-α (LXRα), sterol regulatory element-binding protein1C (SREBP1C), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), cyclooxygenase 2 (COX2), nuclear factor-κB (NF-κB).

1.4.3.4 Direct effect of irisin on pancreas

Different studies revealed a beneficial effect of irisin on pancreatic β-cells through p38-MAPK-irisin-betatrophin signalling pathway. Betatrophin, an insulin regenerating hormone secreted primarily from the liver and plays a critical role in β-cell renewal. Following muscle stimulation there is an increased expression of PGC-1α, which in turn stimulates the expression and cleavage of FNDC5 to generate irisin, subsequently activating the expression of UCP1. The expression of UCP1 promotes WAT browning, increases energy expenditure, promotes insulin regeneration, and completes the building of β-cells [104].

Recently, irisin was considered as an islet hormone that has a novel role in pancreatic islet physiology, exerting local vascular effects [105].

1.4.4 Irisin receptor

The identification of irisin receptor is still a challenge; however, recent studies suggested that the αV family of integrin receptors are likely to be the irisin receptors in thermogenic fats and osteocytes. It was found that most integrin complexes, including integrin β1-α1, showed significant binding with irisin; however, αV/β5 integrins have shown the highest binding affinity [106], [107].

Additionally, it was recently discovered that irisin serves as a novel ligand for integrin αLβ2 (a.k.a. lymphocyte function-associated antigen 1, LFA-1) and α4β7 (a.k.a. lymphocyte Peyer's patch adhesion molecules, LPAM-1) which both of them are exclusively expressed on leukocytes, most prominently on lymphocytes, thereby supporting lymphocyte adhesion and migration in inflammation [108], [109] .

1.4.5 Factors regulating irisin levels

1-Exercise: a series of studies have shown that acute exercise is a potential stimulus to promote the secretion of irisin. It has been reported that circulating irisin concentrations of healthy young adults increase significantly 30 min after acute exercise. Furthermore, a single 40 min of aerobic running can induce minimal increase of serum irisin in both hot (21~25°C) and cold (−5~5°C) environment [110], [111], figure (1-15).

In response to exercise, regulation of irisin depends on the specific training protocol (intensity, duration, type of exercise), age, training status and muscle mass. Additionally, it was found that short sessions of rigorous exercise acutely and transiently increased serum levels of irisin in adults and children, while no differences in irisin levels were found with prolonged (6 weeks) or

chronic (1 year) exercise [112]. Irisin was reported to reach its peak levels after about 60 min of exercise and return to its baseline 6 hrs. later [113].

The effect of exercise on the levels of irisin is linked to the increased expression of PPARGC-1α (or PGC-1α), which regulates mitochondrial biogenesis and functions, as well as gene expression in muscle cells [114]. In addition, intracellular deprivation of ATP after exercise might be a triggering factor for the synthesis and release of irisin from skeletal muscle [101].

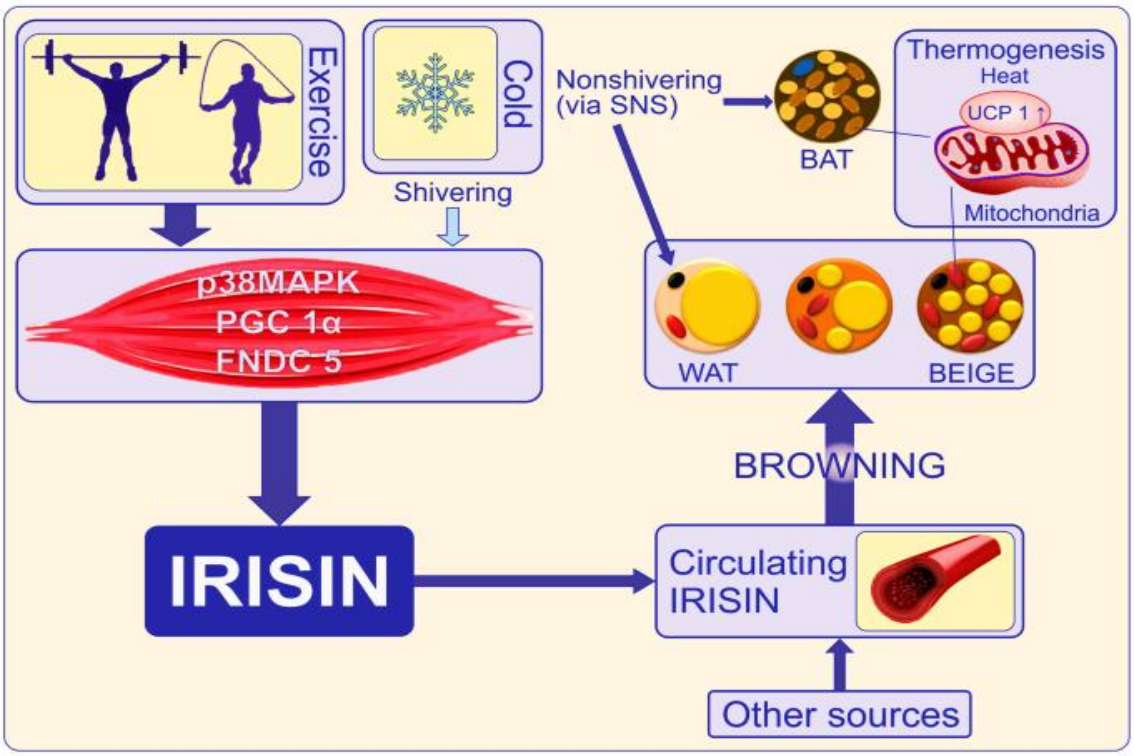

Figure 1-15. The effect of exercise and cold exposure on irisin secretion [90]

2-Obesity: Even though irisin is secreted from skeletal muscle after contraction, it has identified that irisin is also secreted from fat tissue particularly WAT and subcutaneous adipose tissues. Furthermore, like other adipokines, the secretion of irisin from subcutaneous-adipose tissues is influenced by the circulatory levels of irisin [115]. It was also found that improved expression of irisin in adipose tissues and improved function of adipose tissues are achieved by a positive feedback mechanism by circulating irisin levels [116].

3-Leptin: leptin is a satiety adipokine synthesized by the WAT. It was found that leptin increases the expression of FNDC5 in the skeletal muscles which subsequently increase irisin induced muscle growth [117].

In humans, there is still controversy about the effect of leptin on irisin levels. Based on an experimental study on adipose tissue explants from non-obese subjects, a noticeable decline in the expression of FNDC5 and serum irisin concentrations observed after administration of leptin [118]. While another study showed a negative correlation between them [119].

4-Myostatin: known also as growth differentiation factor-8, it is expressed in skeletal muscle and released as GTF-8 superfamily protein. It controls myoblast proliferation by acting as a negative potent regulator of skeletal muscle growth and muscle development [120]. It has been found that myostatin is overexpressed in obese and diabetic patients [121].

Myostatin as well, has an antagonistic effect on insulin action in the post-receptor phase by inhibiting Akt phosphorylation and has an inhibitory effect on irisin secretion [97]. In addition, suppression of myostatin action stimulates conversion of WAT to BAT through the activation of PPAR-γ and elevating the secretion of irisin. As a result, targeting myostatin via the irisin pathway represents a potential therapeutic target for combating insulin resistance and obesity [122].

5- Betatrophin: also known as angiopoietin-like protein 8, is another peptide hormone secreted mainly from the liver, affects glucose and lipid metabolism [100]. A link between the hormones irisin and betatrophin was recently described, in which stimulation of rat adipocytes with irisin increased the mRNA expression of betatrophin. Additionally, reactive oxygen species induced by exercise have been proposed as the initiating stimuli for secretion of irisin, which by acting on WAT could stimulate betatrophin secretion [123].

1.5 C-peptide:

1.5.1 Definition:

C-peptide is a linker chain cleaved from proinsulin in pancreatic β-cells and composed of 31 amino acids. It was believed that C-peptide is an inactive by-product of insulin for decades. However, recent studies have found that it is a bioactive molecule with numerous beneficial effects on different types of cells and tissues. The cleavage of C-peptide is important to produce the mature and functional insulin hormone. C-peptide is used as a biomarker for β-cell function, because it is secreted in similar amount with insulin, escape first pass metabolism by the liver, and has a longer half-life than insulin {insulin has a half-life of about 3 min, C-peptide about 30 min} [124].

C-peptide was known to be decreased significantly in type 1 diabetic patients, however it was found that not all patients exposed to absolute destruction in pancreatic beta cells [125]. Younger children were found to present with more severe symptoms because they have lost an average 80% of their islets, compared to 60% in those 7-14 years old and 40% in those older than 14 years. Destruction of beta cells is complete within 3 years of diagnosis in most young children, especially those with HLA-DR3/4 genotype. It is much slower and often only partial in older patients, 15% of whom have still some β-cell function preserved 10 years after diagnosis [126].

1.5.2 Synthesis and Secretion:

The precursor of insulin prohormone composed of 110- amino acids, which includes a signal peptide, A and B chains, and the linker peptide. The signal peptide is cleaved within the rough endoplasmic reticulum of β-cells, and the cysteine residues of the insulin chains (A and B) are oxidized to generate a disulfide bonds between them. Then, the prohormone is packed into secretory vesicles in the Golgi apparatus, which in turn proteolytically cleaved into distinct

insulin and C-peptide molecules by prohormone convertase 1,2 [127]. Figure (1-16).

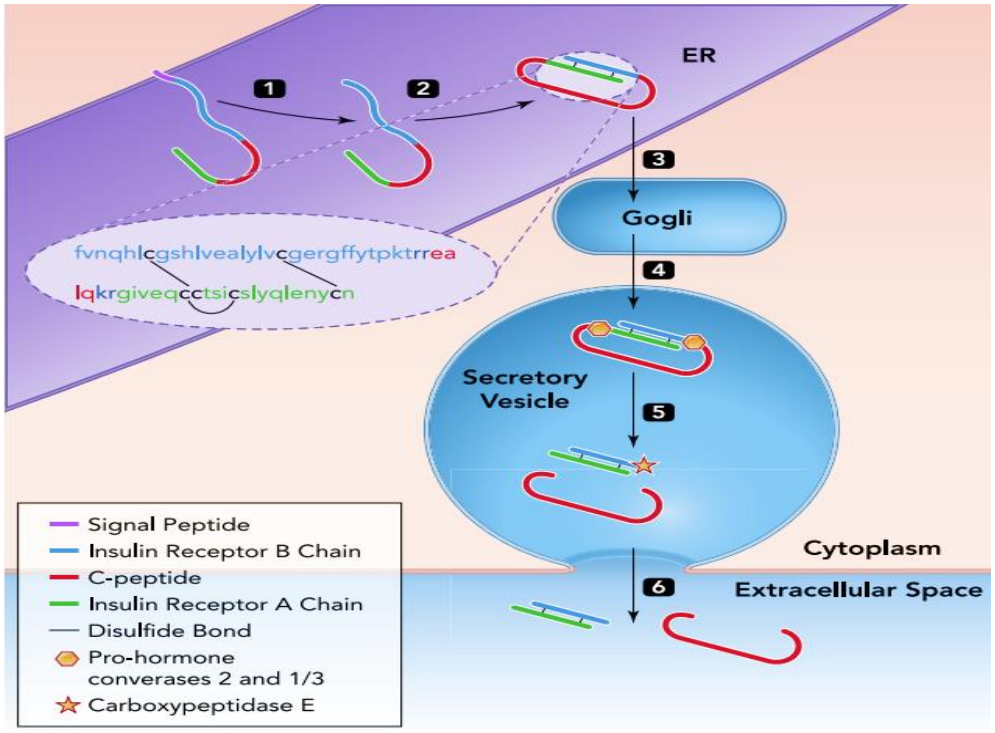

Figure 1-16. Post translational processes of prepro-insulin to produce insulin and C-peptide [128]**.**

Afterwards, C-peptide and the B-chain of insulin undergo to further processing by carboxy-peptide E to remove the terminal basic rsidues and form the mature insulin and C-peptides. The mature products then stored in secretory vesicles until stimulation beta cells by hyperglycaemia. Increased glucose metabolism inside the β-cells results in increased production of ATP, which inturn leads to the lockdown of ATP- sensitive K^+ channels, depolarization of membrane and influx of Ca^{+2} into the cells. Subsequently, the secretory vesicles fuse with the membrane releasing insulin and C-peptide simultaneously into the extracellular space [128]. C-peptide has an essential function in insulin synthesis in that it links the A and B chains in a manner that allows correct folding and interchain disulfide bond formation [129].

1.5.3 Mechanism of action:

Numerous signalling pathways has been shown to be activated by C-peptide, such as PLC, MAPK, PI3/AKT, NF-kB, PKA, and PKC, as shown in figure (1-17). These pathways initiate endothelial nitric oxide synthase (eNOS) and the activity of Na^+/K^+ ATPase and thus trigger the expression of many transcription factors [130], [131]. Involvement of these signalling cascades would imply an interaction of C-peptide with a G-protein coupled receptor (GPR) or receptor complex on the surface of cell membranes. Specifically, GPR146 was identified as a putative receptor for C-peptide [132].

The binding of C-peptide to cell membranes elicits intracellular signalling through G-protein and Ca^+ dependent pathways, resulting in activation and increased expression of eNOS, Na^+/K^+-ATPase and several transcription factors of importance for anti-oxidant, anti-inflammatory and cell protective mechanisms [133].

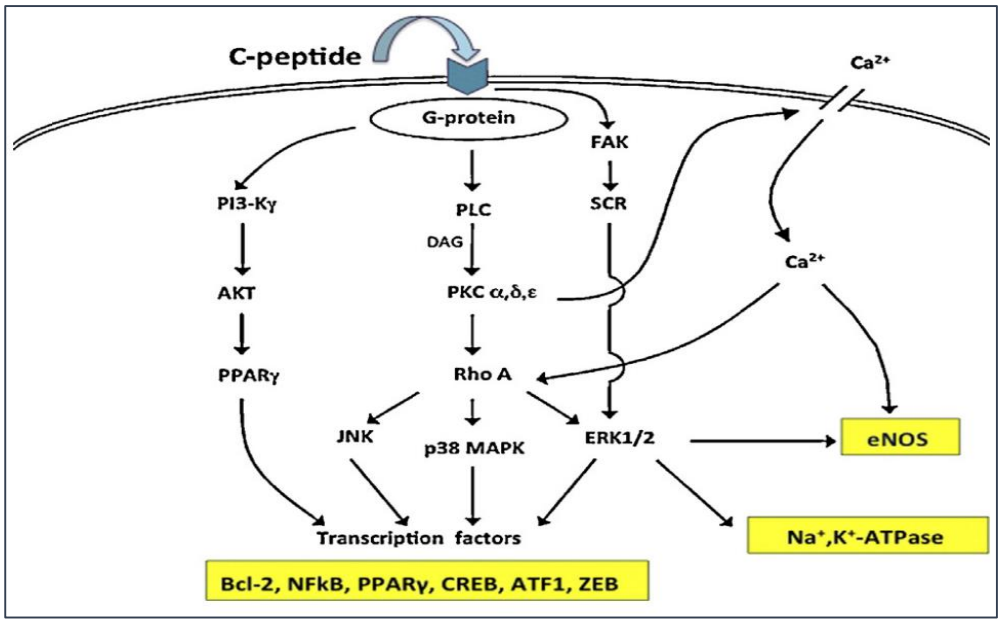

Figure 1-17. Intracellular signalling pathways of C-peptide [133]**.**

Increased eNOS production will decrease ROS and increase vasodilation. Furthermore, C-peptide reduces NF-κB activity to decrease the expression of

cellular adhesion molecule (CAM) and leukocyte interaction, TNF-α mediated apoptosis, and production of inflammatory cytokines, figure (1-18) [12].

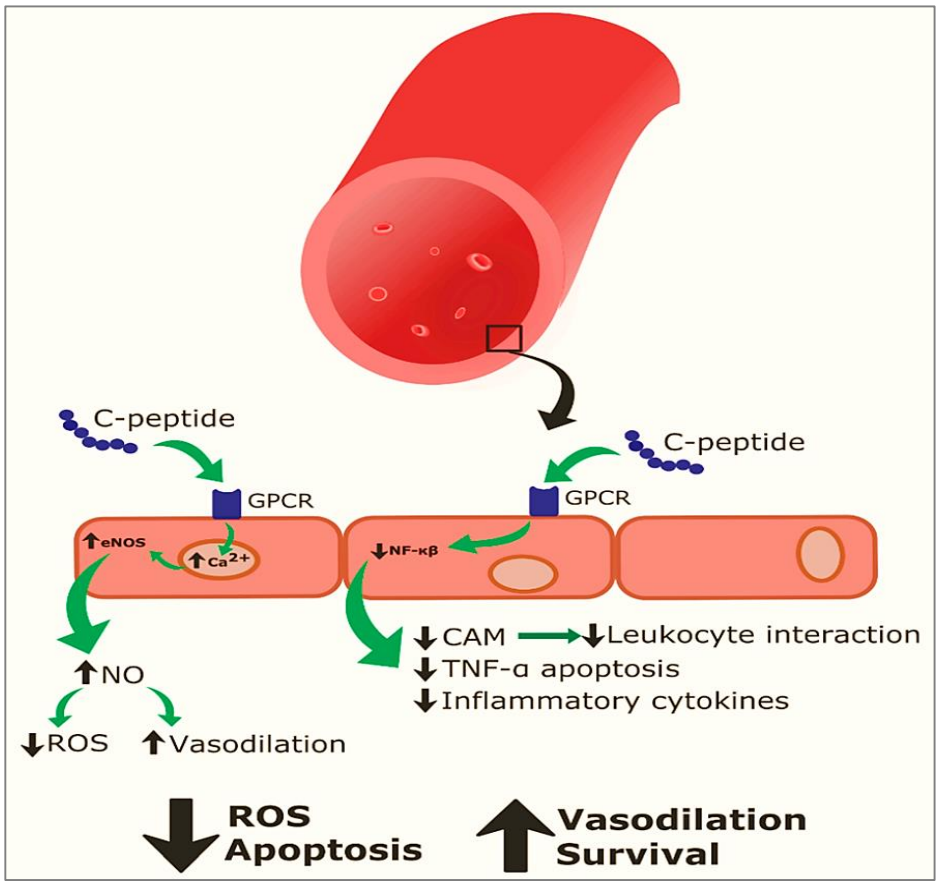

Figure 1-18. The action of C-peptide on endothelial cells [12]**.**

1.5.4 Physiological roles of C-peptide in T1D:

Numerous biological activities of C-peptide have been confirmed in recent years, including its ability to improve blood flow in the skin capillaries of feet, increase oxygen uptake and microvascular blood flow in the exercising forearm, reduce albumin excretion in the urine, and enhance neuronal functions in patients with T1D, figure (1-19) [134].

Furthermore, C-peptide has the capacity to reduce the extent of diabetes-induced functional and structural abnormalities of the kidneys as well as cardiac autonomic and endothelial dysfunction, increase left ventricular blood flow and cardiac contractility, increase muscle and skin blood flow, augment peripheral nerve conduction velocity, and ameliorate nerve structural abnormalities [135].

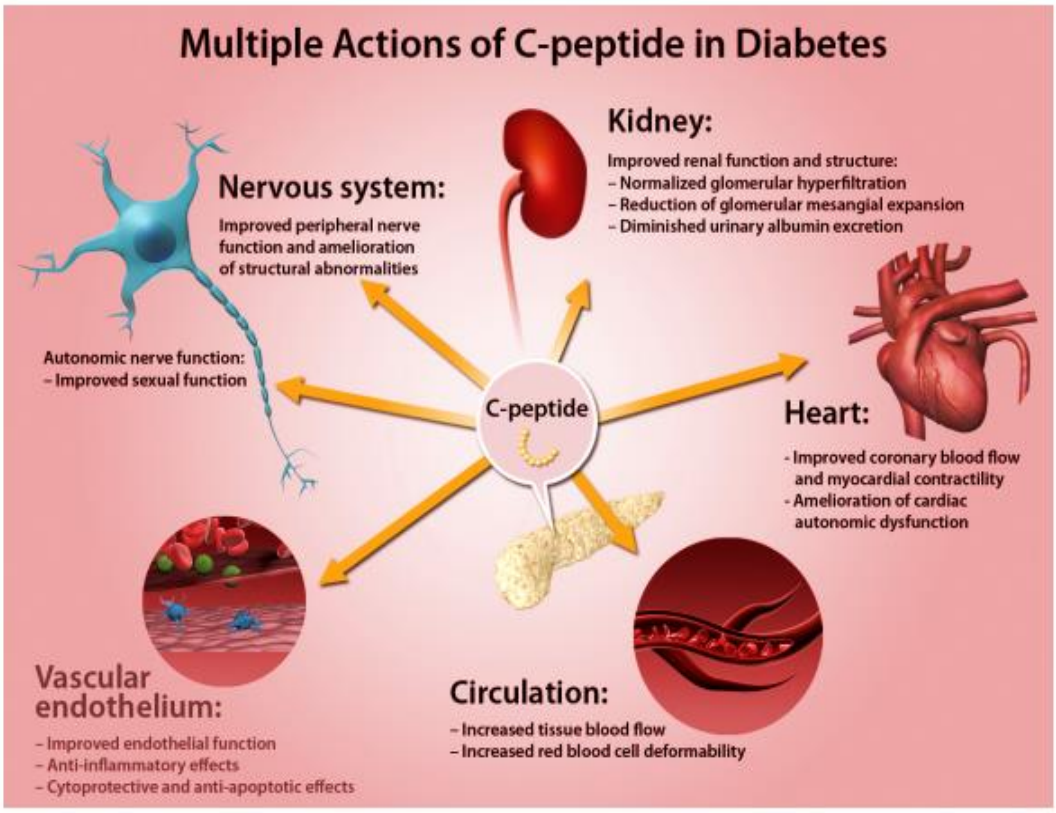

Figure 1-19. Physiological effects of C-peptide [135]

Another study showed that C-peptide was able to activate insulin receptor tyrosine kinase, insulin receptor substrate-1, tyrosine phosphorylation, and p90 Rsk (90-kDa ribosomal 56 protein kinase). Moreover, C-peptide mimics the effects of insulin, such as stimulation of glycogen synthesis and amino acid uptake [136]. Moreover, C -peptide can reduce ROS formation via RAC-1 mediated inhibition of NAD(P)H oxidase, the principal source of ROS generation in endothelial cells after exposure to hyperglycaemia. The anti-apoptotic effect of C-peptide exerted through the inhibition of caspase3 and increasing the production of Bcl-2 [137], figure (1-20).

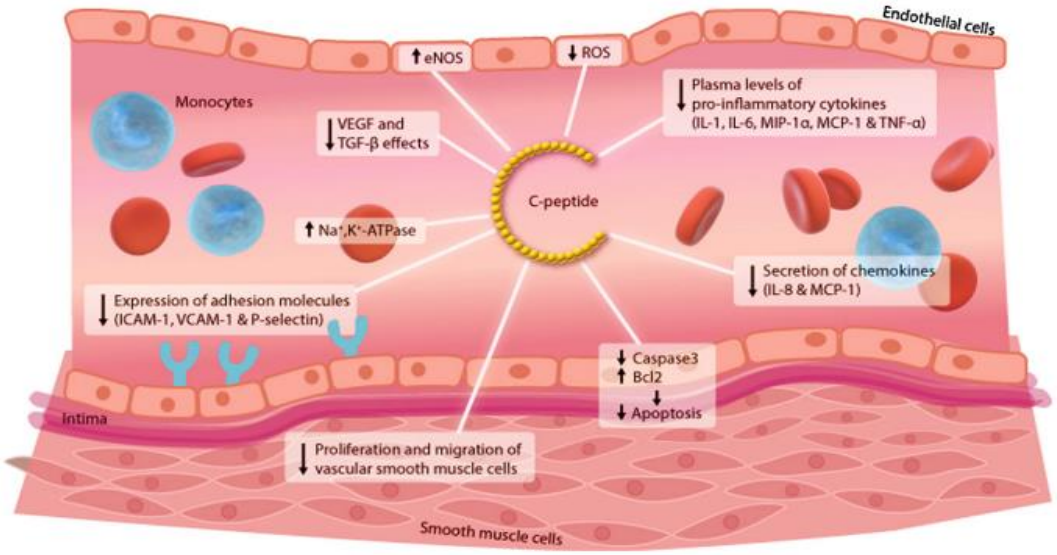

Figure 1-20. Anti-oxidant, anti-inflammatory, and anti-apoptotic effects of C-peptide [133]**.**

1.5.4.1 Direct effect of c-peptide on Na+, K+-ATPase

It has been found in vitro studies that C-peptide has a direct effect on Na^+/ K^+-ATPase in renal tubular cells, and this effect is in a concentration-dependant manner. The property of C-peptide accomplished via the stimulation of PKC, ERK1/2 and the transcription factor ZEB [138], [139]. Studies have found that the reduced activity of Na^+, K^+-ATPase in the RBC of T1D patients, which was proved to have a role in diabetes-complications development, is proportional to the decreased levels of C-peptide, and can be corrected by administration of C-peptide or islet cell transplantation [140].

1.5.4.2 C-peptide and glucose utilization

In previous experimental studies, C-peptide administration did not show any effect on glucose levels [141]; however, after that C-peptide was found to have its ability to stimulate 3-O-methylglucose transport in human muscle strips [142] and showed a marked response in whole body glucose utilization after infusion [143]. This effect shown on both healthy and T1D individuals by a mechanism that is independent on the insulin receptor and of receptor tyrosine kinase activation [144].

Aims of the study:

1- Evaluate the role of irisin in the development of type 1diabetes mellitus.

2- Identify the effect of preptin on insulin through the measurement of C-peptide levels.

3- Determine the relation of C-peptide with irisin and preptin levels.

Chapter Two
Materials and Methods

2. Materials and Methods

2.1 Study design:

The study population in this case-control study composed of a total of 100 subjects, divided into two groups with 50 subjects for each group, one group for type 1 diabetic patients and the other for apparently healthy control, figure 2-1.

The collection of samples started from the participants within the period from September-February (2022-2023). The practical part of this study performed in the Chemistry and Biochemistry department laboratory at the College of Medicine /University of Babylon.

2.1.1 Patients group:

Fifty patients with T1D (already diagnosed by paediatric diabetologist) were collected from the Diabetic Centre of Marjan Medical City Hospital and Babylon Maternity Hospital in Babylon Province.

A. Inclusion criteria:

1- T1DM without complications.
2- Age ≤ 18 years.

B. Exclusion criteria:

1- Patients with diabetic complications (retinopathy, nephropathy, and neuropathy).
2- Nephrotic syndrome
3- Growth hormone deficiency
4- Thyroid dysfunction
5- Cushing syndrome
6- Liver dysfunction
7- Obese participants [BMI-percentile ≥ 95^{th}]

Information of patients were taken directly from them and their parents according to the questionnaire form that mentioned in the appendix.

2.1.2 Control group:

The study includes 50 apparently healthy individuals with age ≤ 18 and RBS less than 11 mmol/l. The participants of the control group were taken from the relatives and neighbours, in addition to neighbours and families of some friends.

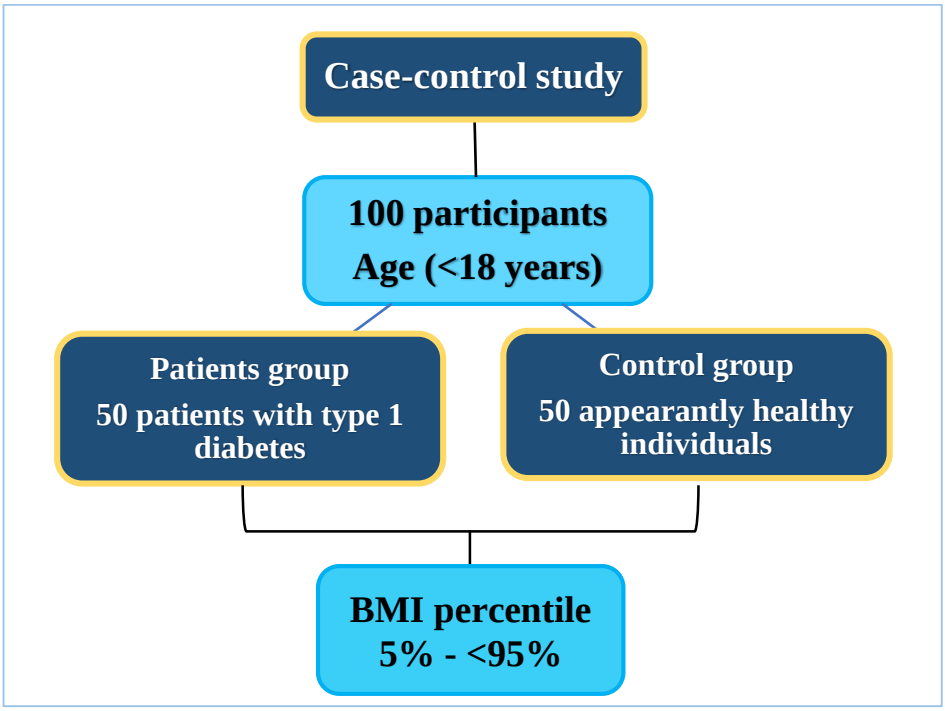

Figure 2-1. Scheme of study design

2.1.3 Ethical approval and consent:

1. Approval by a scientific committee of the College of Medicine (University of Babylon, Iraq) and the Biochemistry Department at the same college.
2. Approval of the scientific committee of Babylon teaching hospital for maternity and children in Hilla city, Babylon province.

3. Approval of the scientific committee of Marjan Medical City Hospital, Babylon province.

The purpose of the study was clearly explained to all participants with their families and written informed consent was obtained.

2.2 Materials and Equipments Utilized in the Study

2.2.1 Chemical and Kits

The chemicals and Kits utilized in the present work have been displayed in table (2-1).

Table (2-1). Chemicals and kits used in this study

Chemicals and kit	Company
Blood glucose kit	**Taytec, Canada**
HbA1c Kit	**Linear, Spain**
Irisin kit	**Bioassay Technology Laboratory, ELISA kit**
Preptin kit	**Bioassay Technology Laboratory, ELISA kit**
C-peptide kit	**Bioassay Technology Laboratory, ELISA kit**

2.2.2 Apparatus and Equipments

The table below shows the instruments used in this study.

Table (2-2): Apparatus and Equipment Used in the study

Apparatus and Equipments	Company
Disposable syringes (5 mL)	Medical jet / Syria
EDTA tube (3ml)	AFCO / Jordan
Gel tube (5mL)	AFCO, (Jordan)
Eppendorf tube (1.5ml,0.2ml)	China
ELISA reader and washer	Biotech / USA
Spectrophotometer	CECIL, (England)
Incubator	Fisher Scient. /Germany
Micropipettes (5 -50µl), (2-20µl), (20-200µl), (100-1000µl)	Slamed, (Germany)
Water Bath	GFL / Germany
Different size tips	China
Distiller	GFL, (Germany)
Plain tube	ASL, Jorden
Weighting scale	USA
Volumetric flask, funnel ,beaker	Schoot, (Germany)
Filter papers	AFCO, Jorden

2.3 Methods

2.3.1 Anthropometric Measures

Body max index of participants calculated as BMI Z-score and BMI percentile and were achieved according to the CDC chart using digital calculator. BMI that is less than 5^{th} percentile is considered underweight, while BMI that is equal or more than 95^{th} percentile is considered obese. Children with BMI equal to 85^{th} up to 95^{th} percentile are considered overweight [145].

2.3.2 Collection of Blood Samples:

Five millilitres of venous blood were drawn from the participants and divided into: two ml in EDTA tube for HbA1c and three ml in gel tube for serum separation. The gel tube left for 20 minutes to be coagulated at room temperature and then centrifuged in 3000 xg for 20 minutes.

The serum of the gel tube used for the measurement of random blood sugar and the rest amount divided into three Eppendorf tubes, then stored in the deep freezer $(-60)^0$C of the Central Blood Bank for later estimation of irisin, preptin, and C-peptide. HbA1c test was measured by an automated analyser.

2.3.3 Limitations of the study

This present study has several limitations including the difficulty in collecting samples in the fasted state, because most of diabetic children are prone to hypoglycaemia and they have to take their daily insulin dose which requires taking a meal after that. In addition, some of the healthy children appears to have diabetes after estimation of random blood sugar without having obvious symptoms. Also, it was difficult to find the patients who meet the criteria of our study within the decided duration of sample collection.

2.3.4 Estimation of random blood glucose levels

Principle: The enzymatic method contained Glucose-oxidase enzyme (GOD) that oxidize glucose to gluconate and hydrogen peroxide (H_2O_2). The formed H_2O_2 is detected by a chromogenic oxygen acceptor, phenol, 4-aminophenazone (4-AP) in the presence of peroxidase, according to the following equation and the procedure was done according to blood glucose kit Taytec.

$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{phenol} + \text{4-AP} \xrightarrow{\text{Peroxidase}} \text{Red Quinone dye} + H_2O$$

Procedure:

As in the protocol prepared by the assay kit of Taytec company:

1- Three plain tube was prepared, and 1 ml of working reagent was added for each tube.

2- A volume of 10 µl from standard solution was added to standard tube, and then 10 µl of sample was added to sample tube. All tubes mixed well, and incubated for 5 minutes in water bath at 37^0 C.

Pipetted into well identified test tubes	Blank	Standard	Sample
Reagent (ml)	1 ml	1 ml	1 ml
Standard (ml)	___	10 µl	___
Sample (ml)	___	___	10 µl

After incubation, the absorbance was measured at 505 nanometre using one-centimetre width cuvette and the concentration was measured by beer lambert law.

Calculation:

Glucose Concentration (mg/dl) = A of sample / A of standard × 100 (Conc. of standard).

2.3.5 Measurement of HbA1c

Haemoglobin A1c measured by using automated analyser (HbA1c Turbidimetric, Linear, Cromatest)

***Principle*:** This method directly determinates the haemoglobin A1c (HbA1c) in whole blood, using an antigen and antibody reaction. Total haemoglobin and HbA1c compete for the unspecific absorption rate to the latex particles (R1). Mouse ant-human HbA1c monoclonal antibody (R2a) binds to the coated particles with HbA1c. The presence of goat anti-mouse IgG polyclonal antibody (R2b) causes the agglutination of the particles (complexes).The amount of agglutination is proportional to the concentration of the HbA1c in the sample and can be measured by turbidimetry [146].

Calculation

- The absorbance values (A) were plotted against the concentration of HbA1c of each calibrator. Plot the absorbance values (A) obtained against the concentration of each calibrator.

-The assay is standardized according to the IFCC (International Federation of Clinical Chemistry) reference method [147], using the following formulas:

HbA1c IFCC (%) = HbA1c (mmol/mol) IFCC / 10

2.3.6 Determination of human irisin

***Principle of the assay*:** This kit is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with human irisin antibody. Irisin present in the sample is added and binds to antibodies coated on the wells, and then biotinylated human irisin antibody is added and binds to irisin in the sample. Then Streptavidin-HRP is added and binds to the biotinylated irisin antibody. After incubation, unbounded Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and colour develops in proportion to the amount of human irisin. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm [148].

Table (2-3). Components of irisin ELISA kit

Components	Quantity
Standard Solution (64ng/ml)	0.5 x 1
Pre-coated ELISA Plate	12*8 well strips x1
Standard Diluent	3ml x 1
Streptavidin-HRP	6ml x 1
Stop Solution	6ml x 1
Substrate solution A	6ml x 1
Substrate solution B	6ml x 1
Wash buffer concentrate (25x)	20ml x 1
Biotinylated human irisin antibody	1ml x 1
User instruction	1
Plate sealer	2 pics
Zipper bag	1 pic

Reagent Preparation

a- All reagents were brought to room temperature before usage

b- The original standard sample was diluted as the following table

32 ng/L	**Standard No.5**	**120µl Original Standard + 120µl Standard Diluent**
16 ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
8 ng/L	Standard No.3	120µl Standard No.5 + 120µl Standard Diluent
4 ng/L	Standard No.2	120µl Standard No.5 + 120µl Standard Diluent
2 ng/L	Standard No.1	120µl Standard No.5 + 120µl Standard Diluent

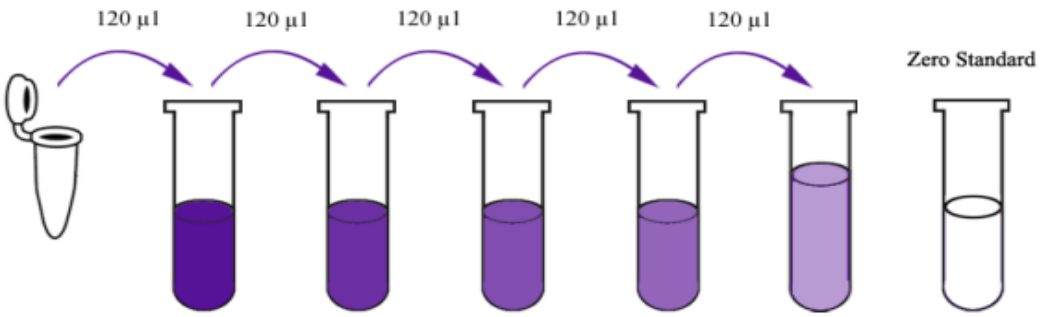

Figure 2-2: Concentration of standards of irisin.

Standard Concentration	**Standard No.5**	**Standard No.4**	**Standard No.3**	**Standard No.2**	**Standard No.1**
64ng/ml	32ng/ml	16ng/ml	8ng/ml	4ng/ml	2ng/ml

-Wash Buffer: 20ml of Wash Buffer (25X) diluted into deionized or distilled water to yield 500 ml of 1x Wash Buffer.

Procedure:

1- All reagents, standard solution, and samples were brought to room temperature and reagents prepared before starting assay procedure.

2- A volume of 50 µl of standard was added to standard well, then a volume of 40 µl of sample was added to sample well followed by 10 µl of anti-Irisin antibody to sample wells.
3- A volume of 50µl streptavidin-HRP was added to sample wells and standard wells except blank control well. Then mixed well, the plate covered with a sealer, and incubated for 60 minutes at 37^0 C
4- After incubation, the plate was washed 5 times with wash buffer. In automated washing, all wells were aspirated and washed 5 times with wash buffer, overfilled with wash buffer. The plate blotted onto paper towels.
5- A volume of 50µl of substrate A and 50µl of substrate B were added to each well. The plate was covered with a new sealer and incubated for 10 min. at 37^0 C in the dark.
6- A volume of 50µl Stop Solution was added to each well. The colour in the wells was changed from blue to yellow.
7- The absorbance was read at 450 nm by using a microtiter plate reader within 15 minutes.

Calculation of results

The amount of the measured biomarker in the serum was determined by plotting the standard curve between the absorbance and standard concentrations in a logarithmic regression type using Excel software, Figure 2-3. Then, the straight-line equation was obtained in the following form:

Absorbance= Slope*Ln (Concentration) + Intercept

Then, Ln (Concentration) = (Absorbance-Intercept)/Slope

Then the unknown concentration of the biomarkers obtained from the following equation:

Concentration of biomarker= 2.718 ^ ((Absorbance-Intercept)/Slope))

Note: the standard concentration of 8 ng/L was excluded from the standard curve **(**the standard curve to be acceptable need at least 4 concentrations, so we excluded one out of six because it has a suspicious optical density**)**.

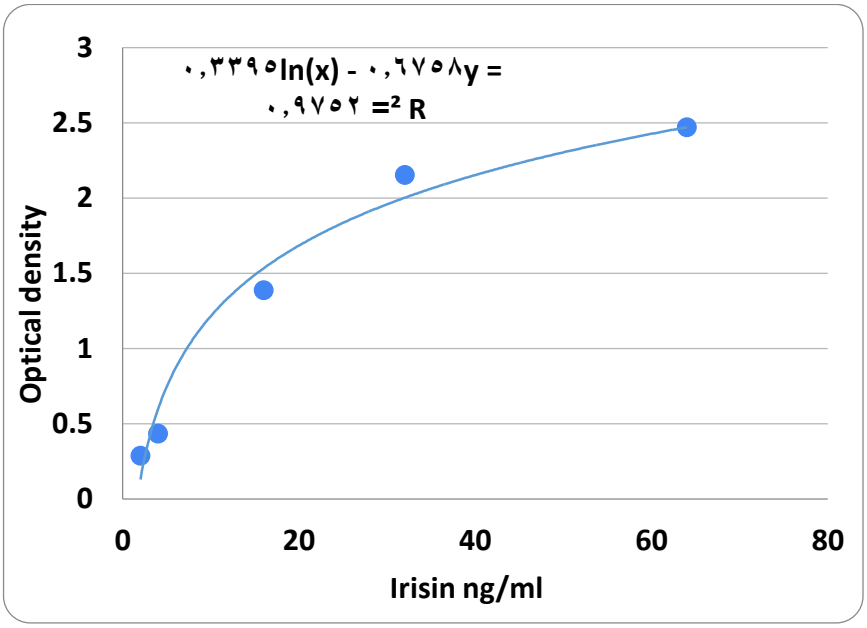

Figure 2-3. Standard curve for irisin.

2.3.7 Measurement of serum preptin levels

Principle:

This kit is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human preptin antibody. Preptin present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human preptin Antibody is added and binds to preptin in the sample. Then Streptavidin-HRP is added and binds to the biotinylated preptin antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and colour develops in proportion to the amount of Human preptin. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm [149].

Table (2-4). Components of ELISA kit for human preptin,

Components	Quantity (96T)
Standard Solution	(4800ng/L) 0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution	A 6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate	(25x) 20ml x1
Biotinylated Human preptin Antibody	1ml x1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

Reagent preparation

a- All reagents were brought to room temperature before usage

b- The original standard sample was diluted as the following table

2400 ng/L	**Standard No.5**	**120µl Original Standard + 120µl Standard Diluent**
1200 ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
600 ng/L	Standard No.3	120µl Standard No.5 + 120µl Standard Diluent
300 ng/L	Standard No.2	120µl Standard No.5 + 120µl Standard Diluent
150 ng/L	Standard No.1	120µl Standard No.5 + 120µl Standard Diluent

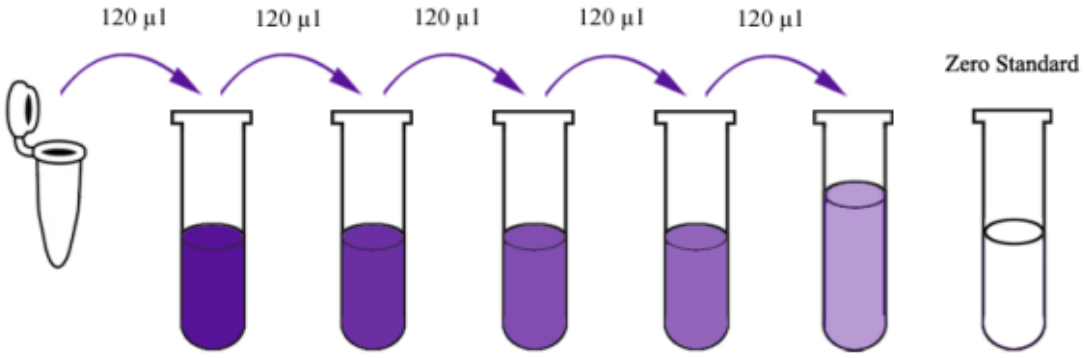

Figure 2-4: Concentration of standards of preptin.

Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
2400ng/L	1200ng/L	600ng/L	300ng/L	150ng/L

Wash Buffer: a volume of 20ml of Wash Buffer (Concentrate 25x) diluted into deionized or distilled water to yield 500 ml of 1x Wash Buffer.

Assay Procedure

1. All reagents, standard solutions and samples were prepared and brought to room temperature before usage.
2. A volume of 50µl standard was added to standard well.
3. A volume of 40µl of serum sample was added to sample wells followed by adding 10µl anti-preptin antibody to sample wells, then 50µl streptavidin-HRP was added to sample wells and standard wells. Then, mixed well, the plate covered with a sealer, and incubated 60 minutes at 37°C.
4. After incubation, the plate was washed 5 times with wash buffer by automated washing. The plate was blotted onto absorbent material (paper towels).
5. A volume of 50µl of substrate solution A and 50µl of substrate solution B was added to each well sequentially. Plate incubated for 10 minutes at 37°C in the dark.

6. A volume of 50µl Stop Solution was added to each well, the blue colour changed into yellow immediately.
7. The optical density (OD value) of each well was determined immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculation of results

The amount of the measured biomarker in the serum was determined by plotting the standard curve between the absorbance and standard concentrations in a logarithmic regression type using Excel software (Figure 2-5). Then, the straight-line equation was obtained in the following form:

Y= mx + b

Absorbance= Slope*Ln (Concentration) + Intercept

Then, Ln (Concentration) = (Absorbance-Intercept)/Slope

Then the unknown concentration of the biomarkers obtained from the following equation:

Concentration of biomarker= 2.718^ ((Absorbance-Intercept)/Slope))

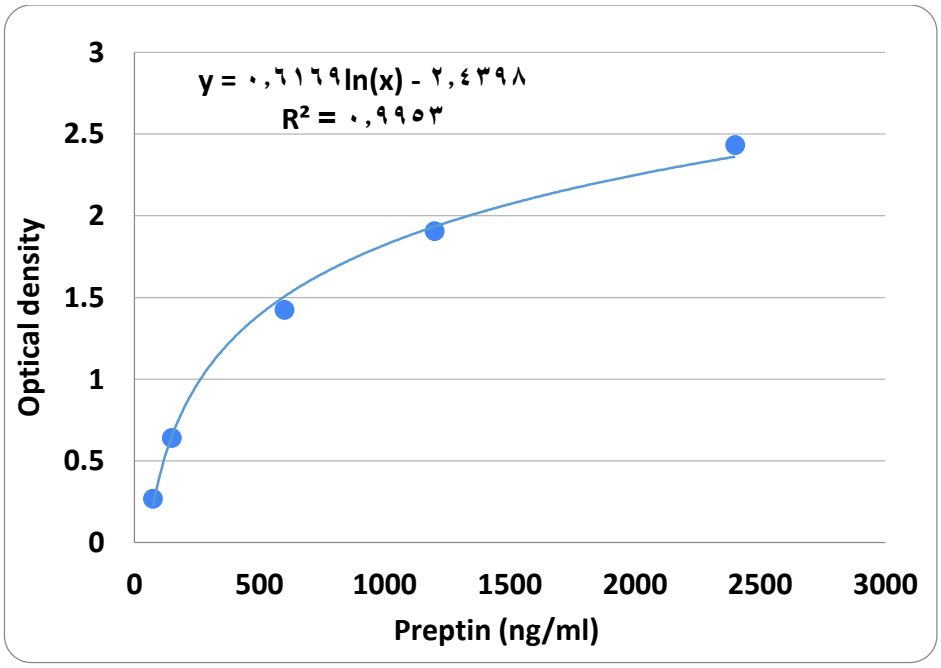

Figure 2-5. Standard curve for preptin.

2.3.8 Measurements of C-peptide levels in serum

***Principle*:**

This kit is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with human C-P antibody. C-P present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human C-P Antibody is added and binds to C-P in the sample. Then Streptavidin-HRP is added and binds to the biotinylated C-P antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and colour develops in proportion to the amount of human C-P. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm [150].

Table 2-5. Components of ELISA kit for human C-peptide:

Components	Quantity
Standard Solution	(32ng/ml) 0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate	(25X) 20ml x1
Biotinylated human C-P Antibody	1ml x1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

Reagents preparation

a- All reagents were brought to room temperature before usage

b- The original standard sample was diluted as the following table

16ng/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
8ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
4ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
2ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
1ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent

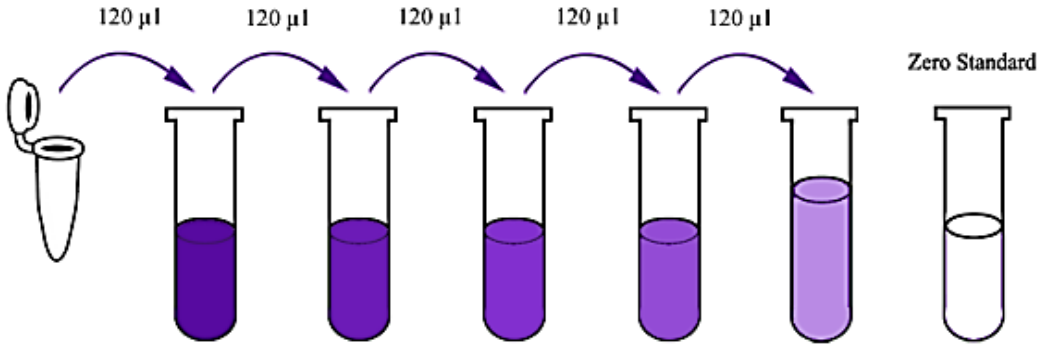

Figure 2-6: Concentration of standards of C-peptide.

Standard Concentration	**Standard No.5**	**Standard No.4**	**Standard No.3**	**Standard No.2**	**Standard No.1**
32ng/ml	16 ng/ml	8 ng/ml	4 ng/ml	2 ng/ml	1 ng/ml

Assay Procedure

1. All reagents, standard solutions and samples were prepared and brought to room temperature before usage.
2. A volume of 50µl of standard was added to standard well.

3. A volume of 40µl sample was added to sample wells followed by adding 10µl of anti-C-P antibody to sample wells, then 50µl of streptavidin-HRP was added to sample wells and standard wells. After that, the plate was mixed well, covered with a sealer, and incubated for 60 minutes at 37°C.
4. After incubation, the plate was washed 5 times with wash buffer by automated washing. Then, the plate was blotted onto absorbent material (paper towels).
5. A volume of 50µl of substrate solution A and 50µl of substrate solution B was added to each well sequentially. Then, the plate was incubated for 10 minutes at 37°C in the dark.
6. A volume of 50µl of Stop Solution was added to each well, the blue colour immediately changed into yellow colour.
7. The optical density (OD value) of each well was determined immediately using a microplate reader setted to 450 nm within 10 minutes after adding the stop solution.

Calculation of results

The amount of the measured biomarker in the serum was determined by plotting the standard curve between the absorbance and standard concentrations in a logarithmic non-linear regression type using Excel software (Figure 2-7). Then, the straight-line equation was obtained in the following form:

Absorbance = Slope * Ln (Concentration) + Intercept

Then, Ln (Concentration) = (Absorbance-Intercept) / Slope

Then the unknown concentration of the biomarkers obtained from the following equation:

Concentration of biomarker= 2.718 ^ ((Absorbance-Intercept)/Slope))

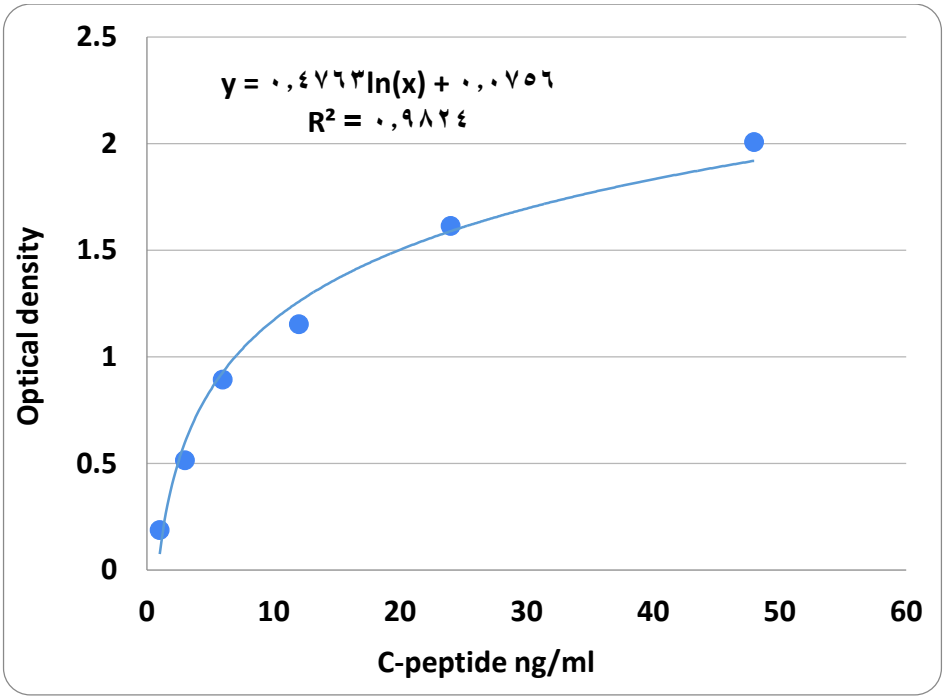

Figure 2-7. Standard curve for C-peptide.

2.4 Statistical analysis

Statistical analysis performed using the Statistical Package for Social Sciences (SPSS IBM, version 28.0). The normality of data distribution was tested using Kolmogorov-Smirnov and Shapiro-Wilk tests. Normally distributed continuous data expressed as mean ±SD, while non-normally distributed data expressed as median (interquartile ranges: 25%-75%). Mann-Whitney U test and Student's t-test were used to compare the median and mean between groups for non-normal and normally distributed data, respectively. Correlations between variables performed by using Spearman correlation. Simple linear regression analysis performed to find the possible prediction on continuous variables. A p-value ≤ 0.05 was considered statistically significant. Results with extreme values were excluded.

Chapter three
Results
and
Discussion

3. Results and Discussion

3.1 Demographic characteristics of the studied groups

3.1.1 Age:

The mean and standard deviation of the age of the studied groups were shown in table 3-1. The range of age in the patients and control group was (2-16) years; with mean ± SD (10.11 ± 3.6), (10.10 ± 3.9), respectively. The difference in the mean of age was statistically insignificant, which belongs to the matching in the range of age between T1DM patients and control group in order to eliminate its effect on other parameters.

Table 3-1. Comparison of age in the studied groups using Student's t-test.

	Group	Number	Mean ± SD	P-value
Age (years)	**Patient**	**42**	**10.11 ± 3.6**	**0.984**
	Control	**42**	**10.10 ± 3.9**	

3.1.2 Distribution of sex in the studied groups.

Among the patients with T1D who contributed to this study, there were 52% boys and 48% girls, as shown in figure (3-1).

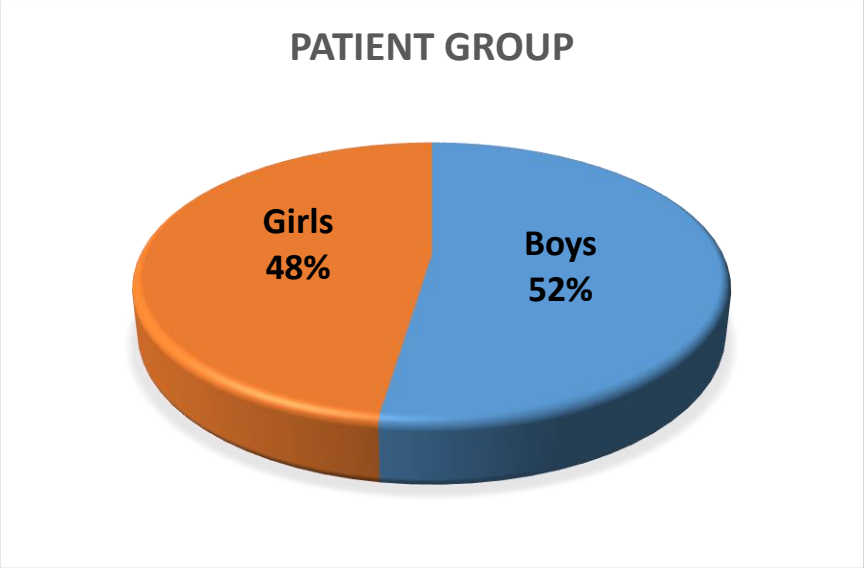

Figure 3-1. Percent of boys and girls in the patient group.

Among the control group there were 57% boys and 43% girls, as shown in figure 3-2.

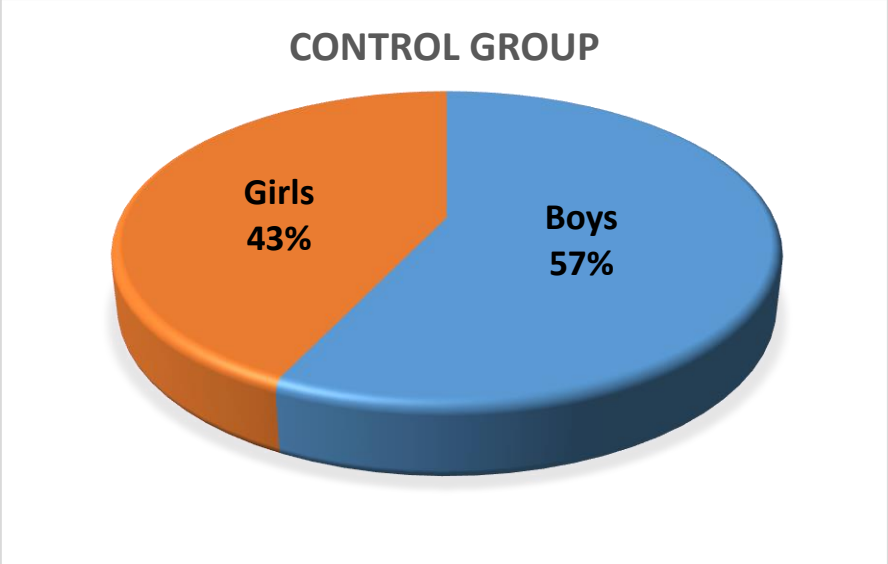

Figure 3-2. Percent of boys and girls in the control group.

The sex distribution of the two studied groups were matched (p-value=0.661). The percentage of boys was higher than girls in patients group because type 1 diabetes is more common in boys rather than in girls [151], and these results comes in agreement with studies done by DiMeglio *et al.* 2018 and Turtinen *et. al.* 2019 [39], [152].

3.1.3 Patients distribution according to residence

The distribution of patients according to residence showed that the majority 62% of patient with T1DM came from rural area. While only 38% of patient with T1DM came from urban area, as shown in Figure (3-3).

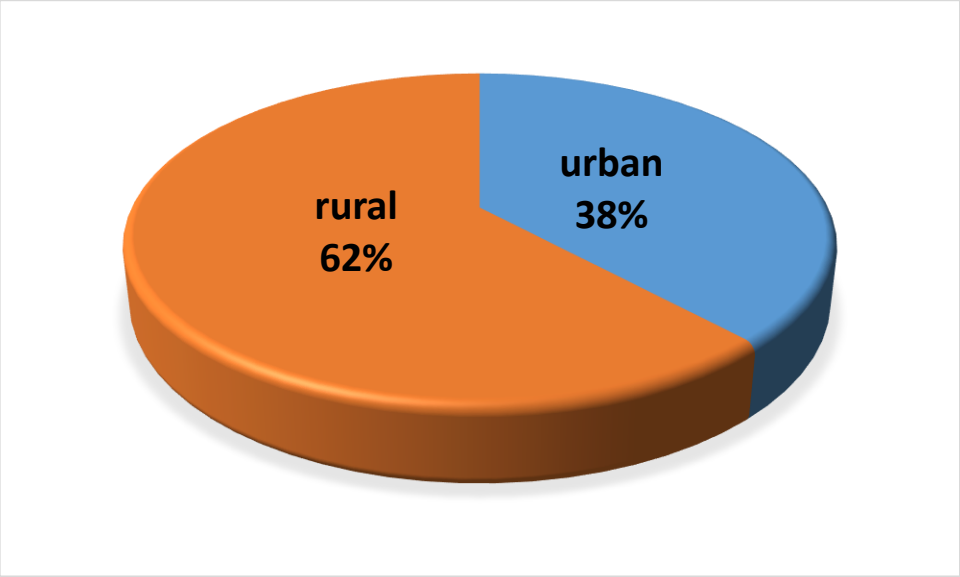

Figure 3-3. Distribution of patients according to residence.

This distribution is might be related to that the individuals of the rural areas visit the hospitals and governmental centres more than the urban individuals.

3.1.4 Distribution of family history of the patients group.

A total of 62% of the patients had a family history of T2DM, 19% had no family history, 14% had both type 1 and type 2 DM, and only 5% had a family history of T1DM, as shown in figure (3-4).

The higher percentage of patients with a family history of diabetes mellitus indicates that the family history is one of the risk factors for type1 diabetes mellitus [39].

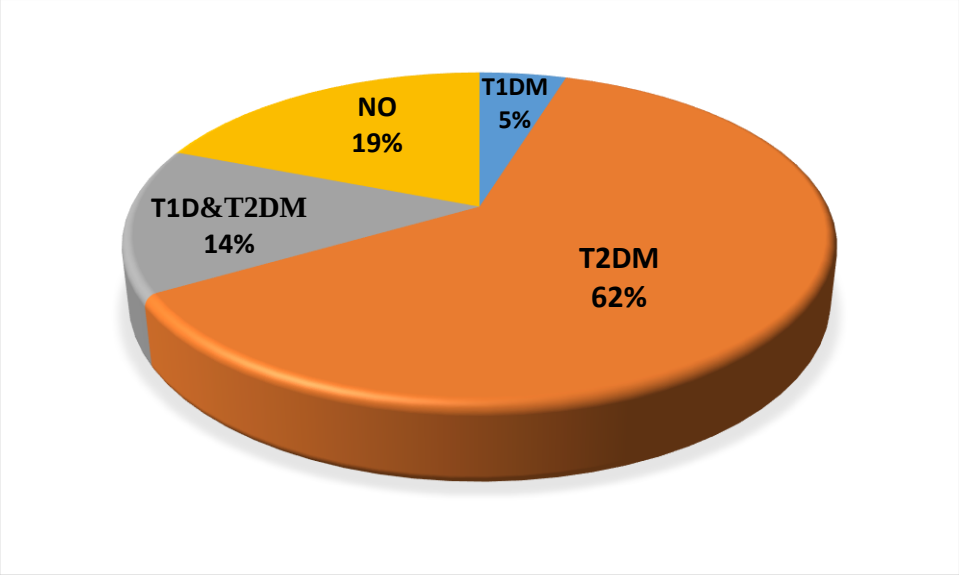

Figure 3-4. Distribution of family history in the patient group

3.1.5 Anthropometric measurements

The comparison of anthropometric measurements between patient and control group are shown in table (3-2). Comparison performed using Mann-Witney U-test.

Table 3-2. Comparison of the anthropometric measurements in the studied groups.

Study variable	Patient group =42 Median (interquartile range)	Control group =42 Median (interquartile range)	P-value
BMI percentile (%)	51.89 (19.15-72.42)	69.51 (21.41-90.86)	0.133
BMI Z score	0.05 (-0.88-0.59)	0.51 (-0.79-1.34)	0.133

There were non-significant differences in BMI percentile (%) and BMI Z score between the patient and control group ($p>0.05$)

3.2 Biochemical parameters

3.2.1 Random blood sugar (RBS) in the studied groups.

There was a significant difference ($p<0.001$) in the median levels of RBS between the patient and control group. Mann-Witney U-test used to compare the median between the studied groups, as shown in Table 3-3.

Table 3-3. Comparison of glucose level in patients and control group.

Study groups	Number	RBS (mmol/l) Median (interquartile ranges)	P-value
Patients	42	13.20 (8.75-19.00)	< 0.001
Control	42	4.20 (3.40-5.00)	

3.2.2 Preptin levels in the studied groups

The current study showed a statistically significant difference in the levels of preptin between the studied groups ($p< 0.05$). Median and interquartile range of the two groups observed in Table (3-4).

Table 3-4. Comparison of preptin levels between the studied groups.

Study groups	Number	Preptin (ng/l) Median (interquartile ranges)	P-value
Patients	42	154.546 (136.86-245.68)	0.026
Control	42	147.449 (121.39-171.99)	

Preptin was significantly increased in the patient group compared with the control group, and this result is in agreement with a study done by Abd El Dayem et. al. 2015 [153]. Preptin was found to be high in patients with type 2 diabetes [79] and polycystic ovary syndrome [154], [155], whom are at high risk of insulin resistance and yet showed a significant positive correlation with HOMA-IR [79].

Even though it is common to have insulin resistance in type 2 diabetes, it was found that this state as well prominent in patients with type 1 diabetes, which is induced by glucose toxicity, lipotoxicity, and defective mitochondrial function [156], [157]. From the shared aspect between T1D and these disorders, the increased susceptibility of having glucose toxicity and defective mitochondrial function raise the probability of higher preptin levels. A study done by Elsaeed W. et. al. 2021[158] disagree with the result of this conducted study, where preptin was significantly reduced in type 1 diabetic patients compared to healthy individuals.

3.2.2.1 Preptin levels and sex

Regarding sex there was non-significant difference in preptin levels between girls and boys of the patients group, as shown in Table (3-5). This result comes in agreement with the study of Kalayci *et. al.* 2018 [79] which was conducted on patients with type 2 diabetes.

Table 3-5. Comparison of the preptin levels according to sex

Study groups	Sex	Number	Preptin (ng/l) Median (interquartile ranges)	P-value
Patients	**Girls**	**20**	**147.701 (129.47-211.35)**	**0.442**
	Boys	**22**	**165.672 (143.90-309.99)**	

3.2.2.2 Preptin levels and age

When the patient group divided into two groups according to age (GroupI- ≤ 10 years, GroupII- > 10 years), there was a non-significant difference between the studied groups (p>0.05), as shown in Ttable (3-6).

Table 3-6. Comparison of preptin levels of patient group according to age.

Patient subgroup	Number	Preptin (ng/l) Median (interquartile ranges)	P-value
GroupI. (≤ 10 years)	21	156.308 (139.48-388.89)	0.242
GroupII. (> 10 years)	21	149.132 (126.74-236.67)	

3.2.2.3 Preptin Levels and Duration of the Disease

On the other hand, preptin levels were non-statistically different between the diabetic patients with duration of disease (<5years), compared to patients with duration of (≥5years) with a p-value >0.05, as shown in Table (3-7).

Table 3-7. Comparison of preptin levels in patient group according to the duration of the disease.

Patient subgroup	Number	Preptin (ng/l) Median (interquartile ranges)	P-value
GroupI. (<5 years)	31	149.858 (130.57-222.19)	0.308
GroupII. (≥5years)	11	220.402 (136.41-679.89)	

3.2.3 Irisin levels in the studied groups

The results of the present study demonstrated an increased levels of irisin in the patients group compared to the control group (p<0.05), as shown in Table (3-8).

Table 3-8. Comparison of irisin levels between the studied groups.

Study groups	Number	Irisin (ng/ml) Median (interquartile range)	P-value
Patients	42	5.357 (4.22-7.18)	0.013
Control	42	4.349 (4.01-5.92)	

Irisin levels in the diabetic patients were significantly higher than the healthy control group ($p<0.05$), which is consistent with the studies of Faienza et. al. 2018 and Ates et. al. 2017 [159], [160], which were conducted on T1D patients. This result is contrary to the study of Tentolouris et. al. 2018 [161] where the patients had reduced levels of irisin, however, their study was done on adult type 1 diabetic patients with a long duration of disease that may affect the result. There is still no obvious reason related to the higher levels of irisin in patients with T1D, nevertheless, different factors might be implicated. One of these factors include glucagon, which stimulates PGC1α expression through glucagon-Ca^{+2}-CREB, PKA pathways [162], [163]. As a result, this pathway leads to increased irisin expression. Moreover, ATP deprivation in the skeletal muscles that is increased in T1D patients is might be another stimulus for irisin synthesis and secretion. In addition, it was found that in the muscles of type 1 diabetic patients there is an increased Ca^{+} exposure due to the hyperactive Ca^{+2} kinetics [164], [165]. And this might increase irisin synthesis through Ca^{+2}-AMPK-PGC1α or Ca+-calcineurin/CaMKs-CREB, NFAT, MEF2C, MEF2D pathways, as revealed in Figure (1-10).

3.2.3.1 Irisin levels and sex.

Regarding sex there was a non-significant difference between boys and girls in irisin levels of the patients group ($p>0.05$), as shown in Table 3-9.

Table 3-9. Comparison of irisin levels between boys and girls in the patients group.

Study group	Sex	Number	Irisin (ng/ml) Median (interquartile range)	P-value
Patients	Girls	20	5.629 (4.15-7.82)	0.980
	Boys	22	5.357 (4.25-8.17)	

And this is in agreement with other studies done by Faienza *et. al.* 2018 and Reinehr *et. al.* 2015 [159], [166].

3.2.3.2 Irisin levels and age.

According to the age, there was a non-significant difference in irisin levels between the studied groups (p>0.05), as shown in Table 3-10.

Table 3-10. Comparison of irisin levels according to age in diabetic patients.

Patient subgroup	Number	Irisin (ng/ml) Median (interquartile range)	P-value
GroupI (≤ 10 years)	21	5.295 (4.22-10.98)	0.782
GroupII (> 10 years)	21	5.420 (2.19-6.69)	

3.2.3.3 Irisin levels and duration of the disease.

The results also revealed a non-significant (p>0.05) difference in the median of irisin regarding the duration of the disease between group I and group II. As shown in Table 3-11.

Table 3-11. Comparison of irisin levels regarding to the duration of disease.

Patient subgroup	Number	Irisin (ng/ml) Median (interquartile range)	P-value
GroupI (<5 years)	31	5.156 (4.21-6.51)	0.098
GroupII (≥5years)	11	6.199 (5.42-18.98)	

3.2.4 C-peptide levels in the studied groups

The present study observed a significant difference in the median levels of C-peptide between the patient and control groups (p=0.05), as shown in Table 3-12.

Table 3-12. Comparison of C-peptide between the studied groups.

Study groups	Number	C-peptide (ng/ml) Median (interquartile ranges)	P-value
Patients	**42**	**2.651 (2.13-3.23)**	**0.050**
Control	**42**	**3.156 (2.34-5.29)**	

The levels of C-peptide were significantly decreased in the patients with T1D compared with the control group ($p_=$ 0.05). This result is related to the fact that, type 1 diabetes caused by an autoimmune damage to the pancreatic beta cells. And as it is synthesized in beta cells and cleaved from insulin prohormone, reduced beta cell mass results in reduced insulin levels and thus C-peptide levels [167].

3.2.4.1 C-peptide levels and sex

Regarding sex there was a non-significant difference in the levels of C-peptide between girls and boys of the patient group (p>0.05), as shown in table 3-13.

Table 3-13. Comparison of C-peptide levels according to sex of the patient group

Study group	Sex	Number	C-peptide (ng/ml) Median (interquartile ranges)	P-value
Patients	**Girls**	**20**	**2.515 (2.23-3.23)**	**0.925**
	Boys	**22**	**3.109 (2.10-3.31)**	

3.2.4.2 C-peptide levels and age

C-peptide levels did not show a significant difference according to age when a comparison performed between the age group I (≤ 10 years) and age group II (> 10 years) of the diabetic patients, with a p-value >0.05, as shown in Table 3-14.

Table 3-14. Comparison of C-peptide level of patients according to age.

Patient Subgroup	Number	C-peptide (ng/ml) Median (interquartile ranges)	P-value
GroupI. (≤ 10 years)	21	2.537 (2.10-3.25)	0.568
GroupII. (> 10 years)	21	3.109 (2.20-3.23)	

3.2.4.3 C-peptide levels and duration of the disease

Furthermore, C-peptide levels reduced when the duration of disease increased (≥5 years) with statistically significant difference between the two groups, as shown in Table 3-15.

Table 3-15. Comparison of C-peptide levels of diabetic patients according to duration of disease.

Patient subgroup	Number	C-peptide (ng/ml) Median (interquartile ranges)	P-value
GroupI. (<5 years)	31	3.112 (2.32-3.27)	0.026
GroupII. (≥5years)	11	2.134 (1.23—3.11)	

Type 1 diabetes mellitus is caused by an autoimmune destruction in pancreatic beta cells, however, in some patients autoimmunity may persist beyond the time of diagnosis and so the destruction of beta cells continued [34]. The result of the current study showed a considerable decline in C-peptide levels as the duration of disease increased and this is in agreement with studies done by Davis et. al. 2015 and Carr et. al. 2022 [168], [169].

3.3 Spearman correlation of preptin and irisin with other parameters

3.3.1 Correlation between preptin and other parameters.

The results showed that preptin significantly correlated with irisin and C-peptide, while there was a non-significant correlation with RBS, age, BMI Z.score, BMI percentile, duration of the disease, and HbA1c, as shown in Table 3-16.

Table 3-16. Spearman correlation between preptin and other parameters

Study parameters	r	P-value
Age (years)	-0.157	0.321
BMI Z.score	0.119	0.453
BMI percentile	0.118	0.456
Duration	-0.025	0.758
RBS	0.014	0.875
Irisin	**0.779**	**0.000****
C-peptide	**0.781**	**0.000****
HbA1c	-0.036	0.820

*Correlation is significant <0.05

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

3.3.1.1 Correlations between preptin and irisin

Serum preptin levels showed a significant positive correlation with irisin ($r=0.779$, $p<0.001$), as shown in Figure (3-5).

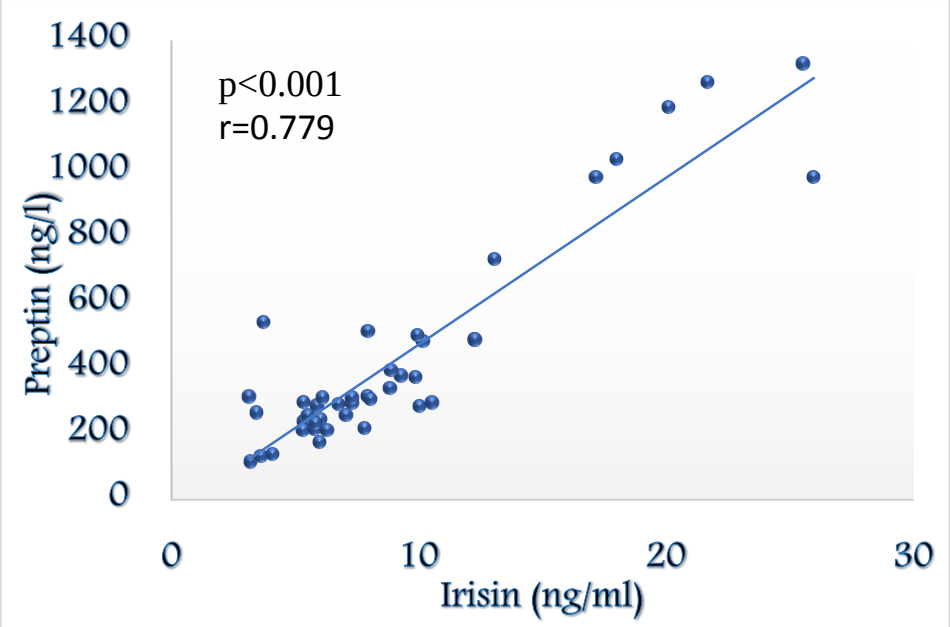

Figure 3-5. Correlation between prpetin and irisin.

To the best of our knowledge, this is the first study that determines the correlation between irisin and preptin in patients with type 1 diabetes. These results support the study of Zhang Y. *et al.* 2014 [170] who found that irisin has the ability to promote the expression and synthesis of betatrophin in the liver, a newly discovered hormone that promotes β-cells regeneration and proliferation, through the p38-PGC-1α pathway [104]. Thus, irisin might promotes preptin secretion indirectly from pancreatic β-cells through betatrophin.

3.3.1.2 Correlation between preptin and C-peptide.

There was a positive significant correlation between preptin and C-peptide ($r=0.781$, $p<0.001$), as shown in Table (3-16). This may be related to the fact that both preptin and C-peptide secreted from pancreatic β-cells in response to hyperglycaemia, thus, as C-peptide released with insulin in response to increased glucose levels, preptin is released at the same time to augment insulin secretion, Figure 3-6.

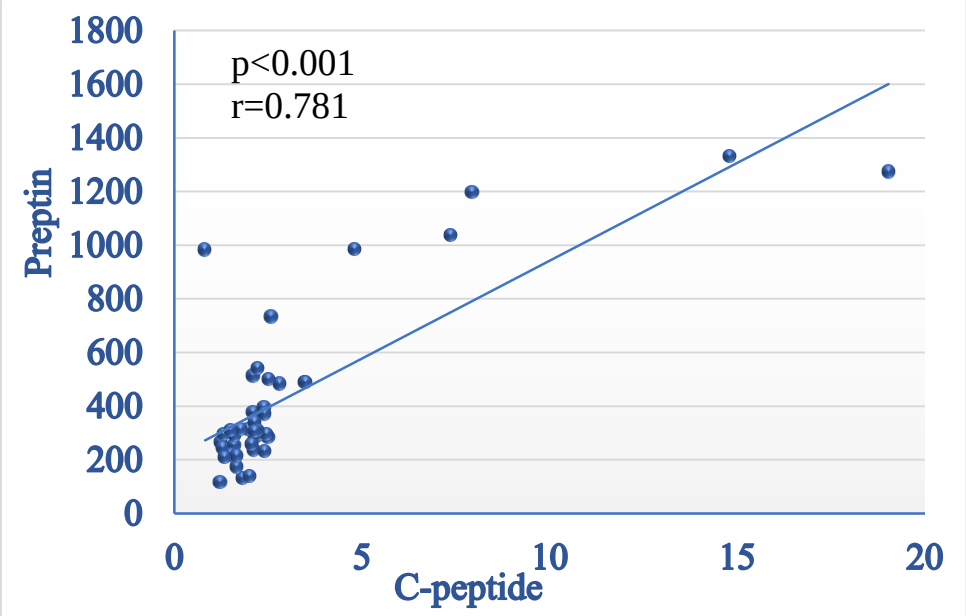

Figure 3-6. Correlation between prpetin and C-peptide.

3.3.1.4 Linear regression analysis with preptin as a dependent variable

In linear regression analysis, only irisin showed a significant association with preptin, which means that irisin could be used as a predictor for preptin levels and so residual β-cells, as revealed in Table 3-17.

Table 3-17. Linear regression analysis with preptin as a dependent variable

Coefficients [a]					
Model	**Unstandardized Coefficients**		**Standardized Coefficients**	**t**	**Sig.**
	B	**Std. Error**	**Beta**		
(Constant)	51.870	17.632		2.942	.006
Irisin	20.514	3.558	.725	5.766	.000
a. Dependent Variable: preptin (ng/l)					

It is observed from the table of coefficients and the regression equation (figure 3-7) that each one unit (1ng) increase in irisin levels will increase the preptin mean levels by 20.5 ng.

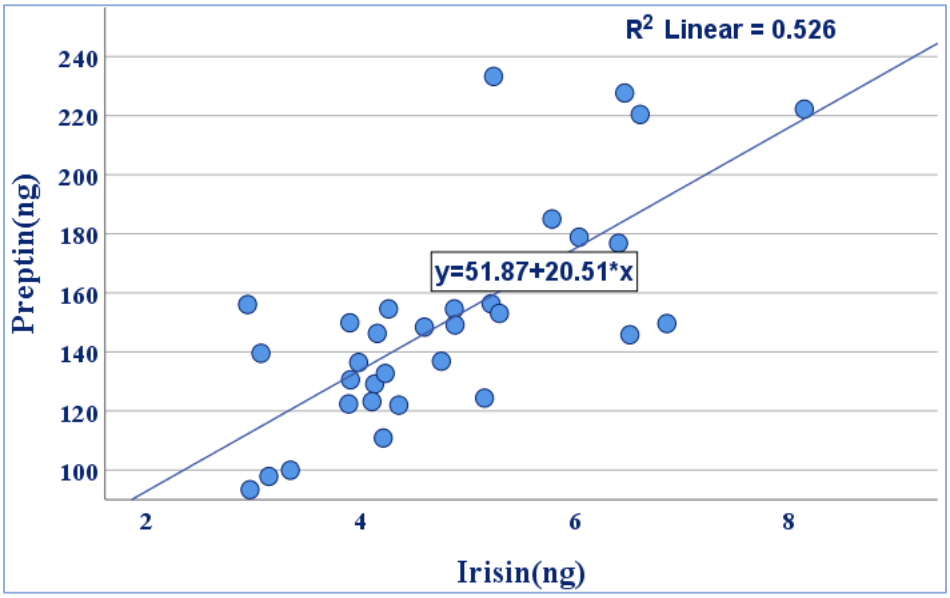

Figure 3-7. Association between preptin and irisin with regression equation

3.3.2 Correlation between irisin and other parameters

The results showed a significant correlation between irisin and C-peptide, and non-significant correlation with RBS, age, BMI Z.score, BMI percentile, duration of the disease, and HbA1c, as shown in Table 3-18.

Table 3-18. Correlation between irisin and other parameters

Study parameters	r	P-value
Age (years)	-0.096	0.546
BMI Z.score	0.115	0.467
BMI percentile	0.114	0.473
Duration	-0.110	0.487
RBS	-0.072	0.649
C-peptide	**0.836**	**0.000****
HbA1c	-0.106	0.502

*Correlation is significant <0.05.
**Correlation is significant at the 0.01 level (2-tailed).

3.3.2.1 Correlation between irisin and C-peptide

Irisin was positively correlated with C-peptide (r=0.836, p<0.001), as shown in Figure 3-7.

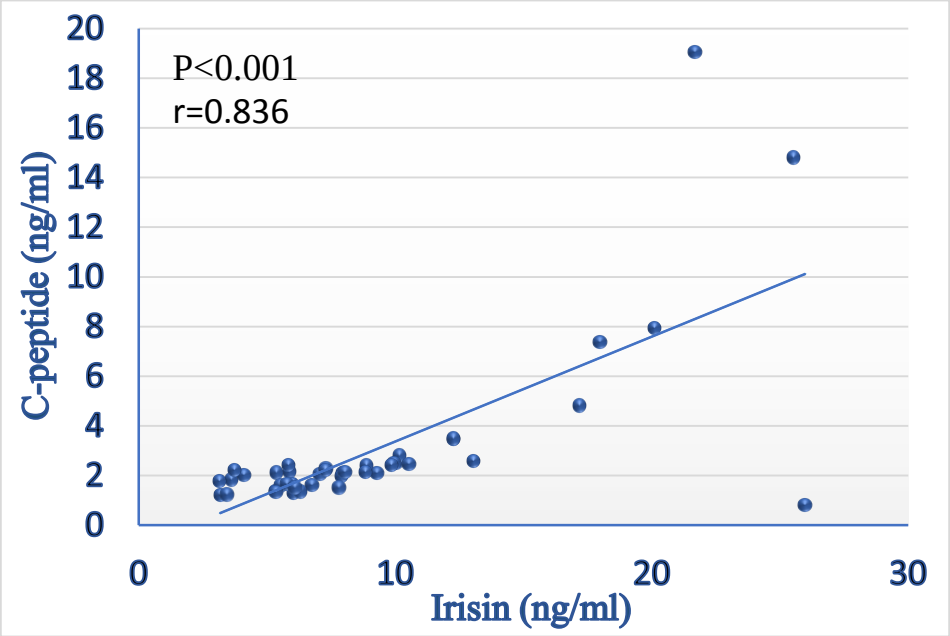

Figure 3-8. Correlation between irisin and C-peptide.

This significant correlation may be due to betatrophin, which is a hormone secreted from the liver and adipose tissue, has recently reported to play a role in beta cell proliferation and regeneration. Since irisin induce betatrophin synthesis through p38-MAPK pathway [104], [171], the positive correlation between irisin and C-peptide might be related to the role of irisin to stimulate betatrophin secretion and β-cells regeneration.

3.3.3 Spearman correlation between C-peptide and other parameters

There was a non-significant correlation between C-peptide with RBS, age, BMI Z.score, BMI percentile, duration of disease and HbA1c, as shown in Table 3-19.

Table 3-19. Spearman correlation between C-peptide and other parameters

Study Parameters	r	P-value
Age (years)	0.031	0.844
BMI Z.score,	0.064	0.688
BMI percentile	0.062	0.697
Duration	-0.024	0.881
RBS	0.015	0.927
HbA1c	0.003	0.983

3.3.3.2 Linear regression analysis with C-peptide as a dependant variable.

Irisin was an independent predictor for C-peptide with a p-value (<0.001) in linear regression analysis, as shown in Table 3-20.

Table 3-20. Linear regression analysis with C-peptide as a dependant variable.

Coefficients [a]					
Model	**Unstandardized Coefficients**		**Standardized Coefficients**	**t**	**Sig.**
	B	**Std. Error**	**Beta**		
(Constant)	.872	.332		2.624	.014
Irisin	.459	.067	.781	6.840	.000
a. Dependent Variable: c-peptide ng/ml					

It is revealed from the table of coefficients and the equation of regression analysis (Figure 3-9) that each one unit (1ng) increase in irisin levels will increase 0.46 ng of C-peptide mean levels.

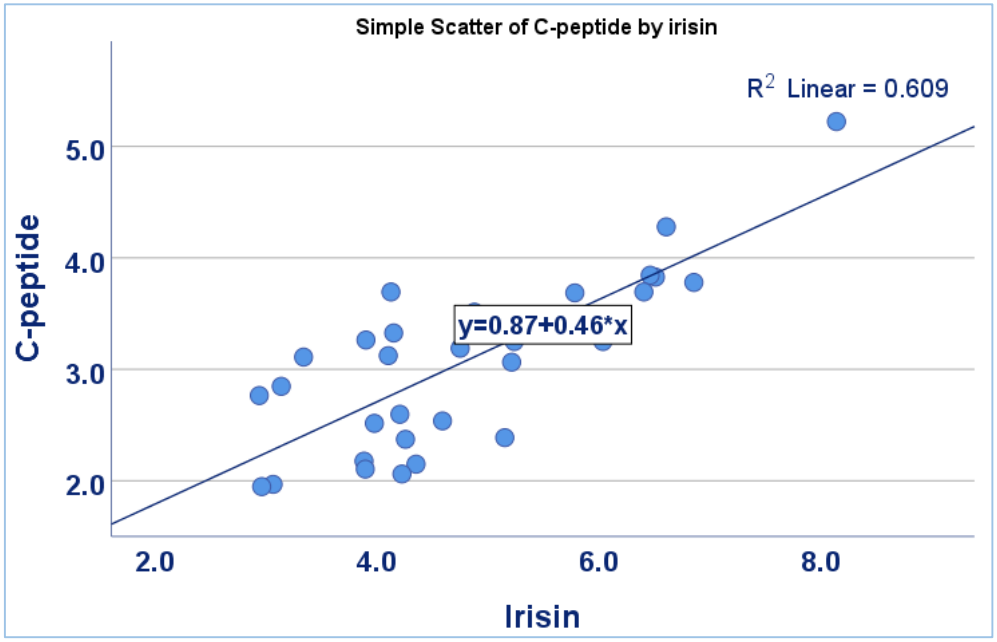

Figure 3-9. Association between irisin and C-peptide with regression equation

3.3.4 Correlation between demographic characteristics and glucose levels.

The results also observed a significant correlation between age and BMI with a p-value <0.001. In addition, there was a significant association between HbA1c and RBS (p=0.028), as shown in Table 3-21.

Table 3-21. Correlation between demographic characteristics and glucose levels.

Study Parameters		r	p-value
Age (years)	BMI	0.566	0.000
	Duration	0.279	0.073
HbA1c (%)	RBS	0.339	0.028

The positive correlation between age and BMI belongs to the fact that, normally, as the age increases, the growth of muscle increases in addition to the increased bone mass and fat mass. The positive association between HbA1c and RBS (Figure 3-10) is in agreement with Beck et. al. study 2019 [172]. Haemoglobin A1C is one of the glycated proteins composed of haemoglobin and glucose, and it indicates how much glucose exists in the blood over the 120 days of the red blood cells' life cycle.

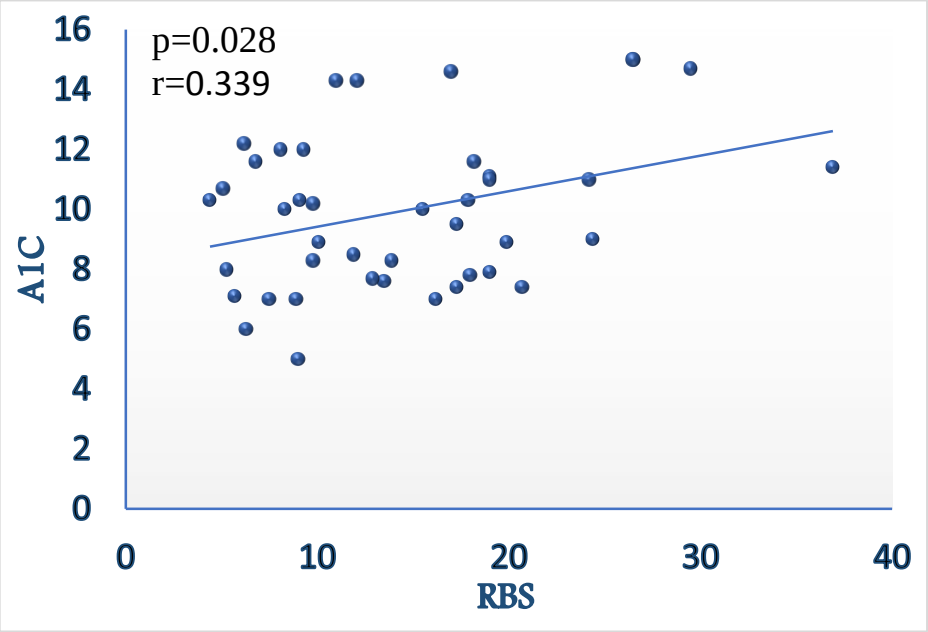

Figure 3-10. Correlation between HbA1c and RBS.

Conclusions

1- Irisin is correlated significantly with preptin, and this result support that there is a relationship between irisin and pancreatic β-cells, and so the development of diabetes mellitus.

2- Through the strong association between preptin and C-peptide, preptin could be used as a physiologic amplifier for the second phase insulin secretion.

3- Irisin is a strong predictor for preptin and C-peptide levels, and thus residual β-cells, therefore, it might be used as a predictive marker with C-peptide for T1D.

4- According to the regression analysis, irisin raised preptin levels that were released from all sources, not only from pancreatic beta cells.

Recommendations

1- Determine betatrophin levels and its correlation with irisin levels in type 1 diabetes mellitus.

2- Assess the correlation between irisin and complications in type 1 diabetes mellitus.

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Appendix

The questionnaire form of this study

Number:	
Family number:	
Name:	
Age:	
Sex: -	Male: Female:
Family history: -	DM Type1: DM type2:
Weight(kg): Height(cm):	BMI:
Residence:	Rural: Urban:
Biochemical tests: -	Random blood glucose: HbA1c:
Coeliac disease:	
Thyroid disease:	T4: TSH: T3:
Fasting:	Yes: no:
Taken insulin:	Yes: no:
Other biochemical tests:	
Other diseases:	

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامــعة بابـــل
كليـــة الطـــب
فرع الكيمياء والكيمياء الحياتية

البربتين والايريسين كمؤشرات ايضية حيوية في مرض السكري من النوع الاول عند الاحداث

رسالة مقدمة إلى

مجلس كلية الطب/ جامعة بابل كجزء من متطلبات نيل درجة الماجستير في العلوم/ الكيمياء الحياتية السريرية

من قبل الطالبة

زينة عامر حفظي

بكلوريوس تحليلات مرضية/ كلية التقنيات الصحية والطبية / جامعة الفرات الاوسط التقنية

أشراف

الاستاذ الدكتور
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د. رحاب فيصل لفتة

١٤٤٥ هجرية

٢٠٢٣ ميلادية

الخلاصة

داء السكري من النوع الأول هو مرض مناعي ذاتي مزمن يتميز بنقصان الانسولين كنتيجة لتحطم خلايا بيتا في البنكرياس مؤديا الى زيادة سكر الدم. انه يحدث بشكل شائع عند الأطفال والمراهقين والشباب. يتطور ارتفاع سكر الدم بالتدريج مع نقصان مستويات الانسولين، مما يجعل المرضى عرضة للجهد التاكسدي و مضاعفات السكري. وبسبب عدم وجود العلاج النهائي للمرض، يعتمد المرضى على حقن الانسولين بشكل دائم.

البربتين هو ببتيد هرمون تم اكتشافه حديثًا مشتق من IGF-II. يتم تصنيع البربتين بشكل أساسي في خلايا البنكرياس وينفرز بالتزامن مع الأنسولين استجابةً لزيادة نسبة السكر في الدم. نظرًا لأن البربتين له دور في مضاعفة إفراز الأنسولين ، يمكن استخدامه كمحفز فسيولوجي لإفراز الأنسولين بوساطة الجلوكوز.

ايريسين عبارة عن ميوكين جديد ينتج عند اداء التمارين الرياضية، يفرز من عضلات الإنسان و هو المسؤول عن الآثار المفيدة للتمارين مثل التنظيم الحراري وفقدان الوزن. نظرًا لوجود فوائد كبيرة للايريسين الناتج من ممارسة التمارين الرياضية ، وخاصة في توازن الجلوكوز ، فإننا نقترح أنه يمكن استخدام الإيريسين كعلاج في المرضى الذين يعانون من مرض السكري من النوع الاول لزيادة استخدام الجلوكوز من قبل الخلايا.

سي-ببتيد عبارة عن سلسلة رابطة مشتق من الأنسولين في خلايا البنكرياس نوع بيتا. لقد اكتشف ان السي-ببتيد لديه عدة تاثيرات مفيدة على العديد من انواع الخلايا والانسجة . نظرًا لأن إفراز السي-ببتيد يكون متساويًا مع الأنسولين، وله عمر نصفي أطول من الأنسولين، ويتجاوز عملية التمثيل الغذائي في الكبد، فإنه يستخدم كعلامة سريرية لوظيفة خلايا بيتا البنكرياسية.

تهدف الدراسة الحالية إلى التحقق من مستويات الإريسين والبربتين في المرضى الذين يعانون من داء السكري من النوع الاول وتحديد ارتباطها بمستويات السي-ببتيد. بالإضافة إلى تحديد ارتباط هذه الهرمونات بمستويات الجلوكوز، لتحديد امكانية استخدام هذه الماركرات كعلاجًا مشتركًا مع الأنسولين.

تم جمع العينات من ١ سبتمبر ٢٠٢٢ حتى ١٣ فبراير ٢٠٢٣. تم تنفيذ الجانب العملي من الدراسة في مختبر قسم الكيمياء والكيمياء الحيوية السريرية في كلية الطب / جامعة بابل.

تضمنت الدراسة الحالية ١٠٠ مشارك في الفئة العمرية (٢-١٦سنة) ، مقسمة إلى مجموعتين: ٥٠ مشارك معافى ظاهريا و ٥٠ مريضًا مصابًا بـ مرض السكري من النوع الاول. تم تقدير مستويات

الجلوكوز في جميع المشاركين باستخدام مقياس الطيف الضوئي بينما تم قياس مستويات البربتين والإريسين والسي-ببتيد باستخدام تقنية الاليزا. تم قياس الهيموغلوبين A1C باستخدام المحلل الآلي.

أظهرت الدراسة انخفاضًا معنويًا في مستويات السي-ببتيد في مجموعة المرضى ($P\leq0.05$). كما اظهرت ارتفاع مستويات سكر الدم العشوائي بشكل كبير في مجموعة المرضى ($P <0.001$).و كانت مستويات البريبتين والإريسين مرتفعة بشكل معنوي في مجموعة المرضى مقارنة بالاصحاء ($P <0.05$).

بالإضافة إلى ذلك ، أظهرت هذه الدراسة ارتباطًا إيجابيًا معنويًا بين البربتين والايريسين ($P<0.001$). يرتبط البريبتين والإيريسين أيضًا ارتباطًا وثيقًا وإيجابيًا بالسي-ببتيد($P<0.001$). في تحليل الانحدار، كان الايريسين مؤشرًا قويا لمستويات البربتين و السي-ببتيد. كان هناك أيضًا ارتباط معنوي موجب بين HbA1c و السكر العشوائي($P = 0.028$)

نستنتج بان الارتباط الإيجابي بين البربتين والإيريسين يدعم وجود علاقة بين إيريسين وخلايا بيتا في البنكرياس ، وبالتالي تطور مرض السكري. يعتبر الايريسين مؤشرا قويا لمستويات البربتين ، وبالتالي خلايا بيتا المتبقية، لذلك يمكن استخدامه كعلامة تنبؤية مع السي ببتيد لمرض السكري من النوع الاول. و من خلال العلاقة القوية بين البربتين والسي ببتيد فانه من المحتمل ان يستخدم البربتين كمضخم فسيولوجي للطور الثاني من افراز الانسولين.