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Assessment of Pentosidine, Leptin, Adeponectine and TNF-α Levels in Iraqi Diabetic Patients with Retinopathy

A Thesis

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

By

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2022 A. D.

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ أَنتَ

الْعَلِيمُ الْحَكِيمُ

صدق الله العلي العظيم

سوره البقرة (32)

Dedication

I dedication this work to:

My parents for Encouragement and support

My brother and my sisters

for their kindness with me

and to all my friends that help me

Jassim

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Thanks and praise to Allah Almighty and his Messenger, may Allah bless him and grant him peace.

Either after:

It is a pleasure to express my thanks to my supervisor **Dr. Ban Mahmood Shaker Al-joda** and **Dr. Qassem Kadhim Al-Rubaie** for their supervision and continuous encouragement throughout the work.

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Jassim Mohammed

Supervisor certification

we certify that this thesis was prepared under our supervision at the College of Medicine, University of Babylon, as partial fulfillment of the requirement for the master degree of science (M.Sc.) in clinical biochemistry.

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

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COMMITTEE CERTIFICATION

We, the examination committee, certify that we have read the thesis entitled " **Assessment of Pentosidine, Leptin, Adeponectine and TNF-α Levels in Iraqi Diabetic Patients with Retinopathy "** and have examined the student Jassim Mohammed Jabur in its contents and that in our opinion it is accepted as a thesis for degree of Master of Science in clinical biochemistry with excellent estimation.

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Summary

Diabetic retinopathy (DR), the most common retinal vascular consequence of diabetes mellitus (DM), is a leading cause of vision problems in people of working age. DR is usually asymptomatic within the early stages. When left untreated, DR can cause significant vision loss and potentially lead to blindness. Classically, DR was thought to be a retinal microvascular disease. However, mounting data shows that retinal neurodegeneration occurs early in the pathophysiology of DR, perhaps contributing to microvascular anomalies. Diabetic retinopathy (DR) is on track to become the primary global cause of vision loss in many nations as the incidence of diabetes mellitus rises and people with diabetes live longer lives.

The present study was designed to investigate the level of pentosidine, leptin, adeponectine and TNF-α in Iraqi diabetic patients with retinopathy and healthy control subject and to assess the relation of each of them.

This study designed as a case-control study. Ninety patients were involved in this study, divided into two groups (DR group that contain 45 patients, and control groups that contain 45 subject).

All the DR subjects included in the current study were previously diagnosed by an ophthalmologist. Currents study was conducted in of ophthalmology in Imam Sadiq Hospital and private clinic in Hilla city.

The particular side of the study was performed at the laboratory of the Biochemistry Department in College of Medicine / university of Babylon. Enzyme-linked immunosorbent assay (ELISA) was used to measure pentosidine, leptin, adiponectin, TNF, and C-RP, while glucose was assessed using the colorimetric method, HbA1c using ichroma, and ESR using the Westergren method.

The results of current study revealed a significant ($P<0.05$) increase in the serum of pentosidine, leptin, TNF-α, adiponectin and C-RP among DR patient than control. The body mass index(BMI) was significantly increase in patients than healthy control.

In this study significant positive correlation between pentosidine with leptin and TNF-α, and there was significant positive correlation

between leptin and TNF-α, and there was significant positive correlation between adiponectin and TNF-α.

In conclusion, diabetes mellitus and long duration hyperglycemia may be one of the important causes of diabetic retinopathy through its direct role in releasing angiogenic and inflammatory factors in the retina of an eye.

List of Contents

Abbreviation	Details
ABCD	Appropriate Blood Pressure Control in Diabetes
AGE	Advanced glycation end products
Ang-1, Ang-2	Angiopoietins
APN	Adiponectin
AUC	Area under curve
BRB	Blood-retinal barrier BRB
CFPs	Color fundus photographs
CSME	Clinically signifi can't macular edema
CURES	Chennai Urban Rural Epidemiology Study
DCCT	Diabetes Control and Complications Trial
DIRECT	Diabetic Retinopathy Candesartan Trials
DM	Diabetes mellitus
DME	Diabetic macular edema
DME	Diabetic Macular Edema
DR	Diabetic retinopathy
ELISA	Enzyme-linked immunosorbent assay
ETDRS	Early Treatment of Diabetic Retinopathy Study
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes
HIF-1	Hypoxia-inducible factor 1
HRP	Horseradish peroxidase
ICSH	International Committee for Standardization in Haematology
IDF	International Diabetes Federation
IL	Interleukin

IRBP	Interphotoreceptor retinoid-binding protein
MAP	Mitogen-activated protein
MESA	Multi-Ethnic Study of Atherosclerosis
MODY	Maturity-onset diabetes of the young
mRNA	Messenger Ribonucleic acid
MVL	Moderate vision loss
NF-κB	Nuclear factor kappa
NGF	Nerve growth factor
NK	Natural killer
NPDR	Non-proliferative phase of DR
NPV	Negative predictive value
NVD	Neovascularization of the disk
NVE	Neovascularization elsewhere
NVI	Novascularization of the iris
PCh	Phosphocholine
PDGF	Platelet-derived growth factor
PDR	Proliferative phase of DR
PEDF	Pigment epithelium-derived factor
PKC	Protein kinase C
PLA2	Phospholipase A2
PPV	Positive predictive value
RAGE	Advanced glycation end receptors
RAS	Renin-angiotensin system
RNA	Ribonucleic acid
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
SD	Standard deviation

STAT3	Signal transducer and activator of transcription 3
STZ	streptozotocin
T1DM	Type 1 diabetes
T2DM	Mellitus Type 2 diabetes mellitus
TACE	TNF-converting enzyme
UKPDS	The UK Prospective Diabetes Study
VEGF	Vascular endothelial growth factor
VTDR	Vision-Threatening Diabetic Retinopathy
WHO	World Health Organization
xg	Gravitational force
ZO-1	Zonula occludens-1

1 INTRODUCTION

1.1 Diabetes Mellitus (DM)

Diabetes mellitus (DM), one of the chronic diseases with the fastest rate of increase in the world, is characterized by high blood glucose levels and abnormalities in the metabolism of fat and protein. When the pancreas fails to secrete enough insulin or when the cells are unable to correctly employ the insulin that is produced, blood glucose levels rise because the cells are unable to digest blood glucose(1).

The World Health Organization (WHO) predicts that by 2030, the number of individuals living with this disease would have risen to 366 million(2). Meanwhile, the International Diabetes Federation (IDF) predicts that by 2045, there will be 693 million people with diabetes(3).

Type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) are the two most common type of diabetes, both of which are caused by impaired insulin production (T1DM) and/or action (T2DM). T1DM is expected to afflict children and adolescents, but T2DM is thought to affect middle-aged and older individuals who have long-term hyperglycemia as a result of poor lifestyle and nutritional choices(4).

Insulin-producing beta cells and glucagon-secreting alpha cells are the two types of endocrine cells found in the pancreas' Langerhans islets. The glucose environment causes beta and alpha cells to adjust their hormone secretion levels. The glucose levels become excessively unbalanced when insulin and glucagon are not in balance. Insulin deficiency and/or impaired action (insulin resistance) cause hyperglycemia in people with diabetes(4).

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder caused by the destruction of beta cells in the pancreatic islets. T1DM can strike at

any age, however it most commonly strikes in adolescents, with a peak onset around puberty. T1DM is equally frequent in both sexes during childhood, although males are more likely to develop the condition in early adulthood(5).

T2DM is one of the most prevalent metabolic diseases in the world, and it is caused by a combination of mainly two factors: inadequate insulin production by pancreatic -cells and the failure of insulin-sensitive tissues to respond to insulin (6).

Insulin release and action must be precisely timed to satisfy metabolic demand; as a result, the molecular processes involved in insulin production and release, as well as insulin response in tissues, must be properly controlled. As a result, flaws in any of the processes involved might result in a metabolic imbalance, which can lead to T2DM pathogenesis(7).

1.2 Diabetic Retinopathy (DR)

Diabetic retinopathy (DR), the most common retinal vascular consequence of diabetes mellitus (DM), is a leading cause of vision problems in people of working age. DR is usually asymptomatic within the early stages. When left untreated, DR can cause significant vision loss and potentially lead to blindness(8).

Classically, DR was thought to be a retinal microvascular disease. However, mounting data shows that retinal neurodegeneration occurs early in the pathophysiology of DR, perhaps contributing to microvascular anomalies(9).

There are numerous clinical studies proving that chronically imbalanced glucose in the blood damages retina microvasculature, causes the blood-retinal barrier (BRB) to break down, fluid leakage, and intra-retinal hemorrhage in the early non-proliferative phase of DR (NPDR), and

causes retinal neovascularization in the proliferative phase of DR (PDR) show in figure 1-1[2] [6].

Proliferative diabetic retinopathy (PDR) is a stage of DR in which new blood vessels develop on the retina. The majority of severe vision loss is caused by PDR. Consequently, retinal vessels might become permeable, resulting in retinal swelling known as diabetic macular edema (DME). In diabetes, DME is a primary cause of moderate vision loss (MVL)(11).

These vessels, however, are fragile and prone to bleeding. The blood that collects in the vitreous cavity as a result of these hemorrhaging veins adversely hinders vision. Further complications, such as traction retinal detachment leading to documented blindness, may make this permanent. Without therapy for PDR, it is expected that half of all patients will go blind within five years of diagnosis(12,13).

Diabetic retinopathy progresses from mild nonproliferative abnormalities, which are characterized by increased vascular permeability, to moderate and severe nonproliferative diabetic retinopathy (NPDR), which is characterized by vascular closure, to proliferative diabetic retinopathy (PDR), which is characterized by the formation of new blood vessels on the retina and the posterior surface of the vitreous (13).

Macular edema, which is defined as retinal swelling caused by leaky blood vessels, can occur at any stage of retinopathy. These changes can be accelerated by pregnancy, puberty, blood sugar management, hypertension, and cataract surgery(14).

During the first 20 years after the development of diabetes, nearly all people with type 1 diabetes and 60% of people with type 2 diabetes will develop retinopathy(14).

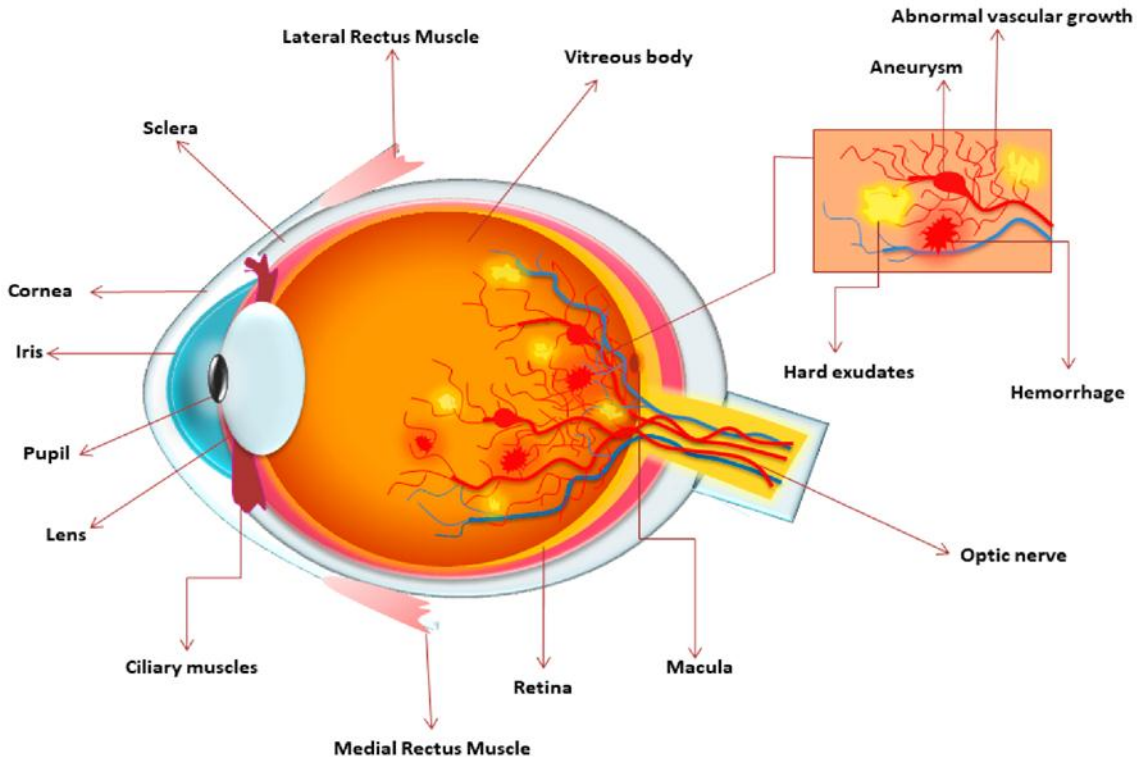


Figure 1-1 Anatomy of complications faced such as retinal vessel hemorrhage and microaneurysms, abnormal vascular development on the retinal surface(15).

Diabetic eye disease manifests itself in a variety of ways, the most common of which are cataracts and diabetic retinopathy (DR). Diabetics are 25 times more likely than the general population to become blind. Diabetic eye disease is the primary cause of blindness in adults under 75 years old in developed countries(13).

DR, on the other hand, is frequently undiscovered until it has progressed to the stage where it poses a serious threat to one's eyesight. Because of low adherence and access to retina screening visits, the current state of DR screening in the real world, which is based on a retina specialist or trained grader assessing color fundus photographs (CFPs), leaves a large proportion of patients undiagnosed and thus receiving medical help too late(16,17). Long-term diabetes, poor glycemic control, and poorly controlled hypertension are all related with the progression and development of DR. (15)(18).

1.2.1 Epidemiology and Prevalence of DR

1.2.2 Global Prevalence

Diabetic retinopathy (DR) is on track to become the primary global cause of vision loss in many nations as the incidence of diabetes mellitus rises and people with diabetes live longer lives(19). In 2010, more than one-third of the world's 285 million diabetics had indications of DR, and a third of these had vision-threatening retinopathy, defined as severe non-proliferative DR or diabetic macular edema (DME)(20). number of people with diabetic retinopathy will increase from 127 million in 2010 to 191 million by 2030(8).

DR accounts for 4.8% of global blindness incidences, according to the World Health Organization (WHO) (37 million).

The most recent estimates of DR prevalence among DM subjects come from a study that pooled data from 35 studies and reported a global prevalence of 34.6% for any DR, with DR risk rising with duration of diabetes(20).

The overall prevalence of any DR higher in those with type 1 77.3% than type 2 25.1% diabetes and increased with duration of diabetes, and values for HbA1c, blood pressure, and cholesterol. according to a pooled study of 22896 adults with diabetes from 35 population-based studies in the United States, Australia, Europe, and Asia as show in figure 1-2 (21,22).

1.2.3 Region Prevalence (Middle East)

Six of the 10 countries with the highest prevalence of diabetes in the world are in the Middle East (Bahrain 25.8%, Kuwait 40%, Iran 29.6%, Jordan up to 64%, Saudi Arabia 19.7%, and Iraq 37%) (23).

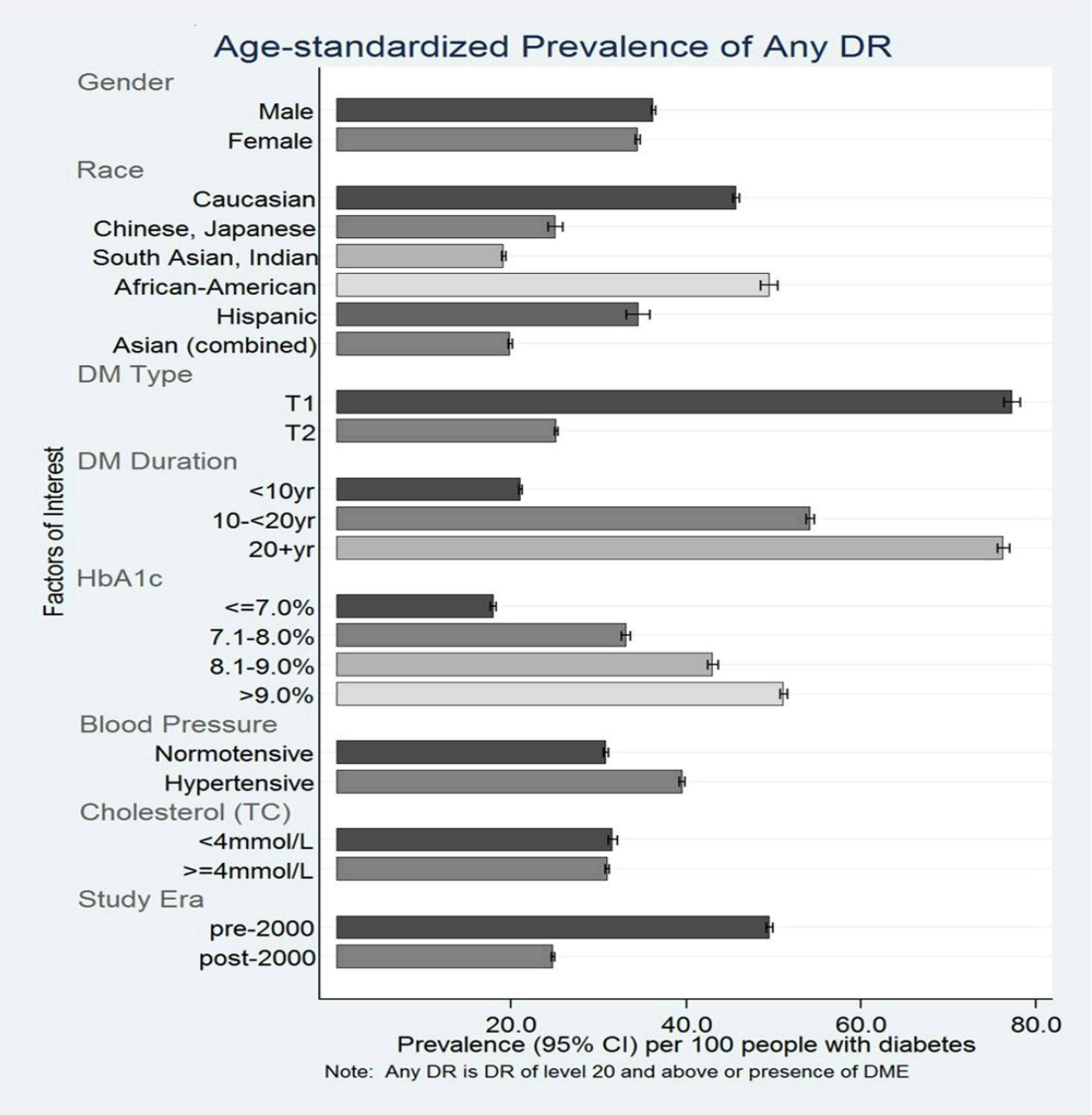


Figure 1-2. Prevalence of any diabetic retinopathy by subgroups of interest(24).

1.2.4 Classification of Diabetic Retinopathy

Progression of DR is related to abnormalities of the retinal microvasculature, including permeability of the blood- retinal barrier (BRB), vascular endothelial cell and pericyte loss, thickening of the vascular basement membrane, subsequent occlusion of capillaries, and retinal neuronal and glial abnormalities(25) .

The pathogenesis of DR is typically asymptomatic, which means that by the time patients detect a loss of vision, the disease may be far progressed. As a result, screening is required to determine the existence and development of the illness(26).

Based on the findings of the multicenter Early Treatment Diabetic Retinopathy Study, the most generally used classification in daily clinical practice to define the DR development phase is (ETDRS)(26,27).

Based on the existence of apparent ophthalmological alterations and the appearance of retinal neovascularization, DR may be classified as Nonproliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR), according to this study. When DR affects the macula, it is termed diabetic macular oedema (DME) and is the commonest cause of blindness in diabetic patients(28,29).

1.2.4.1 Nonproliferative Diabetic Retinopathy

The initial stage of DR, known as NPDR, is characterized by(30):

1- Microaneurysms, which are characterized by the dilatation of microvessels, are the earliest clinically apparent alterations in DR

2- Hemorrhages develop when weaker capillaries burst. They might appear as little dots or bigger blot hemorrhages in the retina's tightly packed deeper layers.

3- Hard exudates consist of lipoproteins and other proteins leaking through abnormal retinal vessels. Lipoproteins and other proteins spilling from damaged retinal vessels make up hard exudates. They appear as waxy or glossy yellow lipid deposits that form a circinate pattern around the foci of leaky capillaries and microaneurysms.

4- Cotton wool patches occur as white lesions with hazy edges in advanced NPDR stages due to vascular occlusion(31).

Depending on the quantity and severity of these alterations, the NPDR can be classified as mild, moderate, severe, and very severe (Figure 1-3) (28,32,33).

International Clinical Diabetic Retinopathy Disease Severity scale, has developed an easily understandable scale to classify NPDR(34).

This scale is based on findings observed upon dilated ophthalmoscopy, which includes no apparent retinopathy - no abnormalities, mild NPDR - microaneurysms only, moderate NPDR - more than just microaneurysms but less than severe NPDR and severe NPDR includes any of the following such as 20 intraretinal hemorrhages in each of four quadrants, definite venous beading in two or more quadrants, prominent IRMA in one or more quadrants and no signs of PDR(35,36).

1.2.4.2 Proliferative Diabetic Retinopathy

Proliferative Diabetic Retinopathy is the most advanced stage of DR, and it occurs when there is a significant obstruction of the blood veins in the retina. The neovascularization process is triggered by ischaemic stimuli. However, new vessels are leaky, fragile, and misdirected and cause poor retinal blood flow (31,37).

The higher metabolic requirement of retinal tissue poses the need for neovascularization which is due to the release of angiogenic signals. Retinal detachment and this neovascularization with the proliferation of the fibrovascular tissue is a characteristic of the PDR stage(38).

Early Treatment Diabetic Retinopathy Study also has classified PDR into early PDR and high-risk PDR, Patients with early PDR had a 75% chance of acquiring high-risk PDR in 5 years (Figure 1-3). (37,39).

Proliferative Diabetic Retinopathy occurs as hypoperfusion in the retinal capillary bed gets more severe and extends over the retinal region. Neovascularization develops at the papilla (neovascularization of the disk, NVD) and on the retina outside the papilla in response to ischemia (neovascularization elsewhere, NVE)(40).

The formation of new vessels at the papilla, on the retina, and, lastly, on the iris (neovascularization of the iris, NVI) might be regarded of as a futile attempt to compensate for ischemia. Epiretinal and subhyaloid vitreous hemorrhages can result from papillary and retinal neovascularization, which can be arranged as membranes and cords on the retinal surface(41).

1.2.4.3 Diabetic Macular Edema

Diabetic Macular Edema (DME) is a complication of diabetic retinopathy (DR) caused by the breakdown of outer BRB and the buildup of intracellular and extracellular fluid inside the macular retina(30). DME is the most prevalent cause of blindness among diabetics, and while it can develop at any stage of the illness, it is more likely in the later stages(12,42).

Diabetic macular edema is retinal thickening within two disc diameters of the center of macula. DME patients were categorized into clinically significant macular edema (CSME) or non-CSME by ETDRS. CSME includes any one of the following lesions:

1. Retinal thickening at or within 500 microns from the center of macula.

2. Hard exudates at or within 500 microns from the center of macula associated with thickening of the adjacent retina.

3. An area or areas of retinal thickening at least one-disc area in size, at least a part of which is within one-disc diameter of the center of macula(43).

Mild NPDR	At least one microaneurysm Fewer intraretinal dot or blot haemorrhages	Have only a 5% risk of progressing to PDR within 1 year and only a 15% risk of progressing to high-risk PDR
Moderate NPDR	Microaneurysms and/or intraretinal haemorrhages present in fewer than 4 quadrants of the retina Presence of cotton wool spots, venous beading, and intraretinal microvascular abnormalities	Have a 12-27% risk of progressing to PDR within 1 year and a 33% 5-year risk of progressing to PDR
Severe NPDR	Patients have one of this elements, known as "4-2-1 rule": (i) 4 quadrants of intraretinal haemorrhages or microaneurysms (ii) 2 quadrants of significant venous beading (iii) 1 quadrant of intraretinal microvascular abnormalities	Have a 52% risk of progressing to PDR within 1 year and a 60% 5-year risk of progressing to PDR
Very severe NPDR	Patients have at least two of the elements of the "4-2-1rule"	Have a 75% risk of progressing to PDR within 1 year
Early PDR	Presence of new vessels	
High-risk PDR	Have one of this elements: (i) Neovascularization of disc > 1/3rd to 1/4th disc diameter (ii) NVD <1/3rd to 1/4th disc diameter with vitreous/pre-retinal haemorrhage (iii) Neovascularization with vitreous/pre-retinal haemorrhage	Have a high risk of severe visual loss

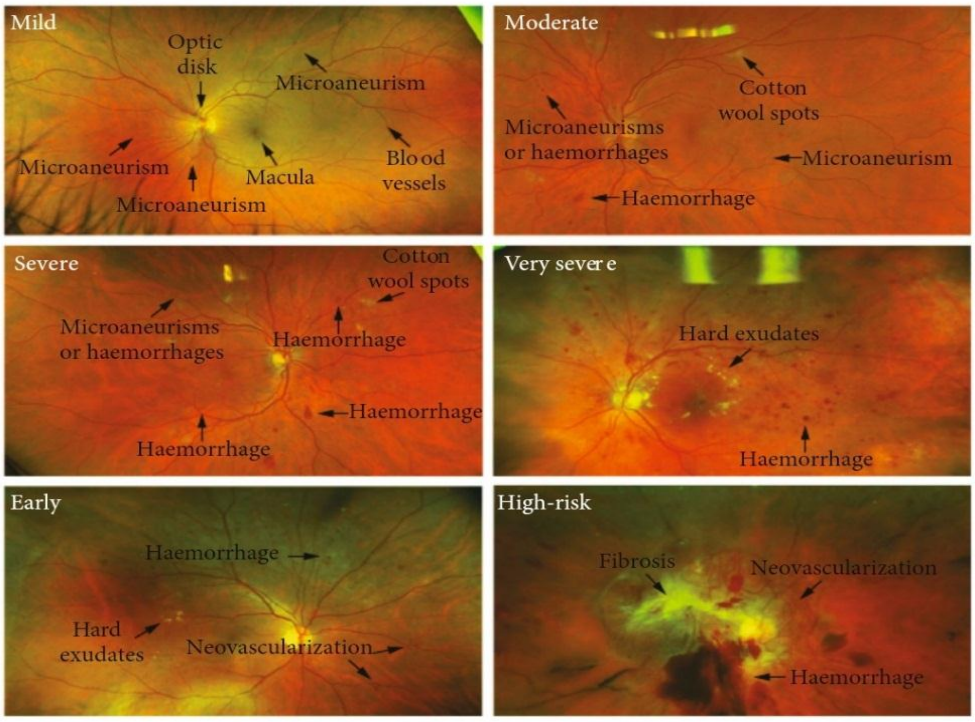


Figure 1-3. From early background DR to PDR and fibrotic tissue, clinical DR progression and fundus images(37).

1.2.5 Etiology and Pathogenesis of Diabetic Retinopathy DR

The cause of DR is unknown. It is a multifactorial illness with a complicated etiology. The precise mechanisms by which elevated blood glucose levels cause diabetic complications are unknown. Regardless, hyperglycemia is known to have metabolic consequences on the retina, causing microvascular damage(44).

Diabetic retinopathy is a retinal microangiopathy. It involves changes in the vascular wall as well as the blood's rheological characteristics. Capillary occlusion is caused by a combination of these conditions, resulting in retinal ischemia and angiographically visible leaking. The loss of pericytes and endothelial cells, as well as the thickening of the basilar membrane, are characteristic histological abnormalities. Microaneurysms, or areas of capillary wall inflating outward, are pathognomonic(45).

In addition, pericyte loss and damage to the endothelium leads to occlusion of capillaries and local ischemia, which activates hypoxia-inducible factor 1 (HIF-1). Subsequently, activation of this factor further increases the expression of the vascular endothelial growth factor (VEGF). Along with the other angiogenic factors Ang-1 and Ang-2, VEGF induces vascular permeability (15).

At the end stage of pathology, neovascularization and neurodegeneration play a crucial role, Neurodegeneration is a result of hyperglycemia-induced downregulation of several vital neurotrophic factors, including NGF (nerve growth factor), PEDF (pigment epithelium-derived factor), IRBP (interphotoreceptor retinoid-binding protein) and somatostatin, whereas neovascularization involves the upregulation of the pro-angiogenic factors VEGF, Ang-1, Ang-2), as well as platelet-derived growth factor (PDGF) and vasoactive hepatocyte growth factors.

Neovascularization generates delicate and permeable blood vessels that are conducive in vitreous hemorrhage. The repetition of such hemorrhages leads to the formation of fibrovascular scars and gliosis, the contraction of which prompts sight-threatening endpoints, namely, PDR and DME (46,47).

1.2.6 Risk factors for progression of diabetic retinopathy

The risk factors for DR may be split into two categories: modifiable and non-modifiable. Hyperglycemia, hypertension, hyperlipidemia, and obesity are all modifiable risk factors. Non-modifiable risk factors for DR onset and progression include diabetes duration, puberty, and pregnancy.

1.2.6.1 modifiable risk factors

1.2.6.1.1 Hyperglycemia

The most common cause of DR is hyperglycemia. The "Diabetes Control and Complications Trial" (DCCT) and "The UK Prospective Diabetes Study" (UKPDS) are two important clinical trials that have revealed a tight link between blood glucose levels and the development and progression of DR. The DCCT was released in 1993 and included data from 1,441 people with type 1 diabetes (T1DM) from 1982 to 1993. When compared to standard treatment, intense hyperglycemia treatment reduced the incidence of DR by 76% and DR progression by 54% in this clinical research(48).

In patients with diabetes, a 1% decrease in HbA1c resulted in a 40% reduction in DR development, a 25% advancement to VTDR, a 25% requirement for laser therapy, and a 15% reduction in blindness(48,49)

Hyperglycemia-induced changes in biochemical pathways, such as increased flux of advanced glycation end products/receptors (AGE/RAGE), polyol pathway, protein kinase C (PKC) activation, and

hexosamine pathway, produce oxidative stress, which causes the rupture of the BRB, pericytes' demise, and increased vascular permeability, which leads to progression to advanced DR stages and the development of diabetes show in figure (1-4) (44,50,51).

Dilatation of blood vessels and alterations in blood flow are the first responses of the retinal blood vessels to hyperglycemia. Abnormal proliferation and permeability of microcirculatory vessels are pathological features of diabetic retinopathy illness. Arterioles, capillaries, and venules are the microvascular bed's smallest functional units. Microvessels, unlike macrovessels, have specific roles in oxygen and micronutrient transport. Main microcirculatory features include permeability to small molecules, regulation of physical dimensions, and functional properties of the basement membrane, which vary in different types of microvascular beds such as the glomeruli, retina, myocardium, skin, and muscle(46,52).

In diabetic patients, these modifications are considered to represent a metabolic autoregulation to increase retinal metabolism(53).

Another symptom of DR's early stages is pericyte loss. Pericyte apoptosis mediated by excessive hyperglycemia has been demonstrated in both in vitro and in vivo experiments(54). Since pericytes provide structural support for capillaries, their loss results in localized outpouching of capillary walls. This process is linked to the production of microaneurysms, which is the first clinical symptom of DR(55).

During the pathophysiology of DR, pericyte loss is accompanied by endothelial cell death and basement membrane thickening, all of which contribute to the BRB's dysfunction(56). In addition, a significant loss of pericytes and endothelial cells causes capillary occlusion and ischemia.

Through the activation of hypoxia-inducible factor 1, retinal ischemia/hypoxia causes VEGF overexpression (HIF-1)(57).

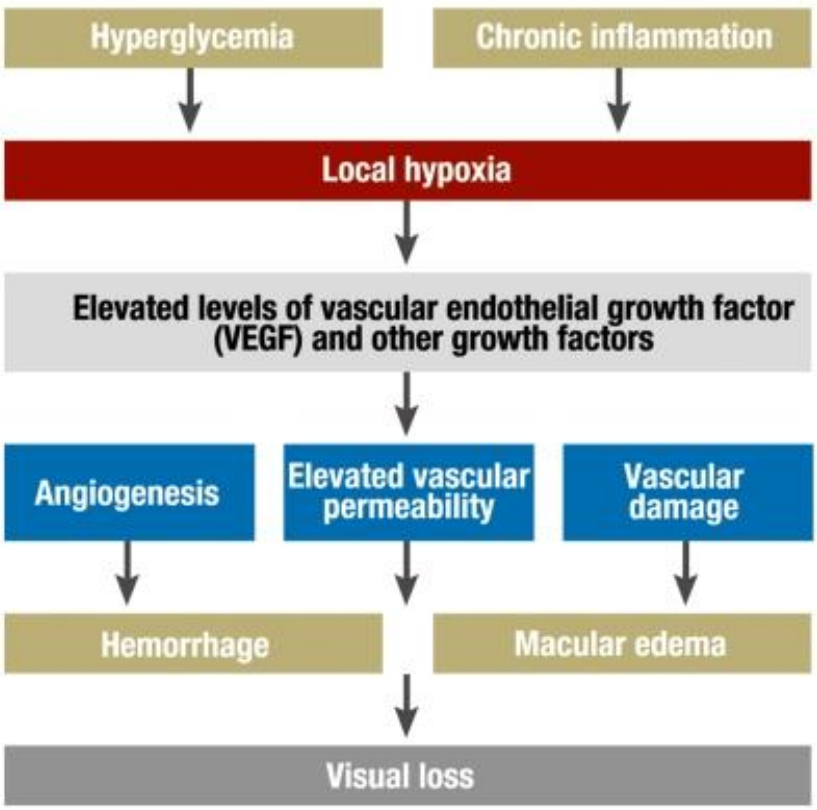


Figure 1-4 Schematic flow- chart for the pathogenesis of diabetic retinopathy(45)**.**

The elevated of phospholipase A2 (PLA2) levels in diabetics cause VEGF overexpression. The phosphorylation of tight junction proteins such as occludin and zonula occludens-1 (ZO-1) by VEGF, a crucial component in the development of PDR and DME, is thought to promote vascular permeability. Furthermore, VEGF increases endothelial cell proliferation by activating mitogen-activated protein (MAP) as an angiogenic agent. The retina of diabetic mice, as well as the vitreous of people with DME and PDR, showed increased VEGF expression(58–60).

1.2.6.1.2 Inflammation

The pathophysiology of DR is complicated by inflammation. In both diabetic animal models and people, chronic low-grade inflammation has been detected at various stages of the disease(15,54,61).

Inflammation is a protective process triggered by injury or stress and mediated by the host immune system. Different and varied stimuli, such as hyperglycemia, growth factors, advanced glycation end products (AGEs), high levels of circulating or vitreous cytokines and chemokines, and reactive oxygen species, can stimulate the inflammatory response of the retinal vasculature (ROS)(62).

Acute inflammation has a number of positive effects, including tissue defense and healing, but chronic inflammation causes structural and molecular changes in the retina, which frequently end in tissue damage and cell death(63).

1.2.6.1.3 Hypertension

Hypertension is a key modifiable risk factor for DR, according to epidemiological research and several clinical trials(48). Each 10-mmHg rise in systolic blood pressure is linked to a 10% increase in the risk of early DR and a 15% increase in the risk of PDR or DME(64–66).

Among the UKPDS, individuals with hypertension who maintained strict blood pressure management had a 37% lower risk of microvascular disease, a 34% lower rate of retinopathy development, and a 47% lower rate of visual acuity deterioration in adults with type 2 diabetes(48). These advantages, however, will not last if blood pressure regulation is not maintained on a long-term basis(67).

Specific blood pressure-lowering drugs that target the renin-angiotensin system (RAS) may also have extra positive benefits on retinopathy, irrespective of their absolute hypotensive effects, according to some clinical investigations. Candesartan, an angiotensin II receptor

blocker, reduced the incidence of retinopathy by two or more steps on the Early Treatment Diabetic Retinopathy Study (ETDRS) scale by 18% or by three or more steps by 35% in type 1 diabetes, and increased retinopathy regression by 34% percent in type 2 diabetes, according to the multicenter Diabetic Retinopathy Candesartan Trials (DIRECT)(68–70).

For individuals with normotensive and normoalbuminuric cases, the EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus found that Lisinopril, an angiotensin converting enzyme inhibitor, might reduce DR development by 50% in the first year and 80% in the second year. The baseline differences in the glycaemic levels of the treatment and control groups, however, complicated this study. Furthermore, neither the UKPDS nor the Appropriate Blood Pressure Control in Diabetes (ABCD) research found that angiotensin-converting enzyme inhibitors were superior to other antihypertensive drugs in preventing DR development(63,69).

However, the improvements were only seen in people with early retinopathy, and there was no influence on the incidence or development of DME. Enalapril and losartan decreased the incidence of retinopathy development in type 1 diabetic patients by 65 percent and 70 percent, respectively, in the Renin-Angiotensin System Study (RASS), regardless of blood pressure changes during the course of the study. This shows that the effect on DR risk reduction isn't mediated by a hypertension effect(19,69).

1.2.6.1.4 Hyperlipidemia

dyslipidemia may have a role in the development of DR, results from population-based research and clinical trials are mixed(71,72). There hasn't been a single lipid metric reliably linked to DR. In the Chennai Urban Rural Epidemiology Study (CURES), total cholesterol was an independent risk

factor for DR, however it was protective against DR in the Singapore Malay Eye Study cohort(73).

The severity of retinopathy was connected with rising triglycerides and inversely associated with HDL cholesterol in type 1 diabetes, but not with total cholesterol levels. The Multi-Ethnic Study of Atherosclerosis (MESA), on the other hand, found no link between serum lipids and DR(74). Triglycerides were shown to be linked to the presence of DR in the lipid panel, while low-density lipoprotein was linked to DME(73).

More recently, a subgroup of the Action to Control Cardiovascular Risk in Diabetes (ACCORD-Eye) study found that fenofibrate reduced the rate of DR progression (6.5% vs. 10.2% with placebo) by at least three steps on the ETDRS Severity Scale after four years in patients with type 2 diabetes who were also taking simvastatin, adding to the growing body of evidence supporting fenofibrate's beneficial effect on DR progression(75)[84] [85].

1.2.6.2 Non-modifiable risk factors

1.2.6.2.1 Diabetes Duration

Diabetes duration is probably the best predictor of retinopathy development and progression. In the WESDR, the prevalence of any retinopathy was 8 percent at 3 years, 25% at 5 years, 60% at 10 years, and 80 percent at 15 years among younger-onset diabetes patients. At three years, the prevalence of PDR was 0%, but by 15 years, it had risen to 25% (46,78). With the passage of time, the incidence of retinopathy rose as well. In the WESDR younger-onset group, the 4-year incidence of developing proliferative retinopathy rose from 0% in the first 5 years to 27.9% in years 13–14 of diabetes. The risk of acquiring PDR has remained consistent during the last 15 years(24,78).

1.2.6.2.2 Puberty and Pregnancy

DR, especially in T1DM, can deteriorate quickly between puberty and pregnancy. When comparing the period before and after menarche in WESDR, there was a 30% increased chance of getting DR. Subjects with T1DM identified after puberty had a considerably shorter time to acquire DR than those diagnosed before puberty(79,80).

Progression of DR in pregnancy was observed to be below in individuals with Cataracts who did not have DR at baseline. 47 percent of individuals with NPDR advanced to a more severe spectrum, with 50% requiring laser therapy.

In the postpartum period, 29% of women had DR regression. As a result, non-mydriatic photography should be used to diagnose T1DM after puberty, early-onset T2DM throughout childhood, and pregnancy. Early pan-retinal photocoagulation therapy and attentive monitoring throughout pregnancy and the postpartum period may assist pregnant women with PDR(81).

1.2.7 Screening for DR

Due to the fact that DR can proceed irreversibly with only a few visual symptoms, all diabetic patients should have their eyes screened and treated as soon as possible. Regular dilated eye exams have long been known to be beneficial in diagnosing and treating vision-threatening DR that is asymptomatic(74). According to the WESDR, diabetic retinopathy screening should be done at the time of diagnosis and then every year after that in those with type 2 diabetes(82).

In practice, eye exams for persons with diabetes are frequently personalized in terms of scheduling and frequency(74). Even in the absence of retinopathy, evaluation at least once a year is indicated in high-risk individuals (e.g., those with diabetes for 10 years or more and on insulin, or those with a poor systemic risk factor profile)(83). Pregnant women with non- gestational diabetes should have a thorough eye examination during the first trimester, with follow-up throughout the pregnancy if retinopathy is present. Regular eye exams may also be beneficial to the management of diabetic patients due to their positive psychological benefits (e.g., education about risk factors, compliance)(84).

A - Ophthalmoscopy

Ophthalmoscopy is the most commonly used technique to screen for DR. When performed by an ophthalmologist, the specificity of direct and indirect ophthalmoscopy was high, but the sensitivity was low (34-50%), particularly for early retinopathy, in comparison to 7-field stereo photographic assessment(40).

B - Digital imaging

Digital imaging makes fundus photography easier and more widely accessible. It may be used to obtain fundus images through non-dilated pupils. Mydriasis is usually necessary in older patients. Single-field fundus photography with interpretation by trained readers could serve as a screening tool to identify patients with DR(41).

1.3 Pentosidine

Pentosidine is a biomarker for advanced glycation end products (AGEs). This broad family of chemicals has a well-characterized and easily detectable member(90).

Pentosidine, which is derived from the pentose ribose, creates fluorescent cross-links between arginine and lysine residues in collagen. It is made when amino acids react with the Maillard reaction products of ribose as show in figure 1-5. It is valuable for detecting accumulated damage to proteins (advanced glycation endproducts) caused by non-enzymatic browning interactions with carbs, despite the fact that it is only present in trace amounts among tissue proteins(86).

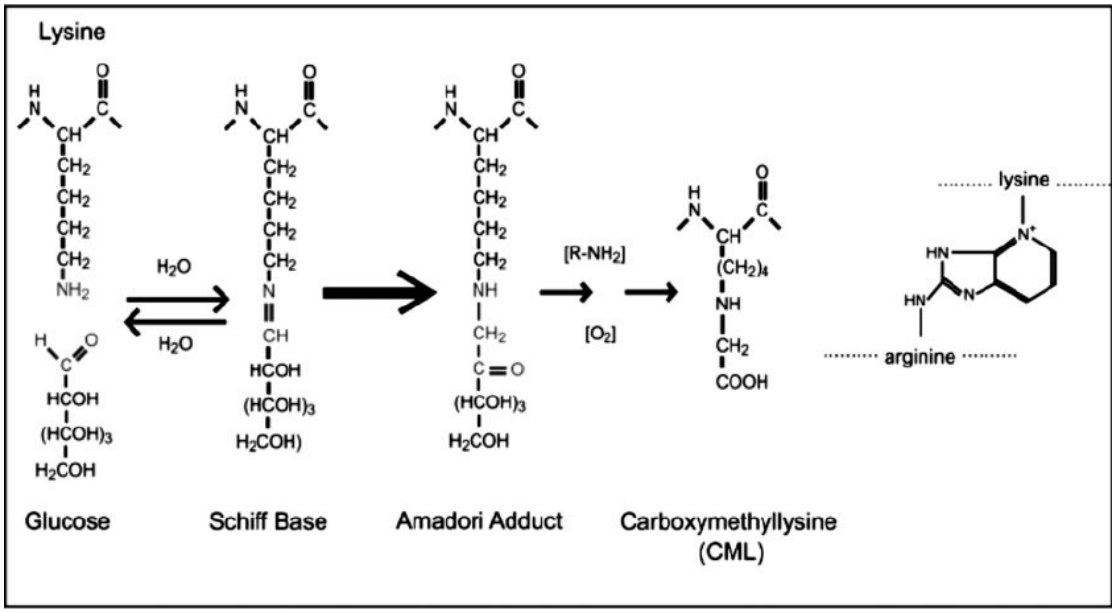


Figure 1-5 Steps leading to the development of advanced glycation end products (AGEs) for N-carboxymethyllysine (92).

Advanced glycation end products (AGEs) are nonenzymatically glycated and oxidized proteins or lipids that come into contact with aldose sugars. Schiff bases and Amadori products are formed as a result of early glycation and oxidation reactions. AGEs are formed when proteins and lipids are further glycated, causing molecular rearrangements(93).

Advanced glycation end products (AGEs) are sugar-derived changes that may have a role in the pathophysiology of age-related illnesses affecting connective tissue, lens, blood vessels, and neurons. Because

tissue damage occurs in diabetes, nearly all in vivo investigations of AGEs have focused on diabetes rather than age-related illnesses(90).

Sugar concentration and protein turnover rate both impact the formation of AGEs. As a result, certain proteins that reach critical levels of AGE modification in areas where diabetes issues occur may flip over too quickly for typical blood glucose levels to cause functional defects, whereas proteins with a longer half-life would be damaged over a longer time(85–87).

1.3.2 Pentosidine in Diabetic Retinopathy

Pentosidine play a key role in the evolution of diabetic retinopathy, since they cause retinal cell malfunction and death(94). The AGE-RAGE axis' various components, including signal transduction, ligand synthesis, and end-point effectors, might represent interesting targets for diabetic retinopathy therapy(95).

The AGE-RAGE interaction appears to play a key role in diabetic retinopathy's long-term inflammation, neurodegeneration, and retinal microvascular dysfunction. According to studies, increased AGE generation in the vitreous may play a role in the development of diabetic retinopathy by increasing bFGF (basic fibroblast growth factor) synthesis by retinal Müller cells(96).

The production of AGEs from the interaction of sugars and carbonyl compounds is very susceptible to the proteins of the human eye, according to studies. AGEs build up over time in the lens and retina, causing vision problems(97).

AGE has been found in the walls of retinal blood vessels, which contributes to vascular obstruction and increased permeability of retinal endothelial cells, resulting in vascular leakage. AGE crosslinking of

proteins in the arterial wall enhances vascular stiffness, while ECM protein alteration reduces pericyte adhesion(98,99).

Binding to RAGE by AGE-modified extracellular proteins also causes retinal damage. Binding to RAGE triggers a series of signaling events that result in increased oxidative stress and the production of local growth factors, cytokines, and adhesion molecules, as well as increased oxidative stress. Ages are harmful to AGE receptors with pericytes, and diabetic retinopathy has been linked to pericyte destruction (100,101).

1.4 Leptin

Leptin is a 16-kD (167-amino acid) a peptide hormone generated mostly by adipocytes, while it is also produced in smaller amounts by other tissues and organs such as the mammary gland, ovary, skeletal muscle, stomach, pituitary gland, and lymphoid tissue, perhaps for local function(102). Leptin is secreted in proportion to the amount of adipose tissue, making it a useful indicator of energy storage. Leptin is pulsatile released into the circulation in humans, and leptin concentrations have a circadian rhythm. Sleep patterns influence levels, with the peak levels occurring between midnight and early morning and the lowest levels occurring in the early to mid-afternoon.(103).

Obese and lean people have comparable pulsatile leptin secretion patterns, but obese people have larger pulse amplitudes and overall higher leptin concentrations than lean people because they have more body fat(104–106).

Women's leptin levels may be greater during the luteal phase of the menstrual cycle, but menopause is associated with a drop in circulating leptin levels. Leptin levels rise as a result of renal failure. These findings show that, in addition to variations in body fat mass and/or distribution, sex

differences in leptin concentration are likely the result of differences in sex hormones, such as estrogen and testosterone(107–110).

Leptin is an adipose-derived endocrine signal that acts on hypothalamic receptors to induce satiety, improve lipid metabolism, and increase energy expenditure(111). Leptin regulates feeding via binding to cell receptors in the arcuate and ventromedial nuclei, as well as other areas of the hypothalamus and dopaminergic neurons in the ventral tegmental area(112).

The fundamental function of the hormone leptin is to regulate adipose tissue mass via effects on appetite, dietary energy usage, physical activity, and energy balance mediated by the central hypothalamus. Leptin's secondary activities are control of energy expenditure, modulation of fetal and maternal metabolism, and those of a permissive factor in puberty, activator of immune cells, activator of beta islet cells, and growth factor outside of the brain, in the periphery of the body(105,113,114).

1.4.1 Leptin in diabetes mllitus and DR

Serum levels of leptin are increased in some diabetic patients, and leptin resistance has been linked to the disease's development and therapy. Leptin regulates insulin synthesis in human pancreatic cells, and blood insulin levels in diabetic people and experimental animals correlate with leptin levels(115). It's also been suggested that leptin has a function in insulin resistance(116,117).

Endothelial cells, fibroblasts, and the retinal pigment epithelium have all been found to carry leptin receptors. Leptin has been proven in recent research to act as an angiogenic factor in vivo, causing endothelial cells to proliferate and migrate. It's also found in higher concentrations in the plasma of people with retinopathy caused by hypertension or diabetes.

Blood serum and vitreous leptin levels have been linked to neovascular eye diseases like proliferative diabetic retinopathy(115,118).

1.5 Adeponectine

Adeponectine the most abundant circulating protein (30 kDa) synthesized only in adipose tissue and acting as a hormone with anti-inflammatory, anti-diabetic, and insulin-sensitizing properties, is known to play an important role in various metabolic processes, including glucose control and fatty acid catabolism, among the numerous adipocytokines(119).

The APM1 gene is the encoding gene for adiponectin (also known as GBP28). APM1 is located on chromosome 3q27, a genetic region linked to diabetes and metabolic syndrome risk(120).

Adiponectin increases glucose uptake in the liver and muscle while decreasing gluconeogenesis in the liver. (121).

Adiponectin levels are notably lower in obese individuals than in lean adults, in contrast to most adipose-related cytokines (leptin, adipsin, resistin, IL-6, etc). Furthermore, it's worth noting that women's adiponectin plasma concentrations were discovered to be substantially greater than men's[131] [132].

1.5.1 Adeponectine Functions

Proliferation, inflammation, and oxidative stress are among biological processes in which adiponectin plays a role. It promotes GLUT-4 translocation in muscle cells, stimulates glucose and fatty acid metabolism, and lowers glucose synthesis in the liver(124–127).

Adiponectin's anti-inflammatory characteristics have been linked to a number of pathways, including its ability to increase IL-10 production and

enhance macrophage polarization toward the anti-inflammatory M2 phenotype. -induce the anti-inflammatory cytokine IL-10 in M2 macrophages; -reduce the expression of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-12(128,129).

Adiponectin is an immunomodulator that targets the immune system directly. Adiponectin works as an immune suppressor molecule, lowering T cell responsiveness, B cell lymphopoiesis, and TNF-production, as well as suppressing macrophage activation and proliferation, according to various in vitro studies(130).

1.5.2 Adeponectine in diabetes mellitus and DR

Diabetes complications It is difficult to correlate APN levels with diabetic retinopathy or other diabetes complications since elevated APN may be a positive and compensating response. Elevated APN levels are linked to retinopathy and nephropathy in type 1 diabetic individuals(131).

In type 2 diabetic patients, high aqueous humor APN levels are observed with proliferative diabetic retinopathy versus non-diabetic controls, possibly due to increased permeability of the blood-retinal-barrier with diabetic retinopathy progression.

Much lower brain and aqueous humor APN levels are reported compared with circulating APN levels, suggesting a role of the blood brain barrier and blood retinal barrier in APN homeostasis. There is also the possibility of induction of local APN expression in the diabetic retina(132).

APN is involved in systemic lipid metabolism, and APN deficiency reduces the expression of important lipogenic genes in the liver. The systemic lipid profile may be impacted by APN, which may then have an effect on the structure and function of the retinal vasculature(133).

Although some reports indicate that high serum APN levels correlate with the severity of diabetic retinopathy progression especially in the proliferative phase, there is increasing evidence suggesting that APN improves insulin-resistance and supports vascular maintenance indicating a potentially protective role of APN in diabetic retinopathy in type 2 diabetic patients. However, there is limited knowledge of the effect of administering APN in diabetic retinopathy(134).

1.6 TNF-α

TNF- α also known as cachectin, is a powerful pro-inflammatory cytokine that is expressed as a 26 kDa membrane-bound protein that is then cleaved by TNF-converting enzyme (TACE) to release a soluble 17 kDa monomer that forms homotrimers in circulation(135). It was first discovered in the serum of endotoxin-treated mice as a mediator of transplantable tumor necrosis. TNF-α is cytocidal or cytostatic for some tumor cells in tissue culture, according to subsequent research(136). Furthermore, new research suggests that TNF-α may have a role in either the pathogenesis of infection, tissue injury, and inflammation or as a helpful mediator in host defense, immunology, and tissue homeostasis(137,138).

TNF is not normally detectable in healthy people, but it is discovered at higher serum and tissue levels in inflammatory and infectious diseases, and serum levels are linked to infection severity(139,140).

Although monocyte/macrophage cells are the primary producers of TNF in inflammatory diseases, TNF can also be produced by mast cells, T and B lymphocytes, natural killer (NK) cells, neutrophils, endothelial cells, smooth and cardiac muscle cells, fibroblasts, and osteoclasts.(141,142).

Pro-TNF is a protein that is produced on the plasma membrane and may be cleaved in the extracellular domain by matrix metalloproteinases, resulting in the release of a soluble 17 kDA form(143).

1.6.1 Role of TNF-α in Diabetic Retinopathy

TNF-alpha is a pro-inflammatory cytokine linked to insulin resistance and chronic inflammation. The molecular mechanisms of TNF-alpha function have recently been extensively studied. TNF-alpha levels in the blood have been found to be higher in both animals and humans in numerous studies(144,145).

TNF- α levels in diabetes plasma have been demonstrated to promote leukocyte-endothelial cell adhesion. TNF-α promotes angiogenesis in vivo, according to research. TNF- α is also essential for VEGF-induced endothelial hyper permeability[156][158].

TNF-alpha is a key mediator of the retinal vasculature's leukocytosis generated by VEGF, interleukin-1 alpha, and platelet-activating factor, as well as cell death/apoptosis in retinal neurons and vascular endothelial cells in DR. These findings imply that TNF-alpha-mediated retinal leukocytosis and apoptosis contribute to the breakdown of the blood-retinal barrier (BRB) in DR. Patients with proliferative diabetic retinopathy had higher levels of soluble TNF receptors in their serum and vitreous, according to previous research(149–152).

These findings suggest a relationship between inflammation and insulin resistance, which is supported by evidence that TNF-alpha suppression reduces pathologic processes associated with the onset of early DR, such as BRB breakdown(153).

1.7 C-Reactive Protein (CRP)

C-reactive protein (CRP) is a ring-shaped pentameric protein found in blood plasma whose levels rise in response to inflammation. It's a hepatic acute-phase protein that rises in response to macrophages and T cells secreting interleukin-6(154).

Tillet and Francis discovered this highly conserved plasma protein in 1930 while researching the sera of patients with acute Pneumococcus infection. It was called after its reaction with the capsular (C)-polysaccharide of Pneumococcus. CRP binds to polysaccharides on bacteria, such as phosphocholine (PCh), and activates C1q, triggering the classical complement cascade of innate immunity. CRP belongs to the pentraxin family, which also contains serum amyloid A and other structurally similar compounds(155–157).

CRP binds to phosphocholine on the surface of dead or dying cells, as well as some microorganisms. This activates the complement system, allowing macrophages to remove necrotic and apoptotic cells and microorganisms through phagocytosis. Increased levels of IL-6, which is produced by macrophages and adipocytes in response to a variety of acute and chronic inflammatory conditions such as bacterial, viral, or fungal infections; rheumatic and other inflammatory diseases; malignancy; and tissue injury and necrosis, cause this so-called acute phase response. Interleukin-6 and other cytokines are released as a result of these circumstances, causing the liver to produce CRP and fibrinogen(158–161).

On microorganisms, CRP binds to phosphocholine. It is thought to aid complement binding to foreign and damaged cells and enhance macrophage phagocytosis (opsonin-mediated phagocytosis) by macrophages that express a CRP receptor. It functions as an early defense system against infections in innate immunity(162).

1.7.1 Role of C-reactive protein in Diabetic Retinopathy

C-reactive protein (CRP) is an inflammatory biomarker linked to endothelial dysfunction and atherogenesis, as well as macrovascular disease and diabetes-related nonocular microvascular complications(156).

On the other hand, there is little information about a potential connection between CRP and DR, and the few studies that have been done have had conflicting outcomes. Higher CRP levels were associated with early and advanced retinopathy in a population-based cohort of 543 persons with type 1 diabetes mellitus (T1DM) and the Hoorn research, which comprised 625 non-diabetics and patients with type 2 diabetes mellitus (T2DM). In a prospective clinic-based trial comprising 328 T2DM participants, a higher CRP level was substantially related with a higher probability of baseline DR but not with DR development throughout a ten-year follow-up(157).

1.8 Glycated hemoglobin (HbA1c)

Glycated hemoglobin is made by adding glucose to the amino groups of hemoglobin without using enzymes. HbA1c is a type of glycated hemoglobin that results from the attachment of glucose to the hemoglobin -chain's N-terminal valine(163).

HbA1c and hemoglobin glycated at places other than the N-terminus of the beta chain, such as epsilon amino groups on lysine residues, are included in total glycated hemoglobin. The concentration of HbA1c is affected by both the amount of glucose in the blood and the erythrocyte's life span. HbA1c shows the integrated glucose concentration over the previous 8–12 weeks since erythrocytes are in the circulation for about 120 days As a result, it is free of the huge variations in blood glucose concentrations that occur on a daily basis(164).

1.9 Erythrocyte Sedimentation Rate (ESR)

The erythrocyte sedimentation rate (also known as the sed rate or ESR) is a popular hematological test that can be used to detect and track an increase in inflammatory activity in the body caused by one or more illnesses such as autoimmune disease, infections, or malignancies. The ESR isn't specific to any one condition; instead, it's utilized in conjunction with other tests to identify whether or not there's been an increase in inflammatory activity(165,166).

1.11 Aim of study

1- Assessment of pentosidine, leptin, adiponectin and TNF-α in the patients with diabetic retinopathy compared with control

2- Estimate the correlation between pentosidine, leptin, adiponectin and TNF-α.

3- Study the correlation between all parameter and diabetic retinopathy severity.

2. Materials and Methods

2.1 Subjects

This study was conducted over a period of 10 months from November 2021 until August 2022. Sample collected from the clinic of ophthalmology in Imam Sadiq Hospital and Ophthalmology center in Hilla city. The particular side of the study was performed at the laboratory of the Biochemistry Department in the College of Medicine / university of Babylon.

2.1.1 Study Design

Present study is designed as case control study

2.1.2 Sample Size

The sample size was determined according to the Fisher formula for sample size (167) which is:

$$n = \frac{Z^2 P(1-P)}{d^2}$$

Where n= sample size

Z= Z statistic for the level of confidence interval 95% which = 1.96.

P= prevalence

The global prevalence of SS is 3% (168).

d= precision (in proportion of one; if 5%, d = 0.05).

n= $(1.96)^2$ *0.04*0.97 / 0.0025 = 45

2.1.3 Study Population

This study including 90 subjects. This subjects were divided into two group, the first group includes 45 patients with diabetic retinopathy and the

second group includes 45 apparently healthy peoples, the age ware ranged between (43-77) years.

2.1.4 Control Group

Forty-five control subjects were involved in this study. All these subjects were apparently healthy, the age ware ranged between (43-77) years.

2.1.3. Patients Group

Forty-five patients with DR who attend the ophthalmology clinic in Imam Sadiq Hospital and private clinic in Hilla city during the study period and met the inclusion and exclusion criteria were involved in this study, these patients included in this study whose age ranged (43-77) years. All patients were pre-diagnosed by the physicians at the ophthalmology center.

2.1.5 Inclusion criteria

All medically previously diagnosed DR patients of any age.

2.1.6 Exclusion criteria

Patients with hypertension.

Patients on steroids treatments.

Autoimmune disease.

Patients with COVD-19

2.1.7 Ethical issues

All subjects involved in this work are informed and the agreement will obtained verbally from each one before the collection of samples. This study was approved by the committee on publication ethics at college of medicine, University of Babylon.

2.1.8 Blood Sampling

Venous blood samples were drawn from diabetic retinopathy and control subjects by using disposable syringe (5 ml) in the sitting position. For mL of blood was obtained from each subject by vein puncture and pushed slowly into two tube (1.5 ml blood in EDTA tube for HbA1c and 2 ml blood in sodium citrate for ESR study and 2.5 ml blood in gel tube for ELISA and RBS study). blood in gel tube allowed to clot at room temperature for 10 -15 minutes and then centrifuged at 2000 xg for approximately 10minutes then serum was obtained and then the blood serum was isolated, split into aliquots an Eppendorf tube stored at (-20°c) until analysis (measure pentosidine, leptin, adeponectine TNF-α and CRP).

2.2 Chemicals

Chemical and kits that were used in this study were listed in table **2-1**:

Table 2-1: Chemical substances used in the study

No	chemical substances	Origin
1	Human Pentosidine ELISA kit	Bioassay Technology Laboratory (china)
2	Human Leptin ELSIA kit	Bioassay Technology Laboratory (china)
3	Human Adeponectine ELISA kit	Bioassay Technology Laboratory (china)
4	Human TNF-α ELISA kit	Bioassay Technology Laboratory (china)
5	Human CRP ELISA kit	Bioassay Technology Laboratory (china)
6	Glucose kit	Biolabo (France)
7	HbA1c	Ichroma (korea)

2.3 Instruments and Equipment's

The instrument that used in this study were showing in the Table **2-2**.

Table 2-2: instruments and equipment's used in this study

No.	Instruments and Materials	Origin
1	Deep Freeze	Angelantoni (Italy)
2	Centrifuge	ROTOFIX 32 A (Germany)
3	ELISA Reader	Biotek, (USA)
4	ELISA Washer	Biotek, (USA)
5	Distiller	GFL, (Germany)
6	Spectrophotometer	DP-303 (japan)
7	Blue and yellow tips	JRL, (Lebanon)
8	Incubator	Fisher Cient, (Germany)
9	Water bath	Grant, (Germany)
10	Micropipettes (5-50µl), (2-20µl),(20-200µl) , (100-1000µl)	Slamed, (Germany)
11	Multichannel micropipette(0-250 µl)	Slamed, (Germany)
12	Printer	Epson, (Indonesia)
13	Test tube with Separating gel	AFCO, (Jordan)
14	Plain tube	China
15	Eppendorf tube (1.5ml)	China
16	Filter papers	China
17	Disposable syringes	Universal ,(china)
18	Volumetric flask, funnel ,beaker	Schoot,(Germany)

2.4 Methods

2.4.1 Determination of Serum Pentosidine Level

Pentosidine level was measured by enzyme linked immunosorbent assay kit. Components of Pentosidine ELISA kits in Table **2-3**.

2.4.1.1 Principle

The Sandwich-ELISA technique is used in this ELISA kit. The plate has been pre-coated with human pentosidine antibody. pentosidine present in the sample was added and binds to antibodies coated on the wells. After removing any unbound substances, a biotinylated human pentosidine antibody was added to wells and binds to pentosidine in the sample. After washing, Streptavidin- Horseradish Peroxidase (HRP) was added to wells and binds to the biotinylated pentosidine antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step. Substrate solution was added to wells and color develops in proportion to the amount of human pentosidine bound. The color development was stopped and the intensity of the color was measured at 450 nm.

Table 2-3: Components of pentosidine ELISA kits.

Components	**Quantity (96T)**
Standard solution (24ng/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

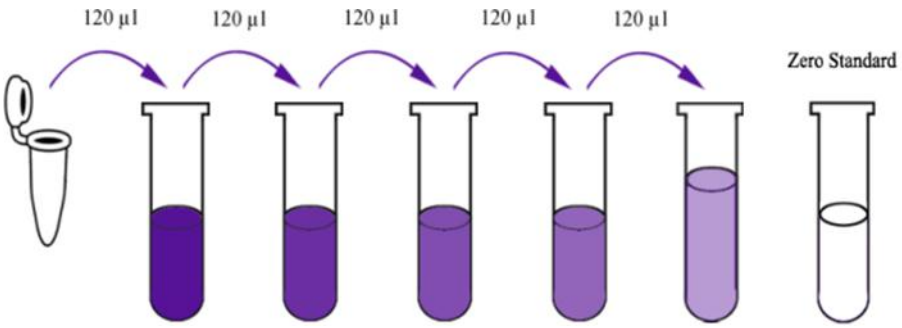
Substrate solution B	6ml x1
Wash buffer Concentrate (25x)	20ml x1
Biotinylated Human PTD antibody	1ml x1
User instruction	1
Plate sealer	2 pics

2.4.1.2 Reagent preparation

A-Before use, all reagents need to be elevated to room temperature.

B-The original standard sample was diluted as the following Table

12ng/mL	Standard No.5	120µl Original standard + 120µl Standard diluent
6ng/mL	Standard No.4	120µul Standard No.5 + 120µl Standard diluent
3ng/mL	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
1.5ng/mL	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
0.75ng/mL	Standard No.1	120µl Standard No.2 + 120µl Standard diluent



Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
24ng/mL	12ng/mL	6ng/mL	3ng/mL	1.5ng/mL	0.75ng/mL

Figure 2-1: Concentration of standards of pentosidine.

C- A volume of 20 ml of concentrated (1x) Wash Buffer were diluted into 500 ml of Wash Buffer concentrate (25x) with deionized or distilled water. When crystals have made in the concentrates, mix gently until the crystals have fully dissolved.

2.4.1.3 Assay Procedure:

1. The assay was performed at room temperature.

2. A volume of 50µL of standard was added to well standard.

3. A volume of 40µL from sample were added and then added 10µL of anti- Pentosidine antibody to sample well, then 50µL of Streptavidin-HRP to both sample and standard wells. Mix well. The over plate covered with sealer and incubated for 60 min at 37 °c.

4.The sealer was eliminated and wash the plate 5 times over with a wash buffer. For each wash, soak wells for 30 sec. to 1 min., with at least 0.35 mL wash buffer. Aspire for automatic washing of all wells, by washing with wash buffer 5 times, filling wells with wash buffer. The plate was blotted into paper towels or other absorbing material.

5. A volume of 50µL substrate solution A added and then 50µL substrate solution B for each wells. The coated plate incubated with new sealer for 10 min. at 37°c in dark media.

6. To each well 50µL of stop solution was added, and the color change from the blue to yellow immediately.

7. The optical density (OD value) of each well identified directly after applying the stop solution by utilize a microplate reader set at 450 nm within 10 min.

Detection range: 0.05 – 20 ng/mL

Sensitivity: 0.025 ng/mL

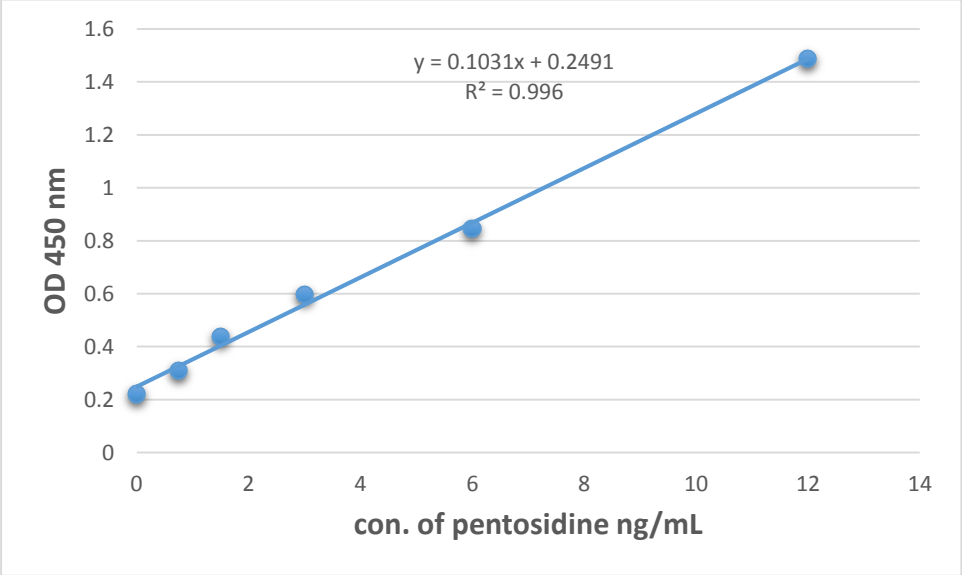


Figure 2-2: Standard Curve of Pentosidine.

2.4.2 Determination of Serum Leptin Level

Leptin level was measured by enzyme linked immunosorbent assay kit. Components of Pentosidine ELISA kits in Table 2-4.

2.4.2.1 Principle

The Sandwich-ELISA technique is used in this ELISA kit. The plate has been pre-coated with human leptin antibody. leptin present in the sample was added and binds to antibodies coated on the wells. After

removing any unbound substances, a biotinylated human leptin antibody was added to wells and binds to leptin in the sample. After washing, Streptavidin- Horseradish Peroxidase (HRP) was added to wells and binds to the biotinylated leptin antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step. Substrate solution was added to wells and color develops in proportion to the amount of human leptin bound. The color development was stopped and the intensity of the color was measured at 450 nm.

Table 2-4: Components of LEPIN ELISA kits

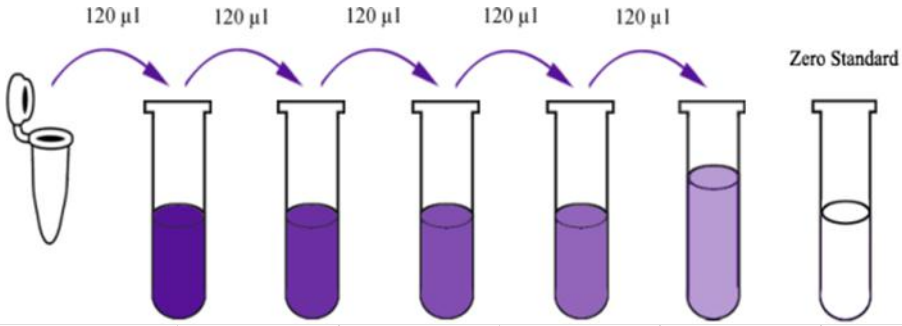
Components	**Quantity (96T)**
Standard solution (12.8ng/L)	0.5ml x1
Pre-coated ELISA plate	12 * 8 well strips x1
Standard diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop solution	6ml x1
Substrate solution A	6ml x1
Substrate solution B	6ml x1
Wash buffer Concentrate (25x)	20ml x1
Biotinylated Human LEP antibody	1ml x1
User instruction	1
Plate sealer	2 pics

2.4.2.2 Reagent preparation

A-Before use, all reagents need to be elevated to room temperature.

B-The original standard sample was diluted as the following table:

6.4ng/mL	Standard No.5	120µl Original standard + 120µl Standard diluent
3.2ng/mL	Standard No.4	120µl Standard No.5 + 120µl Standard diluent
1.6ng/mL	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
0.8ng/mL	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
0.4ng/mL	Standard No.1	120µl Standard No.2 + 120µl Standard diluent



Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
12.8ng/mL	6.4ng/mL	3.2ng/mL	1.6ng/mL	0.8ng/mL	0.4ng/mL

Figure 2-3: Concentration of standards of leptin.

C- A volume of 20 ml of concentrated (1x) Wash Buffer were diluted into 500 ml of Wash Buffer concentrate (25x) with deionized or distilled water.

When crystals have made in the concentrates, mix gently until the crystals have fully dissolved. Procedure.

2.4.2.3 Procedure

1. The assay was performed at room temperature.

2. A volume of 50µL of standard was added to well standard.

3. A volume of 40µL from sample were added and then added 10µL of anti- leptin antibody to sample well, then 50µL of Streptavidin-HRP to both sample and standard wells. Mix well. The over plate covered with sealer and incubated for 60 min at 37 °c.

4.The sealer was eliminated and wash the plate 5 times over with a wash buffer. For each wash, soak wells for 30 sec. to 1 min., with at least 0.35 mL wash buffer. Aspire for automatic washing of all wells, by washing with wash buffer 5 times, filling wells with wash buffer. The plate was blotted into paper towels or other absorbing material.

5. A volume of 50µL substrate solution A added and then 50µL substrate solution B for each wells. The coated plate incubated with new sealer for 10 min. at 37°c in dark media.

6. To each well 50µL of stop solution was added, and the color change from the blue to yellow immediately.

7. The optical density (OD value) of each well identified directly after applying the stop solution by utilize a microplate reader set at 450 nm within 10 min.

Detection range: 0.05 – 10 ng/ml

Sensitivity: 0.021 ng/ml

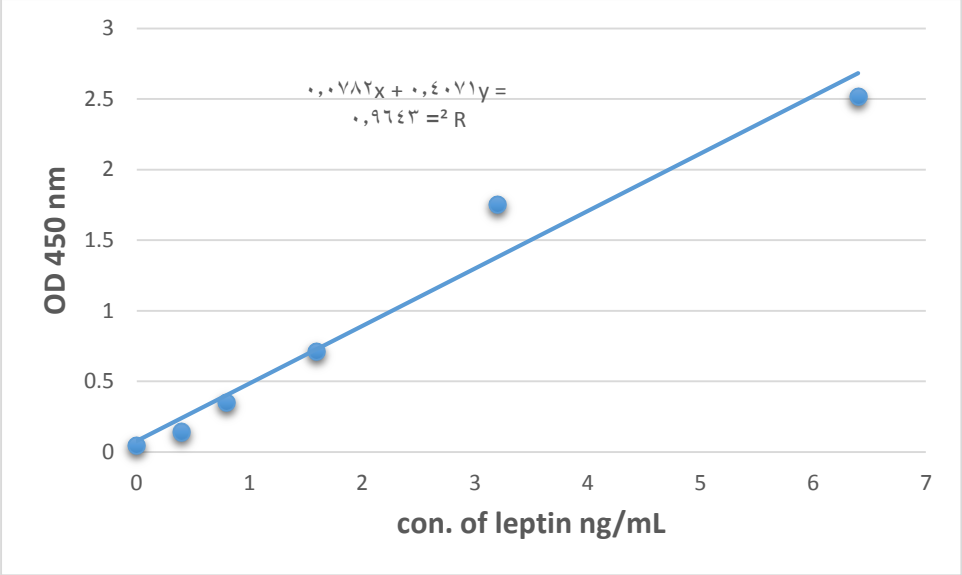


Figure 2-4: Standard curve of leptin.

2.4.3 Determination of Serum Adiponectin

Adiponectin level was measured by enzyme linked immunosorbent assay kit. Components of Pentosidine ELISA kits in Table 2-5.

2.4.3.1 Principle

The Sandwich-ELISA technique is used in this ELISA kit. The plate has been pre-coated with human adiponectin antibody. adiponectin present in the sample was added and binds to antibodies coated on the wells. After removing any unbound substances, a biotinylated human adiponectin antibody was added to wells and binds to adiponectin in the sample. After washing, Streptavidin- Horseradish Peroxidase (HRP) was added to wells and binds to the biotinylated adiponectin antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step. Substrate solution was added to wells and color develops in proportion to the amount of human adiponectin bound. The color development was stopped and the intensity of the color was measured at 450 nm.

Table 2-5 Components of adiponectin

Components	Quantity (96T)
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 APM1 antibody	1ml x1
User instruction	1
Plate sealer	2 pics

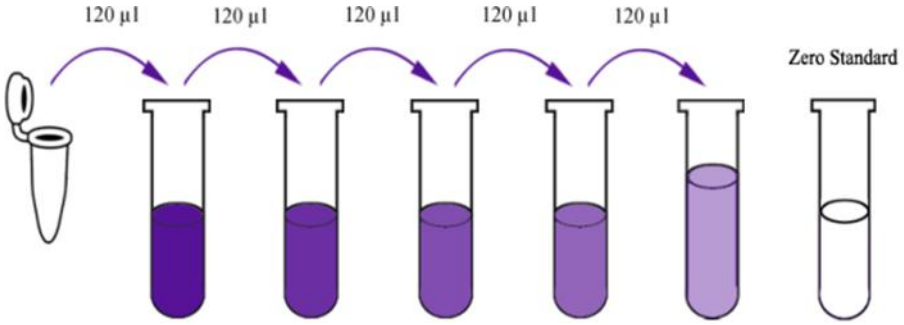
2.4.3.2 Reagent Preparation

A-Before use, all reagents need to be elevated to room temperature.

B-The original standard sample was diluted as the following table**:**

32ngm/L	Standard No.5	120µl Original standard + 120ul Standard diluent
16ng/mL	Standard No.4	120µl Standard No.5 + 120µl Standard diluent

8ng/mL	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
4ng/mL	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
2ng/mL	Standard No.1	120µl Standard No.2 + 120µl Standard diluent



Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
64ng/mL	32ngm/L	16ng/mL	8ng/mL	4ng/mL	2ng/mL

Figure 2-5: Concentration of standards of adiponectin.

C- A volume of 20 ml of concentrated (1x) Wash Buffer were diluted into 500 ml of Wash Buffer concentrate (25x) with deionized or distilled water. When crystals have made in the concentrates, mix gently until the crystals have fully dissolved.

2.4.3.3 Procedure

1. The assay was performed at room temperature.

2. A volume of 50µL of standard was added to well standard.

3. A volume of 40µL from sample were added and then added 10µL of anti- adiponectin antibody to sample well, then 50µL of Streptavidin-HRP to both sample and standard wells. Mix well. The over plate covered with sealer and incubated for 60 min at 37 °c.

4.The sealer was eliminated and wash the plate 5 times over with a wash buffer. For each wash, soak wells for 30 sec. to 1 min., with at least 0.35 mL wash buffer. Aspire for automatic washing of all wells, by washing with wash buffer 5 times, filling wells with wash buffer. The plate was blotted into paper towels or other absorbing material.

5. A volume of 50µL substrate solution A added and then 50µL substrate solution B for each wells. The coated plate incubated with new sealer for 10 min. at 37°c in dark media.

6. To each well 50µL of stop solution was added, and the color change from the blue to yellow immediately.

7. The optical density (OD value) of each well identified directly after applying the stop solution by utilize a microplate reader set at 450 nm within 10 min.

Detection range: 0.2 – 100 mg/L

Sensitivity: 0.11 mg/L

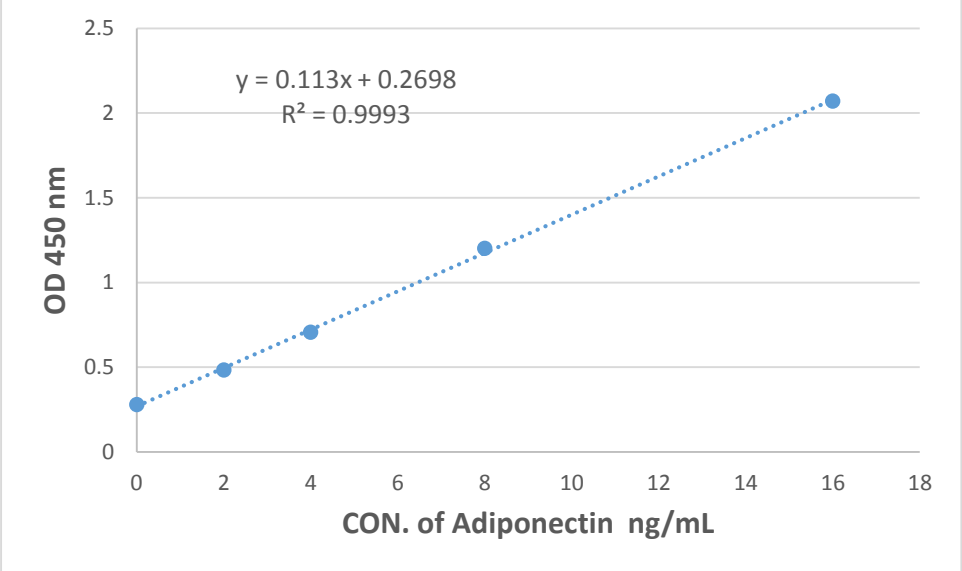


Figure 2-6: Standard curve for ADP

2.4.4 Determination of serum TNF-α level

TNF-α level was measured by enzyme linked immunosorbent assay kit. Components of Pentosidine ELISA kits in Table 2-6.

2.4.4.1 Principle

The Sandwich-ELISA technique is used in this ELISA kit. The plate has been pre-coated with human TNF-α antibody. TNF-α present in the sample was added and binds to antibodies coated on the wells. After removing any unbound substances, a biotinylated human TNF-α antibody was added to wells and binds to TNF-α in the sample. After washing, Streptavidin- Horseradish Peroxidase (HRP) was added to wells and binds to the biotinylated leptin antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step. Substrate solution was added to wells and color develops in proportion to the amount of human TNF-α bound. The color development was stopped and the intensity of the color was measured at 450 nm.

Table 2-6 Components of TNF-α ELISA kits

Components	Quantity (96T)
Standard solution (960ng/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

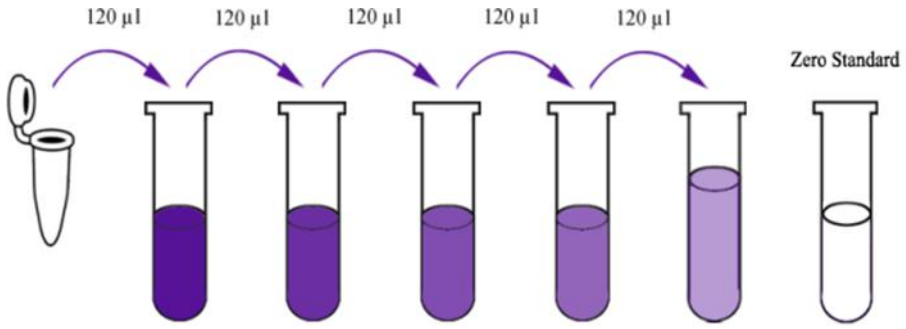
Substrate solution B	6ml x1
Wash buffer Concentrate (25x)	20ml x1
Biotinylated Human TNFA antibody	1ml x1
User instruction	1
Plate sealer	2 pics

2.4.4.2 Reagent preparation

A-Before use, all reagents need to be elevated to room temperature.

B-The original standard sample was diluted as the following table:

480ng/L	Standard No.5	120µl Original standard + 120µl Standard diluent
240ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard diluent
120ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
60ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
30ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard diluent



Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
960ng/L	480ng/L	240ng/L	120ng/L	60ng/L	30ng/L

Figure 2-7: Concentration of standards of TNF-α

C- A volume of 20 ml of concentrated (1x) Wash Buffer were diluted into 500 ml of Wash Buffer concentrate (25x) with deionized or distilled water. When crystals have made in the concentrates, mix gently until the crystals have fully dissolved.

2.4.4.3 Procedure

1. The assay was performed at room temperature.

2. A volume of 50µL of standard was added to well standard.

3. A volume of 40µL from sample were added and then added 10µL of anti- TNF-α antibody to sample well, then 50µL of Streptavidin-HRP to both sample and standard wells. Mix well. The over plate covered with sealer and incubated for 60 min at 37 °c.

4.The sealer was eliminated and wash the plate 5 times over with a wash buffer. For each wash, soak wells for 30 sec. to 1 min., with at least 0.35 mL wash buffer. Aspire for automatic washing of all wells, by washing

with wash buffer 5 times, filling wells with wash buffer. The plate was blotted into paper towels or other absorbing material.

5. A volume of 50µL substrate solution A added and then 50µL substrate solution B for each wells. The coated plate incubated with new sealer for 10 min. at 37°c in dark media.

6. To each well 50µL of stop solution was added, and the color change from the blue to yellow immediately.

7. The optical density (OD value) of each well identified directly after applying the stop solution by utilize a microplate reader set at 450 nm within 10 min.

Stander range: 3-900 ng/L

Sensitivity: 1.52 ng/L

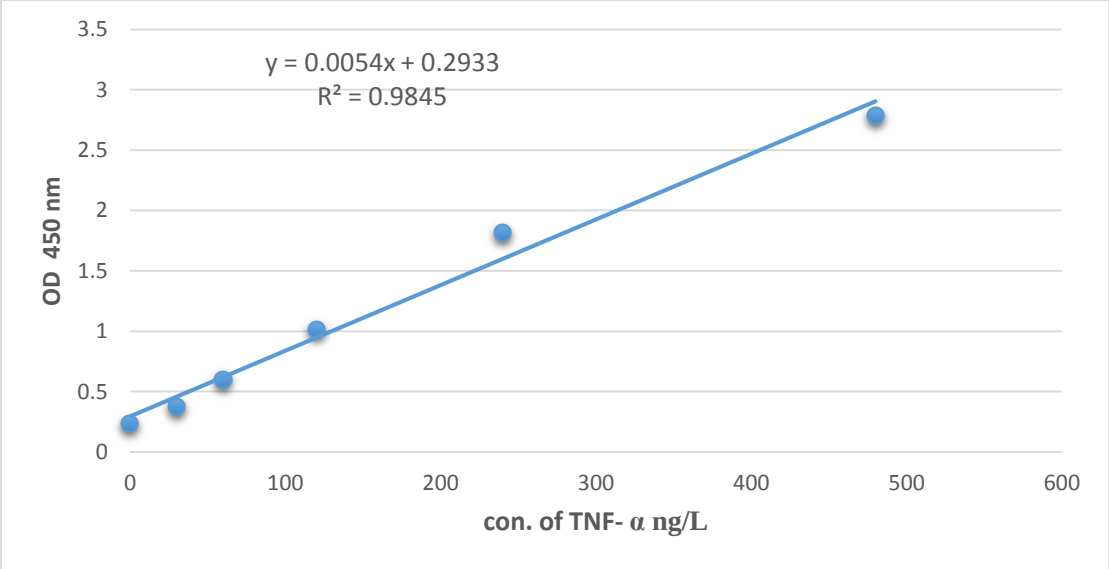


Figure 2-8: Standard curve for TNF-α

2.4.5 Determination of serum C-reactive protein level

C-reactive protein level was measured by enzyme linked immunosorbent assay kit. Components of Pentosidine ELISA kits in Table 2-7.

2.4.5.1 Principle

The Sandwich-ELISA technique is used in this ELISA kit. The plate has been pre-coated with Human CRP antibody. TNF-α present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human CRP Antibody is added and binds to CRP in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated CRP antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human CRP. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Table 2-7 Components of CRP LISA kits

Components	**Quantity (96T)**
Standard solution (6.4ng/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

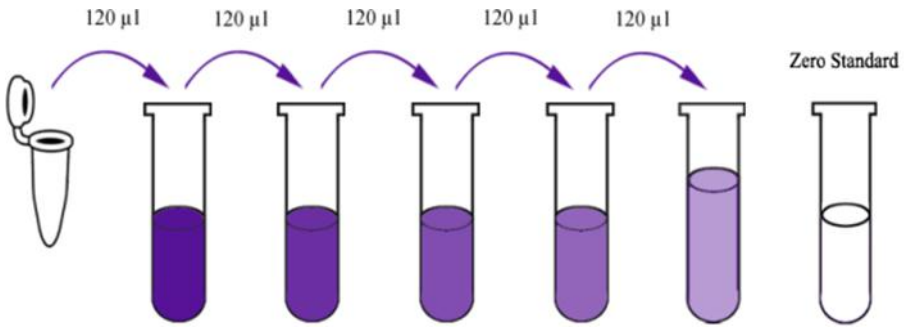
Substrate solution A	6ml x1
Substrate solution B	6ml x1
Wash buffer Concentrate (25x)	20ml x1
Biotinylated Human CRP;PTX1 antibody	1ml x1
User instruction	1
Plate sealer	2 pics

2.4.5.2 Reagent preparation

A-Before use, all reagents need to be elevated to room temperature.

B-The original standard sample was diluted as the following table:

3.2mg/L	Standard No.5	120µl Original standard + 120µl Standard diluent
1.6mg/L	Standard No.4	120µl Standard No.5 + 120µl Standard diluent
0.8mg/L	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
0.4mg/L	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
0.2mg/L	Standard No.1	120µl Standard No.2 + 120µl Standard diluent



Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
6.4mg/L	3.2mg/L	1.6mg/L	0.8mg/L	0.4mg/L	0.2mg/L

Figure 2-9: Concentration of standards of CRP

C- A volume of 20 ml of concentrated (1x) Wash Buffer were diluted into 500 ml of Wash Buffer concentrate (25x) with deionized or distilled water. When crystals have made in the concentrates, mix gently until the crystals have fully dissolved.

2.4.5.3 Procedure

1. The assay was performed at room temperature.

2. A volume of 50µL of standard was added to well standard.

3. A volume of 40µL from sample were added and then added 10µL of anti- TNF-α antibody to sample well, then 50µL of Streptavidin-HRP to both sample and standard wells. Mix well. The over plate covered with sealer and incubated for 60 min at 37 °c.

4.The sealer was eliminated and wash the plate 5 times over with a wash buffer. For each wash, soak wells for 30 sec. to 1 min., with at least 0.35 mL wash buffer. Aspire for automatic washing of all wells, by washing with wash buffer 5 times, filling wells with wash buffer. The plate was blotted into paper towels or other absorbing material.

5. A volume of 50µL substrate solution A added and then 50µL substrate solution B for each wells. The coated plate incubated with new sealer for 10 min. at 37°c in dark media.

6. To each well 50µL of stop solution was added, and the color change from the blue to yellow immediately.

7. The optical density (OD value) of each well identified directly after applying the stop solution by utilize a microplate reader set at 450 nm within 10 min.

Stander range: 0.02-6 mg/L

Sensitivity: 0.012 mg/L

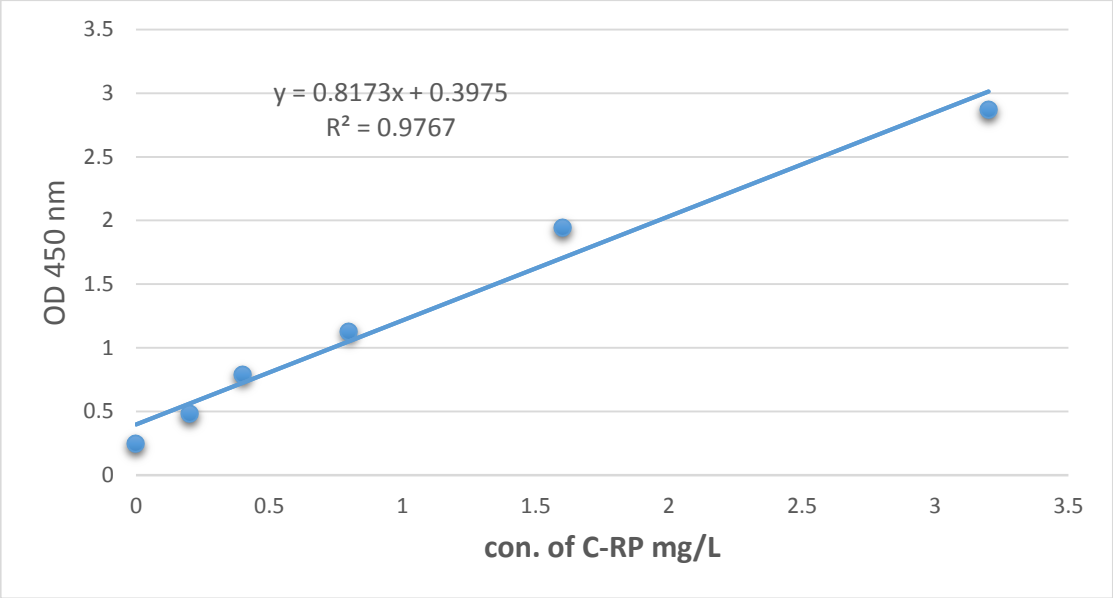


Figure 2-8: Standard curve for C-RP.

2.4.6 Determination of Random Blood Glucose

2.4.6.1 Principle

Glucose oxidase catalyzed the oxidation of glucose to gluconic acid and hydrogen peroxidase. The formed hydrogen peroxide is detected by a chromogenic oxygen acceptor, aphenol and 4-aminophenazone (4-AP) In the presence of peroxidase to form a colored product. The intensity of the

color formed is proportional to the original glucose concentration in the sample.

Table 2-6 Reagents composition of glucose Kit

Reagent 1 (enzyme buffer)	R1	Phosphate Buffer 150 mmol/L Glucose oxidase (GOD) ≥ 20 000 UI/L Peroxidase (POD) ≥1000 UI/L 4-Amino-antipyrine (PAP) 0.8 mmol/L
Reagent 2 (Chromogen)	R2	Chloro-4-phenol 2 mmol/L
Reagent 3 (Standard)	R3	Glucose 100 mg/dL (5.55 mmol/L)

2.4.6.2 Procedure

Allow reagents and specimens at room temperature before using.

Table 2-7 procedure of blood glucose

Pipette into test tubes	**Blank**	**Standard**	**Sample**
Working Reagent (R1+R2)	1mL	1mL	1mL
Distilled water	10 µL		
Standard		10 µL	
Serum			10 µL
The contents were mixed. Let stand for 10 minutes at 37°C or 20 minutes at room temperature. Read absorbance at 500 nm (460-560) against reagent blank. Coloration is stable for 15-20 minutes at 37°C, and then slowly decreases.			

Calculations

The result was calculated as following:

$$\text{Glucose concentration} = \frac{\text{Abs(Assay)}}{\text{Abs(standard)}} \text{ x Standard concentration}$$

(100 mg/dL).

2.4.7 HbA1c Measurement

2.4.7.1 Principle

The test uses a sandwich immunodetection method; the detector antibody in buffer binds to antigen in sample, forming antigen-antibody complexes, and migrates onto nitrocellulose matrix to be captured by the other immobilized-antibody on test strip. The more antigen in sample forms the more antigen-antibody complex and leads to stronger intensity of fluorescence signal on detector antibody. Instrument for ichroma™ tests displays the content of glycated hemoglobin in terms of percent of the total hemoglobin in blood.

2.4.7.2 Procedure

1) Draw 100 µL of hemolysis buffer and transfer it into detection buffer tube.
2) Draw 5 µL of fingertip blood or tube blood using 5 µL capillary tube and put the capillary tube into the detection buffer tube.
3) Close the lid of the detection buffer tube and mix the sample thoroughly by shaking it about 15 times.
4) Take out the cartridge half form i-Chamber slot.
5) Pipette out 75 µL of the sample mixture and load it into a sample well in the test cartridge.
6) Wait till the sample mixture flow appears in the windows.

(about 10 seconds)

7) Insert the cartridge into i-Chamber slot.

8) Leave the cartridge in i-Chamber for 12 minutes before removing.

Scan the sample-loaded cartridge immediately when the incubation time is over. If not, it will cause inexact test result.

9) To scan the sample-loaded cartridge, insert it into the cartridge holder of the instrument for ichroma™ tests. Ensure proper orientation of the cartridge before pushing it all the way inside the cartridge holder. An arrow has been marked on the cartridge especially for this purpose.

10) Press 'Select' button on the instrument for ichroma™ tests to start the scanning process.

11) Instrument for ichroma™ tests will start scanning the sample loaded cartridge immediately.

12) Read the test result on the display screen of the instrument for ichroma™ tests

2.4.8 ESR Measurement

The ESR system consists of a pipette and a tube. An upper sealing cap located on the top of the pipette. The tube with a removable and pierceable rubber stopper, is supplied with pre-filled 3.8% sodium citrate. Once blood and citrate are mixed together, insert the pipette through the stopper andpush to the bottom of the tube, the blood automatically reaches the 0 level.

2.5. Statistical Analysis

This study was a case –control research design. Statistical analysis done by SPSS 22, and Microsoft excel software v. 2016 were used for the data analysis and graphs plotting. No missing values were observed for any

variable. frequency and percentage used for categorical data, mean, median and SD for continuous data. Student t-test was used to compare means between two groups and the correlation test (Pearson test) was performed to find the association between variables. P-value less or equal to 0.05 is consider significant.

The diagnostic value of pentosidine, leptin, and TNF-α was assessed using a Receiver Operating Characteristic (ROC) curve. By choosing the point that is closest to the top-left corner of the ROC curve, one may determine the sensitivity and specificity of a biochemical parameter and calculate the optimal cutoff according to the "Youden Index"(169). Comparing several biomarkers is made easier with the help of the area under the curve (AUC), as shown in Table 2-8

Table 2-10: List of AUC ranges and their classification levels (170).

AUC Range	Classification Level
0.90 - 1.00	Excellent
0.80 - 0.90	Good
0.70 - 0.80	Fair
0.60 - 0.70	Poor
0.50 - 0.60	Failure

3. Results and Discussion

3.1. Demographic Characteristics in Patients and Control

3.1.1. Age

The means and standard deviation of age in patients and control as shown in Table (3-1). Total patients with DR were (45) included in this study whose age ranged (43-75) years; with mean ± SD (60.3 ± 8.0) year. Control group (45) apparently healthy subjects with an age range (48-76) years; with mean ± SD (62.2 ± 7.8) year.

Table 3.1 The means and standard deviation of age in patients and control.

Variable	Study Group	N	Mean ± SD	P-value
Age	control	45	62.2 ± 7.8	0.279
	case	45	60.3 ± 8.0	

P value* < 0.05 was significant
P value* ≥0.05 was non-significant

A non-significant difference in the mean of age between DR patients and the control group could be attributed to the matching of the age range between the subjects of the two studied group. which is particularly important, because the serum level of all biomarker in this study may depend on age. This finding was agreed with a previous study done in Iraq for DR patients(171,172).

3.1.2. Gender Distribution in patients and control.

Amongst forty-five patients with DR who contributed to this study, there were 29(68%) males and 16(23%) females, as shown in Figure 3-1.

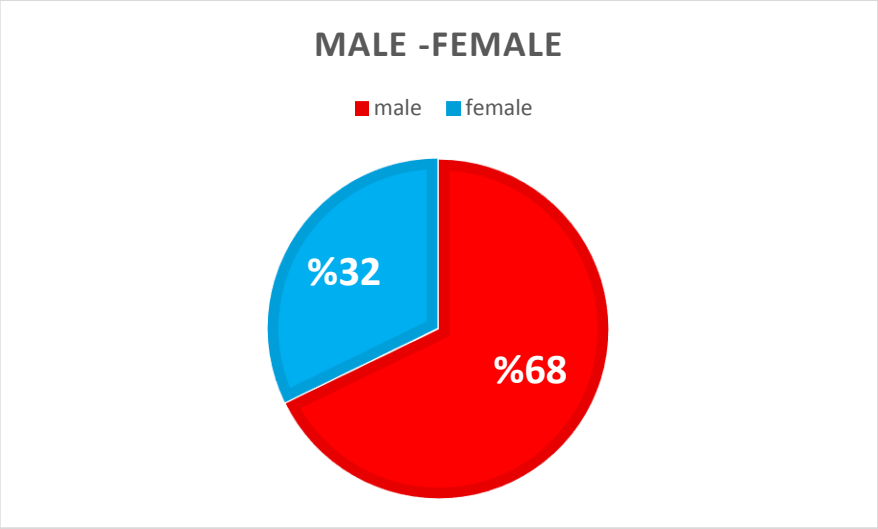


Figure 3-1: Rate of male to female among patients.

Amongst forty-five control who contributed to this study, there were 29 (68%) males and 16(32%) females, as shown in Figure 3-2.

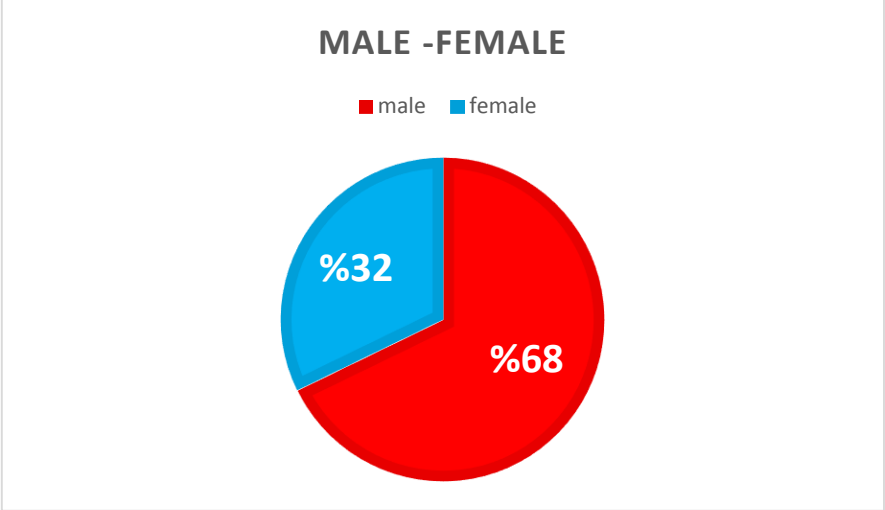


Figure 3-2: Rate of male to female among control group.

Equal number of healthy subjects regarding the gender depends on the number of samples selected, where the same numbers of males and females were taken in this study in order to match between them.

3.1.3. Body Mass Index (BMI) of Participants.

The results of the present study revealed a significant difference (p value ≤ 0.05) in the BMI between control group and patients group. as show in the table 3-2.

Table 3-2: The Body Mass Index (kg /m2) of Participants

Subject	Study group	N	Mean ± SD	P value
BMI(kg/m²)	Control group	45	24.1 ± 4.1	**≤ 0.001**
	Case group	45	28.0 ± 3.2	

The results of this study was agreement with study conducted by (173). Which reported a significant association between high BMI and obesity with DR, and the obese people higher risk to develop DR.

A major cause for that increase in the obesity is particularly due to the sedentary lifestyle with inactivity and unhealthy with too much fat and carbohydrate in diet, as well as excessive sodium consumption favor the development, all of which contribute to overweight and obesity(174).

Other previous study done by (175) reported that the concentration of VEGF has been found to be higher in the vitreous of eyes with DR. Likewise in the serum of obese individuals, elevated antigenic factors including VEGF have been observed partially owing to the presence of oxidative stress

providing additional confirmation of the possible link between obesity and PDR.

Suggests that obesity is associated with both increased local adipose and more generalized systemic inflammation. Adipose tissue is considered to be an active endocrine organ that releases leptin. Plasma leptin levels are seen to be elevated in obesity and correlate positively with both visceral and subcutaneous fat areas. High plasma leptin levels have been found to relate to both hypertensive and diabetic retinopathies. Pertinent to DR, recent findings show that leptin promotes vascular endothelial cell proliferation and angiogenesis in vitro and neovascularization in vivo.

3.2. Biochemical parameters:

3.2.1. Serum Human Pentosidine among DR Patient and Control Group.

Results of the present study revealed that there was a significant difference ($P< 0.05$) in serum concentration of human pentosidine among the DR patient group and control group were Human pentosidine concentration is higher in the DR group than the control. The means, standard deviation, and statistical parameters as shown in the table 3-3

Table 3-3: Comparison of pentosidine level in patients and control groups.

Parameter		N	Mean ± SD	P value
Pentosidine **(ng/ml)**	Control	45	1.4±0.4	0.001
	Case	45	2.9±1.5	

P value* < 0.05 was significant

The results revealed a significant increase in the Pentosidine level of the patients (2.9±1.5ng/ml) compared with the control (1.4±0.4 ng/ml).

The results of current study were agreement with a previous study (176) that reported serum pentosidine levels were significantly increased in diabetic patients with retinopathy and in diabetic patients with nephropathy. And also reported the serum pentosidine levels were an independent risk factor for the presence of diabetic retinopathy. Pentosidine levels were increased markedly with the severity of microangiopathy.

Our findings are consistent with (177) who found that blood pentosidine levels were significantly higher in the patients with PDR than in severe NPDR and mild NPDR subgroups, showing that the blood pentosidine levels increased along with the progression of retinopathy and correlate with the degree of retinopathy.

Pentosidine interact with vascular endothelium via AGE receptors such as RAGE, which can activate NF-κB transcription leading to

enhanced expression of adhesion molecules and secretion of cytokines such as TNF-α and VEGF. Serum-derived AGEs reach pericytes via trans endothelial trafficking or as a result of blood-retinal barrier dysfunction. These serum AGEs may also interact directly with cell surface glycoproteins with potentially damaging effects on membrane integrity and function(178).

3.2.2 Serum Human leptin among DR patient and Control group

Results of the present study revealed that there was a significant difference ($P< 0.05$) in serum concentration of human leptin among the DR patient group and control group were human leptin concentration is higher in the DR group than the control. The means, standard deviation, and statistical parameters as shown in the Table 3-4

Table 3-4: Comparison of leptin level in patients and control groups

Parameter		N	Mean ± SD	P value
Leptin (ng/ml)	Control	45	1.2 ± 0.3	0.006
	Case	45	2.8 ± 0.8	

P value* < 0.05 was significant

The results revealed a significant increase in the leptin level of the patients (2.8 ± 0.8ng/ml) compared with the control (1.2 ± 0.3 ng/ml).

The results of current study were agreement with a previous study(179). that reported serum leptin levels were significantly increased in diabetic patients with retinopathy.

Other study done by(180) that reported the major finding in the present study is that fasting plasma levels of leptin are elevated in diabetic patients with retinopathy, proportionate to the severity of retinopathy.

Plasma leptin concentrations are elevated significantly in obese subjects in proportion to the severity of the disease, suggesting that obese subjects are resistant to the central ant obesity effect of leptin (termed leptin resistance)(111).

It has been recognized that leptin promotes neovascularization and angiogenesis via direct vascular mechanisms(181).

leptin activated STAT3 in retinal endothelial cells, as revealed by increased STAT3 phosphorylation, the leptin-induced VEGF mRNA expression was abolished by adenoviral transfection of dominant-negative STAT3(182). suggest that leptin increases VEGF mRNA expression in retinal endothelial cells by STAT3 activation. This notion is consistent with the presence of STAT3 binding sites in the 5′ flanking region of the human VEGF gene. Since VEGF plays a critical role in the proliferation of retinal endothelial cells, it is likely that leptin stimulates retinal neovascularization through the upregulation of endothelial VEGF and/or synergistically with VEGF(183).

3.2.3 Serum Human Adiponectin among DR Patient and Control group

Results of the present study revealed that there was a significant difference ($P< 0.05$) in serum concentration of Human adiponectin among

the DR patient group and control group, were Human adiponectin concentration is higher in the DR group than the control. The means, standard deviation, and statistical parameters as shown in the table 3-5

Table 3-5: Comparison of adiponectin level in patients and control groups

Parameter		N	Mean ± SD	P value
Adiponectin (mg/l)	Control	45	3.8 ± 0.8	0.002
	Case	45	6.6 ± 1.5	

P value* < 0.05 was significant

The results revealed a significant increase in the adiponectin level of the patients (6.6 ± 1.5 mg/l) compared with the control (3.8 ± 0.8mg/l).

In agreement with our study the other study done by(184) , that reported serum adiponectin concentrations are significantly elevated in patients with DR compared to those without DR, and furthermore, are cross-section ally associated with DR severity.

Other study done by (185) also reported that DR in type 1 diabetes is also associated with elevated adiponectin when compared with non-DR patients.

In a study of Japanese type 2 diabetes patients,(186) found that adiponectin was elevated in patients with PDR. Moreover, adiponectin concentrations are increased in the aqueous humor and plasma of PDR subjects(187).

Adiponectin has been shown to be antigenic. It is unclear whether this plays any role at all in the angiogenesis associated with PDR. Thus, adiponectin exerts differential functions, and its effect in DR, a complication of both type 1 and type 2 diabetes, remains unclear(187).

Adiponectin in DR is a possible role in tissue injury and repair. A number of studies have shown that adiponectin is up-regulated in damaged tissues. For example, adiponectin mRNA is detected in the liver of a mouse model of hepatic injury. and importantly for any putative direct role in retinal physiology or disease, adiponectin receptors were recently demonstrated in the human and mouse retina(188).

3.2.4 S erum human TNF-α among DR patient and Control group.

Results of the present study revealed that there was a significant difference (P< 0.05) in serum concentration of TNf-α among the DR patient and control group were human TNf-α concentration is higher in the DR group than the control group, the means, standard deviation, and statistical parameters as shown in the table 3-6.

Table 3-6: Comparison of TNf-α level in patients and control groups

Parameter		N	Mean ± SD	P value
TNF-α (ng/l)	Control	45	123 ± 18	0.001
	Case	45	140.8 ± 26	

P value* < 0.05 was significant

The results revealed a significant increase in the TNF-α level of the patients (140.8 ± 26 ng/l) compared with the control (123 ± 18 ng/l).

This result of our study is agreement with other previously study done by(189). that reported the TNF-α level of the DR group was significantly higher than that of the control group.

(190). reported that TNF-α level was significantly elevated in patients with PDR compared to patients with NPDR and to patients with no apparent diabetic retinopathy (NDR).

However, some studies drew opposite conclusions Loukovaara et al. has reported that the level of TNF-α had no significant difference the NPDR and PDR patients.

When interpreting these results, the following issues should be taken into consideration. First, TNF-α can simulate the release and synergistic proliferation of IL-6, IL-8, VEGF and platelet derived growth factor (PDGF)(143).

Second, TNF-α can inhibit the formation and development of retinal vascular endothelial cells, promote apoptosis of endothelial cells, destroy normal function of the vascular wall and influence vascular permeability of the retina(141).

Third, TNF-α can induce neovascularization in the eyes.

3.2.5 Serum human C-RP among DR Patient and Control Group.

Results of the present study revealed that there was a no significant difference (P< 0.05) in serum concentration of CRP among the DR patient and control group. The means, standard deviation, and statistical parameters as shown in the table3-7

Table 3-7: Comparison of C-RP level in patients and control groups

Parameter		N	Mean ± SD	P value
C-RP (mg/l)	Control	45	0.7 ± 0.2	0.25
	Case	45	0.8 ± 0.1	

P value* < 0.05 was significant

The results revealed a no significant differences in the C-RP level of the patients (0.8 ± 0.1 mg/l) compared with the control (0.7 ± 0.2 mg/l).

In agreement with our study the other study done by (191) that reported The CRP correlation with diabetic retinopathy is not significant as no considerable elevation was noticed.

Explanation for our findings is indication bias. In other words, persons with DR could have adopted positive behavioral modifications that led to lower CRP and BMI levels. This possibility is supported by the finding that the associations were stronger in the subjects with a history of diabetic medication use (191).

3.2.6 RBS among Patients and Control Group.

Results of the present study reveal that there was a significant (P value < 0.05) difference in serum concentration of RPS among DR and control group, were RPS concentration is much higher in DR group than control group. The means, standard deviation, and statistical parameters as shown in the table 3-8.

Table 3-8: Comparison of RPS level in patients and control groups

Parameter		N	Mean ± SD	P value
RBS mg/dl	control	45	102.9 ± 20	0.000
	Case	45	247 ± 95	

P value* < 0.05 was significant

The results revealed a significant increase in the RBS level of the patients (247 ± 95) compared with the control (102.9 ± 20).

3.2.7 HbA1c among patients and control group

Results of the present study reveal that there was a significant (P value < 0.05) difference in serum concentration of HbA1c among DR and control group, were HbA1c concentration is much higher in DR group than control group. The means, standard deviation, and statistical parameters as shown in the table 3-9.

Table 3-9: Comparison of HbA1c level in patients and control groups

Parameter		N	Mean ± SD	P value
HbA1c %	Control	45	5.4 ± 1.4	0.000
	Case	45	11.9 ± 2.2	

P value* < 0.05 was significant

The results revealed a significant increase in the HbA1c level of the patients (11.9 ± 2.2 %) compared with the control (5.4 ± 1.4%).

The results of this study was agreement with study conducted by(192) that reported The HbA1c level of the DR group was significantly higher than that of the control group.

Other study also(193) reported Type 1 DM, prolonged DM duration 4 years, and diabetic retinopathy are strongly linked to poor HbA1c control.

3.2.8 ESR level among patients and control

Results of the present study reveal that there was a significant (P value < 0.05) difference in serum concentration of ESR among DR and control group, were ESR concentration is much higher in DR group than control group. the means, standard deviation, and statistical parameters as shown in the Table 3-10.

Table 3-10: Comparison of ESR level in patients and control groups.

Parameter		N	Mean ± SD	
ESR	Control	45	15.4 ± 6.9	0.002
	Case	45	20.7 ± 8.4	

P value* < 0.05 was significant

The results revealed a significant increase in the ESR level of the patients (20.7 ± 8.4) compared with the control (15.4 ± 6.9).

The results of this study was agreement with study conducted by(194)Which reported that Statistically significant higher ESR level among patients group than control group, and correlated to degree of severity diabetic retinopathy.

Other previously study positive correlation between the erythrocyte sedimentation rate and the degree of retinopathy, therefore, ESR can also be used as an indicator to evaluate the progress of patients with diabetes(195).

3.3. Personal Correlations between pentosidine, leptin, adiponectin and TNF-α, with other parameters in DR patient.

Table 3-11 correlation between parameters in patients group

Correlations						
			Pentosidine	Leptin	Adiponectin	TNF-α
case	Pentosidine	Pearson Correlation	1	0.436	0.271	0.457
		Sig. (2-tailed)		0.007	0.072	0.001
		N	45	45	45	45
	Leptin	Pearson Correlation	0.436	1	0.157	0.400
		Sig. (2-tailed)	0.007		0.303	0.007
		N	45	45	45	45
	Adiponectin	Pearson Correlation	0.271	0.157	1	0.482
		Sig. (2-tailed)	0.072	0.303		0.002
		N	45	45	45	45
	TNF-α	Pearson Correlation	0.457	0.400	0.482	1
		Sig. (2-tailed)	0.001	0.007	0.002	
		N	45	45	45	45

P value* < 0.05 was significant

3.3.2 Correlation between BMI and glucose

BMI shows a positive correlation with glucose (r=0.131, P=0.015) as seen in figures 3-3. Some studies demonstrated an association between higher glucose levels and the BMI, similar to our study (196)

Glucose synthesizes fatty acids that constitutes body fat content. An increase in blood glucose level will result in increase in BMI causing

increased lipid biosynthesis and hence body weight. Insulin which is secreted from beta cells of islets of langerhans from pancrease act through specific cell receptor of insulin sensitive cells which results in enhanced glucose uptake into the cell. As BMI increases, insulin resistance also increases which results in increased blood glucose level in body. Since body weight is associated with BMI, it may be expected that BMI should correlate with blood glucose levels.

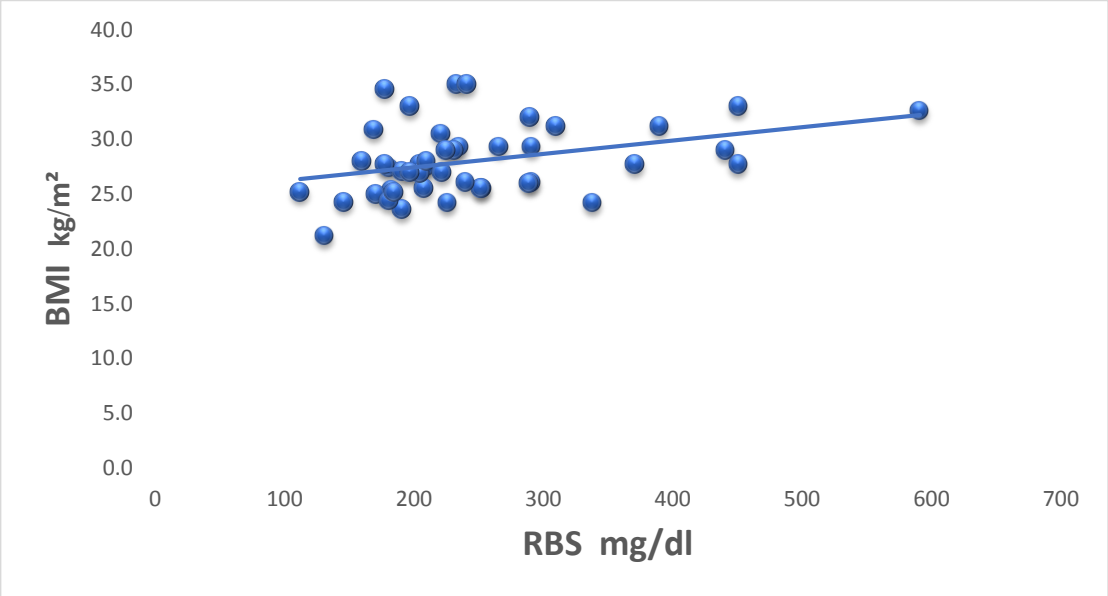


Figure 3-3 Correlations between BMI and glucose

3.3.3 Correlation between pentosidine and leptin.

Serum leptin shows positive correlation with pentosidine (r=0.436, P=0.007) as in Figure 3-4.

Some studies demonstrated an association between higher pentosidine levels and the leptin diabetes mellitus , similar to our study(197,198)

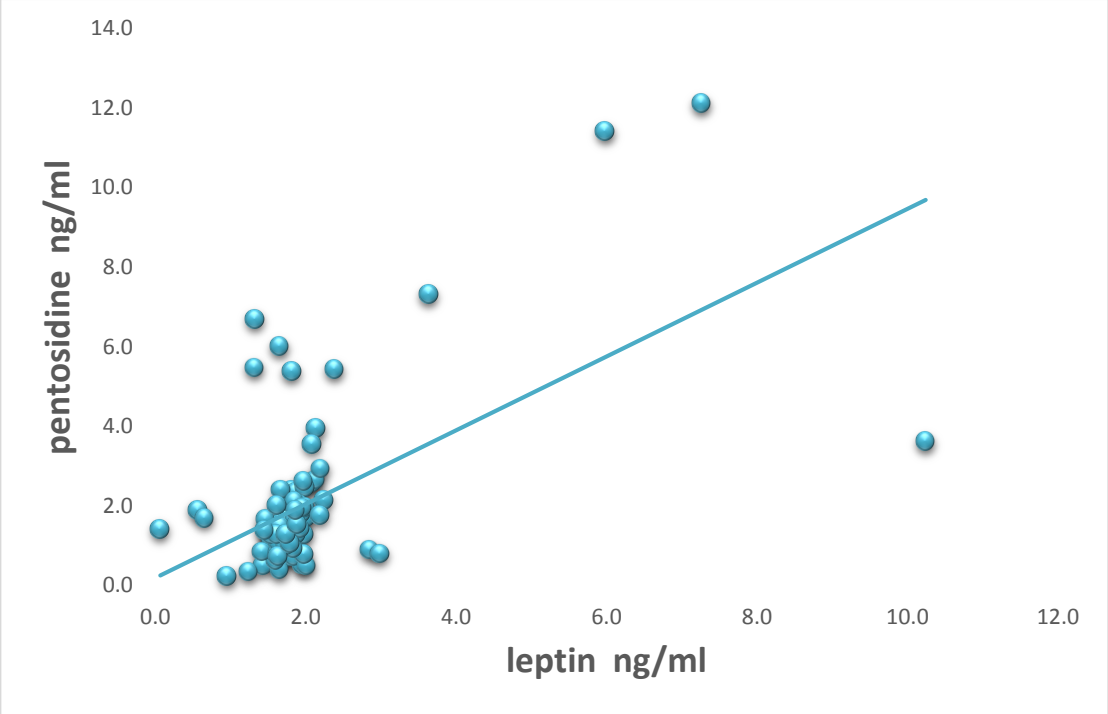


Figure 3-4 Correlations between leptin and pentosidine.

3.3.4 Correlation between Pentosidine and TNF-α.

Serum pentosidine shows a positive correlation with TNF-α (r=0.457, P=0.001) as in figure 3-5.

The binding of pentosidine on cell receptors induces activation of nuclear factor kappa B, resulting in enhanced synthesis of proinflammatory cytokines. Moreover, pentosidine generation may also lead to the formation of new, immunologically relevant epitopes at synovial protein.

Some studies demonstrated an association between higher pentosidine levels and the TNF-α, similar to our study(178).

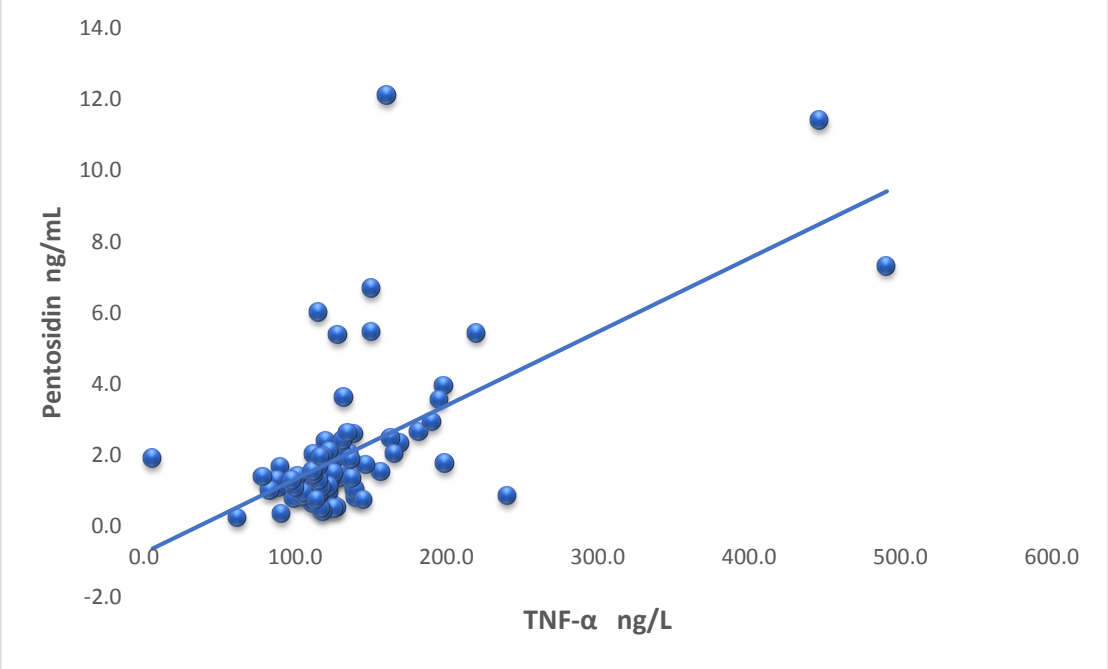


Figure 3-5 Correlations between TNF-α and pentosidine

3.3.5 Correlation between leptin and TNF-α.

Serum leptin showed a positive correlation with TNF-α (r=0.461, P=0.007) as seen in Figure 3-6.

The effect of leptin on cells of the immune system such as macrophages and B cells is typically achieved via stimulation ofTh1 cytokine expression. Regulation of leptin-induced signaling pathways is a key step in immune-related diseases. Interestingly leptin potentiates the production of pro-inflammatory cytokines (including TNF-a) in macrophages in response not only to LPS but also to ozone exposure(199).

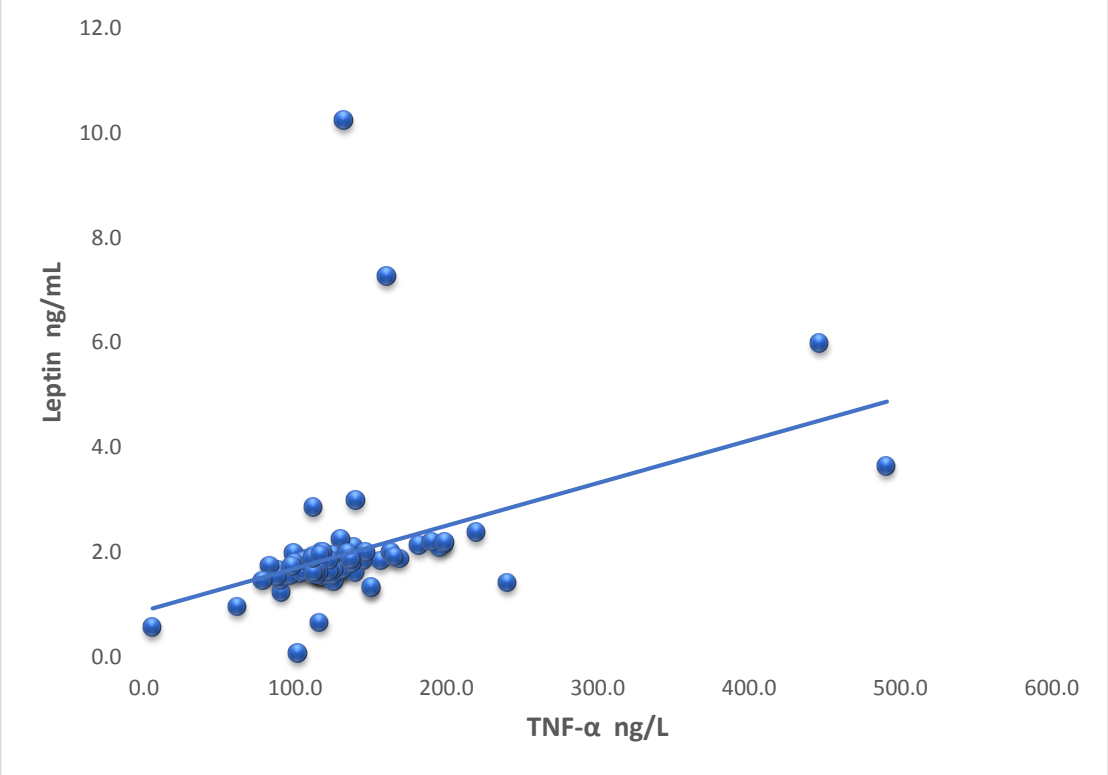


Figure 3-6 Correlations between TNF-α and leptin.

3.3.6 Correlation between Adiponectin and TNF-α.

Serum adiponectin showed a positive correlation with TNF-α (r=0.482, P=0.002) as seen in figure 3-7.

The expression and secretion of adiponectin from adipocytes are significantly induce by TNF-α. Therefore, increased TNF-αmight be partially responsible for the increase adiponectin production in DR(133).

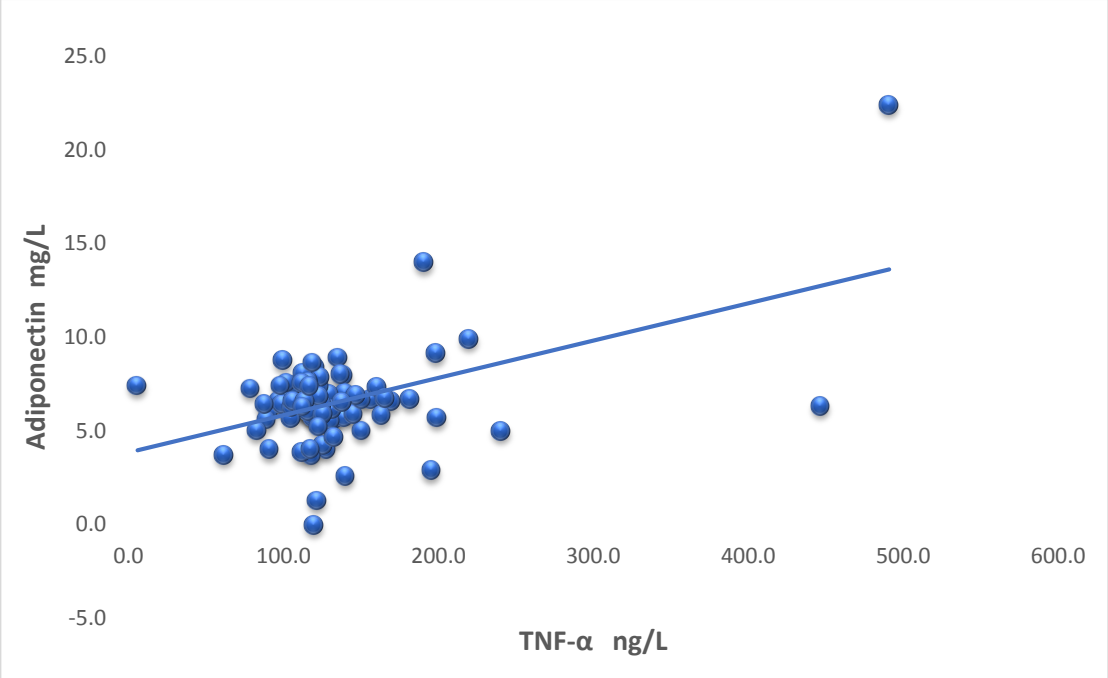


Figure 3-7 Correlations between TNF-α and adiponectin

3.4 ROC Curve of Biochemical Parameters.

3.4.1. ROC curve of pentosidine.

ROC curve for the sensitivity and specificity of pentosidine (ng/ml) for diagnosis of DR, (Cut-off point was ≥ 3.05 (ng/ml), AUC= 0.80 , P value ≤ 0.001), the sensitivity and the specificity was 77 %, 81 % respectively, positive predictive value(PPV) was 71%, negative predictive value(NPV) was 79%, as shown in figure 3-8.

For DR, our result state that good diagnostic value in the diagnosis of DR.

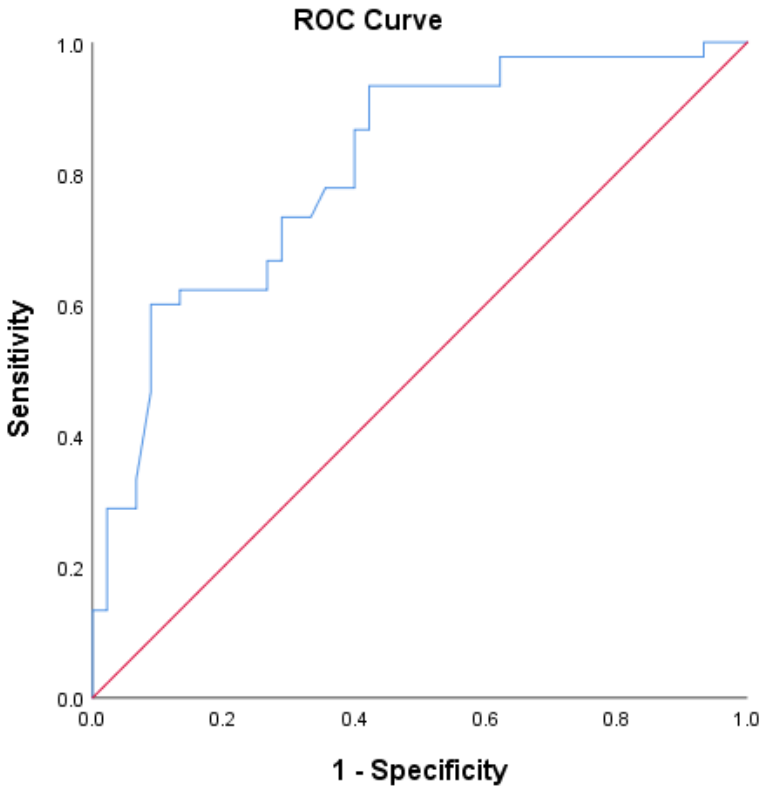


Figure 3-8: ROC Curve of Pentosidine

3.4.2. ROC Curve of Leptin.

ROC curve for the sensitivity and specificity of leptin (ng/ml) for diagnosis of DR, (Cut-off point was ≥ 1.9 (ng/ml), AUC= 0.63 , P value ≤ 0.001), the sensitivity and the specificity was 66 %, 59 % respectively, positive predictive value(PPV) was 79%, negative predictive value(NPV) was 66%, as shown in figure (3-9).

For DR, our result state that poor diagnostic value in the diagnosis of DR.

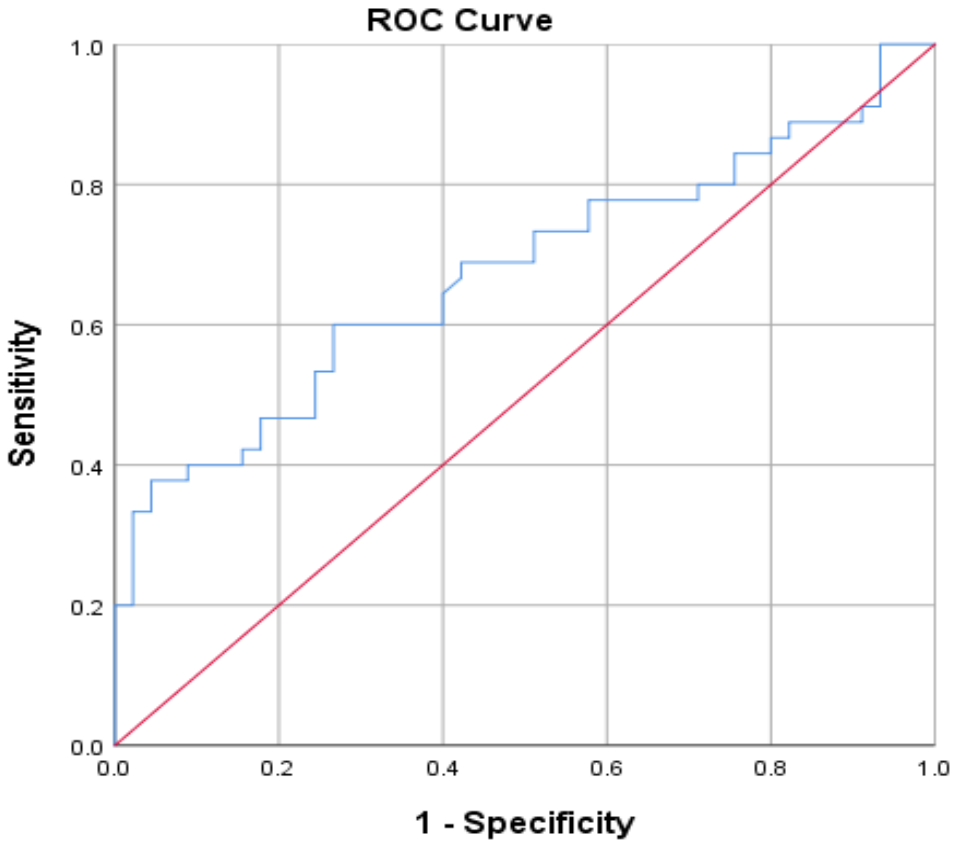


Figure 3-9 Roc curve of leptin

3.4.3 ROC curve of TNF-α.

ROC curve for the sensitivity and specificity of TNF-α (ng/ml) for diagnosis of DR, (Cut-off point was ≤ 121.5 (ng/ml), AUC= 0.77 , P value ≤ 0.001), the sensitivity and the specificity was 77 %, 61 % respectively, positive predictive value(PPV) was 82%, negative predictive value(NPV) was 73%, as shown in figure (3-4).

For DR, our result state that good diagnostic value in the diagnosis of DR.

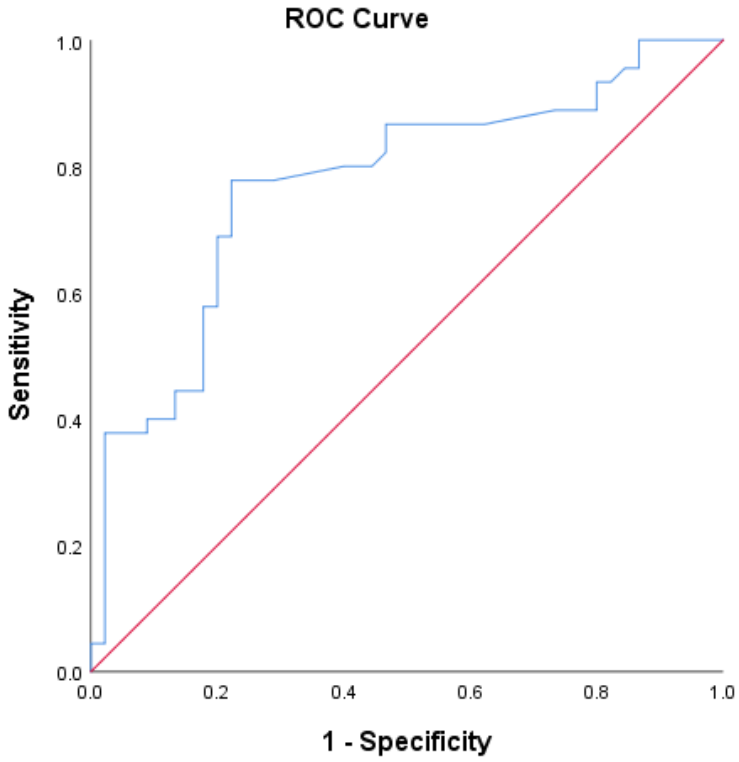


Figure3-10: Roc curve of TNF-α

Conclusions

- Diabetes mellitus and long duration hyperglycemia may be one of the important causes of diabetic retinopathy through its direct role in releasing angiogenic and inflammatory factors in the retina of an eye.
- The biochemical parameters included in this study such as pentosidine, leptin and TNF-α are significantly higher in DR patients than healthy control.
- The BMI was significantly higher in patients than control, so obesity considered as a comorbid for DR patients.
- Pentosidine could be considered as a biomarker of inflammation in DR and is more sensitive and specific than leptin so that it's a good marker for diagnosis of DR patients.
- TNF-α can be used as a new marker for evaluation of disease severity and progression in DR.

Recommendations

- Study these parameters of the patients with DR according to WHO classification.
- Other early markers of diabetic retinopathy (such as, Ang-1, Ang-2, HIF-1 and IAF) are needed to be measured.
- Study on the type 1 diabetic patients

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